Coordination of Post-Translational Modifications of Yeast Transcriptional Activator Gcn4

Akhi Akhter

A THESIS SUBMITTED TO FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF

Master of Science

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

May 2016

Abstract:

Post-translational modifications (PTMs) of transcription factors have emerged as an important regulatory mechanism in eukaryotes. PTMs can modulate the function of a protein, and inappropriate modifications can result in diseases. A protein can become post-translationally modified in several different ways. It can range from a small modification, such as phosphorylation, to more large modifications, such as conjugation with small polypeptides like ubiquitin or SUMO.

Gcn4 is a transcriptional activator that regulates amino acid biosynthetic genes in yeast. Gcn4 is expressed under amino acid starvation conditions, and its degradation depends on phosphorylation, by either Pho85 or Cdk8, which triggers its ubiquitination and subsequent proteolysis by the 26S proteasome. Previous studies showed that Gcn4 becomes sumoylated at two Lys residues (K50, 58), specifically after binding to target gene promoters. However, it is not clear how promoter-associated sumoylation of Gcn4 is coordinated with its phosphorylation and ubiquitination as part of a regulatory cascade of PTMs.

To address this, we examined Gcn4 modifications in individual sumoylation-deficient mutant, phosphorylation-site mutant and ubiquitination-impaired Gcn4 strains. Our results indicate that Gcn4 sumoylation is not dependent on its prior phosphorylation or ubiquitination; however, some degree of Cdk8-mediated phosphorylation and ubiquitination is dependent on sumoylation. Furthermore, we generated a hyper-sumoylated form of Gcn4 that shows significantly reduced occupancy on target DNA, which is dependent on Cdk8. Our results suggest that SUMO modification of promoter-bound Gcn4 triggers its removal by stimulating Cdk8-mediated phosphorylation and subsequent ubiquitination.

Together, this study contributes to the understanding of post-translational modifications of Gcn4, and such information could reveal new targets for directed therapy of diseases or cancer resulting from deregulated signaling pathways.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr Emanuel Rosonina, for giving me a great opportunity to work on many exciting projects in his lab. Without his constant motivation, guidance and help, this thesis would never have been accomplished. His unparalleled expertise in the field was only rivaled by the extreme kindness and understanding he always displayed. I would also like to express my heartfelt gratitude to my advisor, Dr Yi Sheng, for her valuable suggestions and comments, as well as for challenging me to improve my work constantly.

Although it is considerably more challenging than writing this thesis to capture with words the level of support they have provided me, I would like to thank all the members of the Rosonina Lab, past and present: Eva, Justin, Farhan, John, Veroni, Tammi, Russell, Arman and Jessica. Notably, my sincere thanks to John Babu (the legend himself) for his endless suport with everything, lab related or otherwise.

A special thanks to Dr. Peter Cheung and Dr. Mark Bayfield for allowing me to utilize their equipment and to my thesis committee members Dr. Katalin Hudak and Dr. Dorota Crawford for their time and support.

Like a thesis without a discussion, this section would not be complete without expressing a heartfelt gratitude to all my friends from the 3rd floor of LSB and York (too many to mention separately) for their magnanimity, assistance and valuable advice which helped me survive as a graduate student. I would finally like to thank my best friend Sahar, for sharing my happiness and sadness throughout our time together at York.

Last, but by no means the least, thank you Cristalina for all your guidance and the many emails which made me more informed (because of the notifications in them) and wiser (because of the great quotes they contained).

Table of Contents

List of Tables:

List of Figures

Abbreviations

˚**C**	Degree Celsius
AD	Activation domain
Ala / A	Alanine
Arg / R	Arginine
Asn	Asparagine
ATP	Adenosine Tri-Phosphate
BRE	TFIIB-Recognition Element
bp	base-pair or base-pairs
bZIP	Basic Leucine Zipper Domain
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
C-terminus	Carboxy-terminus
Cys / C	Cysteine
DBD	DNA-binding domain
DPE	Downstream Promoter Element
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Glu / E	Glutamic acid
FWD	Forward
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gln / Q	Glutamine
Gly / G	Glycine
GTF	General Transcription Factor
HRP	Horseradish peroxidase
IkBa	Inhibitor of nuclear factor kB alpha
Ile / I	Isoleucine
INR	Initiator element
IP	Immunoprecipitation
kDa	Kilodalton

Lys / K	Lysine
MDa	Megadalton
Med	Mediator
mL	Millilitre
mM	Millimolar
mRNA	Messenger-RNA
ORF	Open reading frame
PBST	Phosphate buffer saline with Tween
PCR	Polymerase chain reaction
Phe / F	Phenylalanine
PIC	Pre-initiation complex
PML	Promyelocytic leukemia
PMSF	Phenylmethylsulfonyl fluoride
REV	Reverse
RNA	Ribonucleic acid
RNAP II	Rna polymerase II
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SC	Synthetic Complete
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis
SENP	SENtrin-specific proteases
Ser / S	Serine
SM	Sulfometuron methyl
SUMO	Small ubiquitin-like modifier
TAF	TBP-associated factors
TBP	TATA-binding protein
TBST	Tris-Buffered Saline with Tween
TCF4	T-cell factor-4
Thr / T	Threonine
Trp / W	Tryptophan

Tyr / Y	Tyrosine
TS	Temperature sensitive
TSS	Transcription start site
Ura / U	Uracil
UAS	Upstream Activating Sequences
Ub	Ubiquitin
µg	Microgram
µL	Microlitre
Ulp	Ubiquitin-like specific protease
Val / V	Valine
X	Unspecified amino acid
WT	Wild type

Chapter 1: Introduction

Gene expression is a highly complex and tightly regulated process that requires an ensemble of factors to properly initiate transcription from a DNA template to generate messenger RNA (mRNA), which can then be translated into a protein. In eukaryotes, appropriate regulation of transcription of protein-encoding genes, mediated by RNA polymerase II (RNAP II) in conjunction with a complex array of general transcription factors (GTFs), is essential for normal cell growth and function. Through the action of transcription factors, various cells containing the same genome can function differently, and the functions of these transcription factors can be regulated by post-translational modifications (PTMs), such as phosphorylation, ubiquitination, and sumoylation (Struhl, 1993). For example, the yeast transcriptional activator of amino acid biosynthetic genes Gcn4, becomes sumoylated during target gene activation which facilitates its subsequent removal from promoters (Rosonina et al., 2012). Defects in transcriptional regulation can lead to developmental problems or to diseases such as cancer (Audic and Hartley, 2004). Therefore, it is essential to understand the mechanisms of transcription and how it is regulated.

1.1 Transcriptional Initiation (Pre-initiation Complex Assembly):

The transcription of eukaryotic RNAP II-dependent genes involves a cascade of events that generally begins with the sequence-specific binding of an activator protein to the regulatory elements proximal to the gene's promoter region (Morse, 2007). In yeast, these gene-specific regulatory elements are known as upstream activating sequences (UAS), while in higher organisms they are known as enhancers, which can be situated hundreds or thousands of base-pairs (bp) from the gene’s transcriptional start site (TSS) (Buchman and Kimmerly, 1988). Binding of

transcriptional activators to their target elements initiates the sequential recruitment of GTFs, RNAP II and the Mediator complex which assemble to form a functional pre-initiation complex (PIC). The PIC is essential for directing specific TSS selection and transcription initiation (Thomas and Chiang, 2006).

Transcriptional activators direct PIC assembly on the core promoter, a sequence of approximately 40-120 bp upstream of the TSS in the yeast *Saccharomyces cerevisiae*, whereas in mammals it is located around 25-30 bp upstream of the TSS (Smale and Kadonaga, 2003; Yang et al., 2007). The core promoter often contains a number of sequence specific motifs including the TATA box, the initiator (INR), TFIIB-recognition element (BRE) and downstream promoter elements (DPE) (Smale & Kadonaga 2003; Juven-Gershon & Kadonaga 2010) (**Figure 1**). Most yeast core promoters do not contain a consensus TATA box, but instead contain 'TATA-like' sequences that differ from the TATA box consensus by up to 2 bases (Basehoar et al., 2004; Rhee and Pugh, 2012).

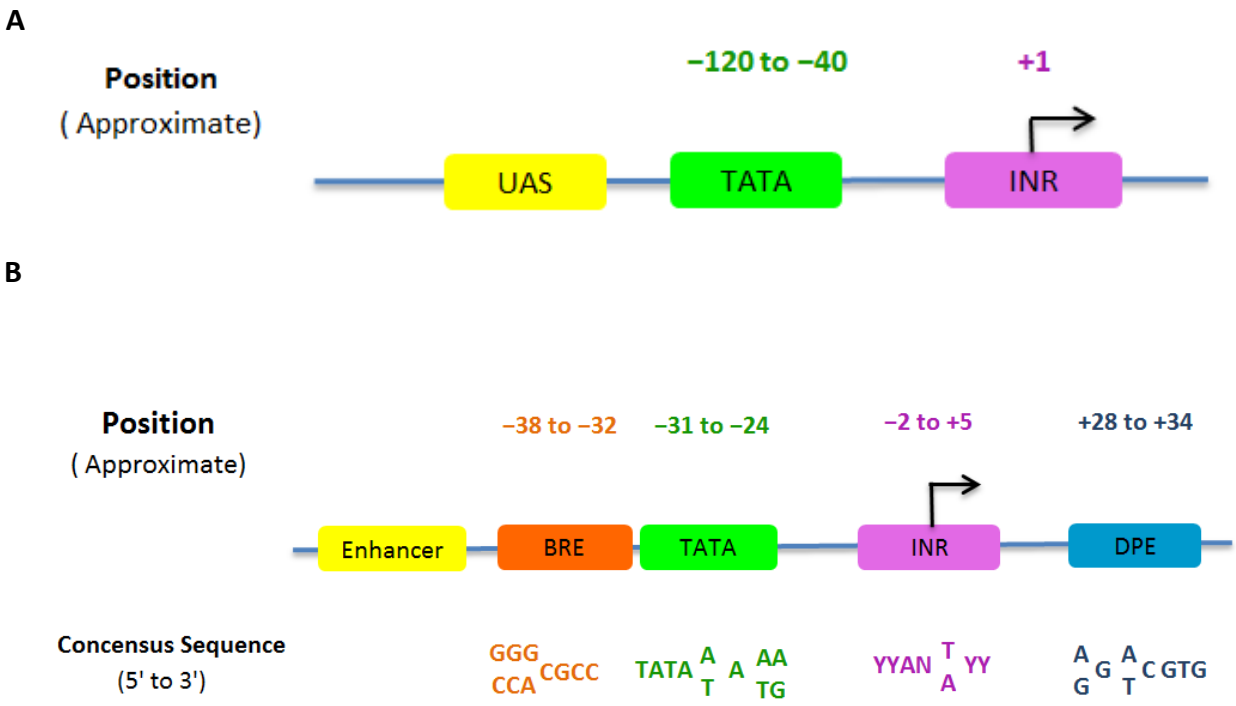


Figure 1. Schematic of the structure and sequence of core promoter elements in eukaryotes. *(A)* A simple core promoter containing a TATA box and initiator (INR) element, with upstream activation sequence (UAS) shown, as found on in yeast (unicellular eukaryotes). *(B)* A complex core promoter containing a TATA box, INR, TFIIB-recognition element (BRE), and downstream promoter elements (DPE) that is typically found in mammals (higher eukaryotes). An enhancer element is also shown. Also indicated are the approximate position of the elements relative to the TSS (above) and their consensus sequences (below). Any particular core promoter may contain all, some or none of these motifs.

In general, the first step in canonical PIC assembly is the sequence-specific binding of TFIID, comprised of the TATA-binding protein (TBP) and a dozen or so evolutionarily conserved RNAPII-specific TBP-associated factors (TAFs), to the TATA box (Klein and Struhl, 1994; Nikolov et al., 1995) (**Figure 2**). This binding induces a dramatic bend in the DNA structure. TFIIA and TFIIB are then recruited, further stabilizing the interaction between TBP and promoter DNA (Flores et al., 1991; Nikolov et al., 1995). TFIIB binds to both TBP and DNA leading to the recruitment of the RNAP II–TFIIF complex (Lee and Hahn, 1995). RNAP II and TFIIF enter the complex together, with TFIIF ensuring the specific and stable binding of the polymerase to DNA (Deng and Roberts, 2007). In the presence of TFIIB and TFIIF, RNAP II orients the DNA template selecting the transcription start site (Bushnell et al., 2004). Finally, TFIIE and TFIIH are recruited to form the transcriptionally competent PIC and, together with the help of RNAP II, catalyze promoter melting and conversion from transcription initiation to elongation (Buratowski et al., 1989; Martinez, 2002; Deng and Roberts, 2007). Upon initiation of transcription, RNAP II moves along the DNA strand, leaving TFIID and TFIIA bound to the TATA box which can then facilitate the binding of additional RNAP II molecules (Van Dyke et al., 1989; Zawel et al., 1995).

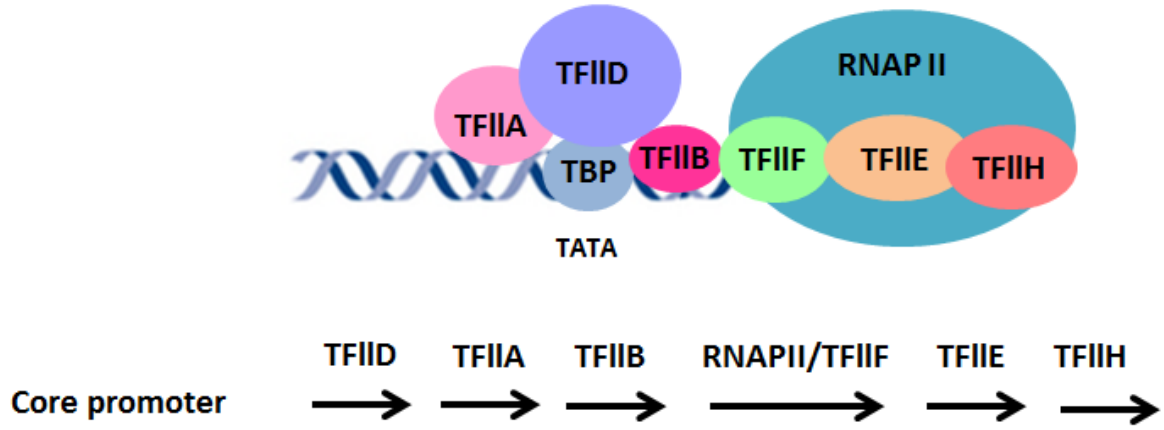


Figure 2: **Schematic of the RNAPII pre-initiation complex (PIC) at the core promoter.** PIC formation occurs by the stepwise recruitment of the general transcription machinery**.** First, TBP (a component of TFIID) binds to the promoter and recruits TFIIA and TFIIB. Then RNAP II and TFIIF are recruited by TFIIB. To complete the PIC assembly, RNAP II recruits TFIIE, which further recruits TFIIH.

1.2 Mediator Component Cdk8:

Mediator is a large, multi-subunit complex that functions as an intermediate between transcriptional activators and the PIC components to coordinate transcription (Cantin et al., 2003; Biddick and Young, 2005; Conaway et al., 2005). Almost all yeast Mediator subunits have homologs in mammals ((Biddick and Young, 2005). However, human Mediator is more complex than its yeast counterpart (Conaway et al., 2005). Mediator may exist in at least two forms: a smaller "core Mediator complex" form and a larger "CDK8 Mediator complex" form. The core

Mediator complex consists of 25 subunits and is 1.2 MDa in size, while the CDK8 Mediator complex contains an additional CDK module and is ~ 2 MDa in size (Wang et al., 2001).

Cdk8, a cyclin-dependent kinase and component of the CDK Mediator complex, is associated with transcriptional regulation from yeast to mammals (Manning et al. 2002; Malumbres et al. 2009). Within the Mediator complex, Cdk8 is part of the CDK module which is composed of Cdk8, Cyclin C, Med12 and Med13 (Borggrefe et al., 2002; Galbraith et al., 2010) (**Figure 3**). Cdk8 phosphorylates several targets, including RNAP II and transcriptional factors (Borggrefe and Davis, 2002; Nemet et al., 2014).

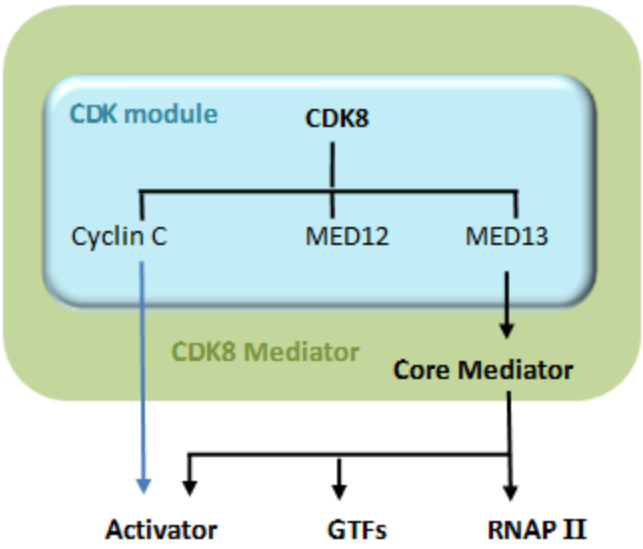


Figure 3. Cdk8 connections map. CDK8 directly interacts with Cyclin C, Med12 and Med13 which form CDK module that connects to the core Mediator through MED13. Through the core mediator, the CDK module interacts with RNAP II and multiple GTFs. The CDK module targets activators directly or through the core mediator.

The functional significance of these Cdk8-mediated phosphorylation events differ depending on the target protein. Studies revealed that Cdk8 responds to numerous intracellular signaling pathways and plays both positive and negative roles in transcriptional regulation (Hengartner et al., 1998; Nemet et al., 2014). For example, Cdk8-dependent phosphorylation of Gal4 is essential for activation of galactose-inducible genes in yeast (Hirst et al., 1999), while in mammals, Cdk8 positively regulates the p53 pathway, the serum response network, and Sip4-dependent transcription (Vincent et al. 2001; Donner et al. 2007; Donner et al. 2010). Furthermore, Cdk8 dependent phosphorylation of histone H3 subsequently stimulates H3K14 acetylation and transcription (Meyer et al., 2008). However, several mechanisms of negative regulation of transcription by Cdk8 are also known. For example, Cdk8 phosphorylates the CTD of RNAP II and prevents the interaction of RNAP II with the core Mediator complex, thereby repressing transcription (Hengartner et al. 1998; Borggrefe & Davis 2002). Additionally, Cdk8-dependent phosphorylation of Cdk7 and Med13 is involved in the inhibition of transcription (Egly and Coin, 2011). Besides transcriptional activation and repression, Cdk8 is also involved in protein turnover. For example, the stability and activity of transcriptional activators, Gcn4 and Phd1, are regulated by Cdk8 dependent phosphorylation, which ultimately signals their rapid degradation (Chi et al., 2001; Raithatha et al., 2012; Rosonina et al., 2012;).

1.3 Transcriptional Activators:

Transcriptional activators are generally sequence-specific DNA binding proteins responsible for the recruitment of multiple complexes that comprise the transcriptional machinery, to the promoter in order to turn on gene expression. Most eukaryotic gene expression is highly regulated by

activators during development or in response to changes in the cellular environment (Ptashne, 1988). Malfunctioning transcriptional activators are associated with a significant percentage of human diseases, including cancers. For example, more than 50% of cancer related cases are associated with mutations in the transcriptional activator p53 (Hainaut and Hollstein, 2000).

In general, activators have a modular structure, typically comprising a DNA-binding domain (DBD) and one or more activation domains (AD). Normally, the DBDs consist of a wide variety of structural folds including zinc fingers, zinc clusters, leucine zippers and the helix-turn-helix motif that interact with DNA with high specificity (Marmorstein et al., 1992; Luscombe et al., 2000). DBDs are responsible for binding to their cognate DNA sequence in the promoter of target genes. In contrast, since ADs do not share recognizable motifs or structure among eukaryotes, they are often classified depending on their overall amino acid composition: as Pro-rich, Gln/Asn-rich, or acid rich ADs (Hoy et al., 1993; Gerber et al., 1994; Luscombe et al., 2000). Activation domains usually bind to coactivators which initiates a cascade of events including targeted chromatin remodeling and stimulation of both the PIC formation and transcription elongation that leads to productive transcription (Kobayashi et al., 1995; Thomas and Chiang, 2015) (**Figure 4**). The function of most ADs is conserved among eukaryotes, even though DNA-binding sequences and some key activator targets, such as co-activators, are not. In addition, ADs are regulated by post-translational modifications such as ubiquitination, acetylation, phosphorylation, sumoylation, methylation and glycosylation which further regulate the timing of transcriptional activators on target gene promoters (Chi et al., 2001; Lipford et al., 2005).

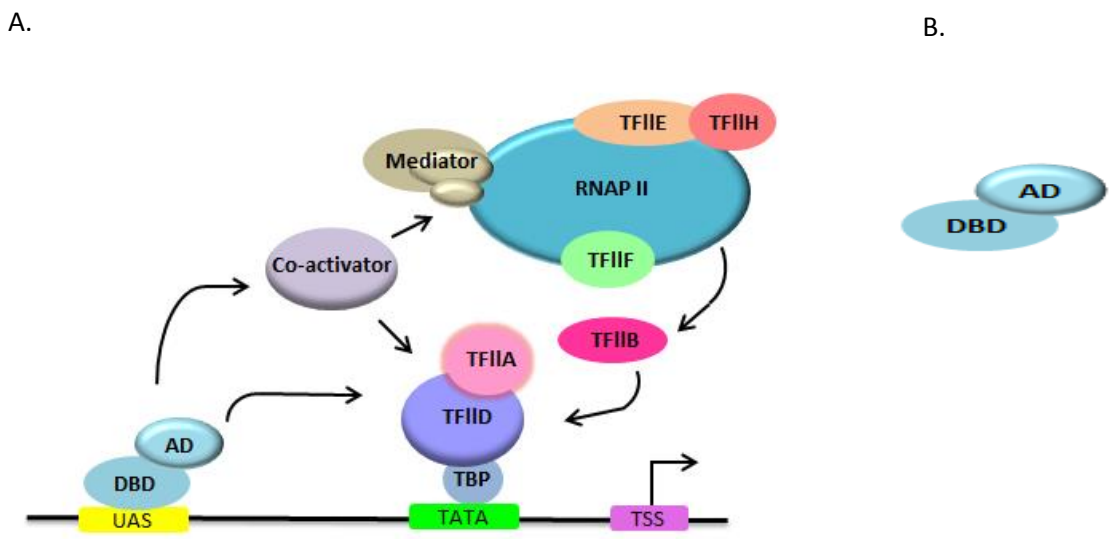


Figure 4. General model of Activator-Mediated Stimulation of PIC Assembly. ***(A)*** Transcriptional activators recruit the transcriptional machinery to a target gene promoter. ***(B)*** An activator is usually composed of at least two modular domains, an activation domain (AD) and a DNA-binding domain (DBD).

Generally, strong transcriptional activators are very unstable proteins due to ubiquitin (Ub)-mediated proteolysis through the 26S proteasome. For example, the yeast transcription factors Gcn4, Gal4 and Ino2/4 and the mammalian transcription factors p53, Jun, Myc and Fos are all short-lived proteins that are regulated by Ub-mediated proteolysis (Chowdary et al., 1994; Treier et al., 1994; Salghetti et al., 1999; Lipford et al., 2005). Ub-mediated proteolysis destroys its targets with extreme precision since destruction by the 26S proteasome generally depends on recognition of polyubiquitination of target proteins (Kornitzer et al., 1994; Salghetti et al., 2000). However, studies have shown that in the majority of cases, interactions of the activation domains of activators with the basal transcriptional machinery are necessary for their destruction. Studies also revealed

that numerous transcriptional activators are destroyed because of their ability to initiate transcription (Salghetti et al., 2000). This is thought to be important for allowing transcription factor turnover on promoters, which allows gene activation levels to reflect transcription factor abundance (Salghetti et al., 2000). However, not all activation domains are capable of being targeted by Ub-mediated proteolysis. For example, activation domains that are rich in Pro or Gln residues do not signal protein turnover, whereas acidic residue-rich activation domains often do signal Ub-mediated destruction (Rogers et al., 1986; Rechsteiner, 1988; Salghetti et al., 2000).

1.4 Sumoylation:

Sumoylation is a conserved and reversible post-translational modification that has been implicated in processes ranging from transcriptional regulation to DNA damage repair to the immune response (Zhao, 2007; Cubeñas-Potts and Matunis, 2013). Similar to ubiquitination, protein sumoylation involves the covalent and reversible attachment of the small ubiquitin-like modifier (SUMO) peptide to specific Lys residues, usually determined by a consensus sumoylation motif, ΨKxE (where Ψ is a large hydrophobic residue, and x is any amino acid), on target proteins (Rodriguez et al., 2001; Geiss-Friedlander and Melchior, 2007).

SUMO peptides are small (~12 kDa) and share a similar three-dimensional structure with ubiquitin (Geiss-Friedlander and Melchior, 2007) (**Figure 5**). However, they share less than 20% amino acid sequence identity with ubiquitin and are different in their overall surface-charge distribution. In addition, SUMO proteins carry an unstructured stretch of 10-25 amino acids at their N-termini that is not found in ubiquitin. SUMO proteins are conserved in all eukaryotes; budding yeast express a single SUMO isoform, encoded by the essential *SMT3* gene, whereas three main isoforms are

found in mammalian cells, SUMO-1, SUMO-2 and SUMO-3 (Geiss-Friedlander and Melchior, 2007; Meluh and Koshland, 1995). SUMO -2 and SUMO-3 are almost 95% identical and are often collectively referred to as SUMO-2/3, whereas SUMO-1 is approximately 48% identical to SUMO-2 and 46% identical to SUMO-3 (Miteva et al., 2000; Saitoh, 2000). All three main mammalian SUMO isoforms are about 45% identical to the yeast Smt3 protein (Wang and Dasso, 2009). SUMO-1 does not form polysumoylated chains whereas the mammalian SUMO-2/3 and yeast Smt3 do (Tatham et al., 2001).

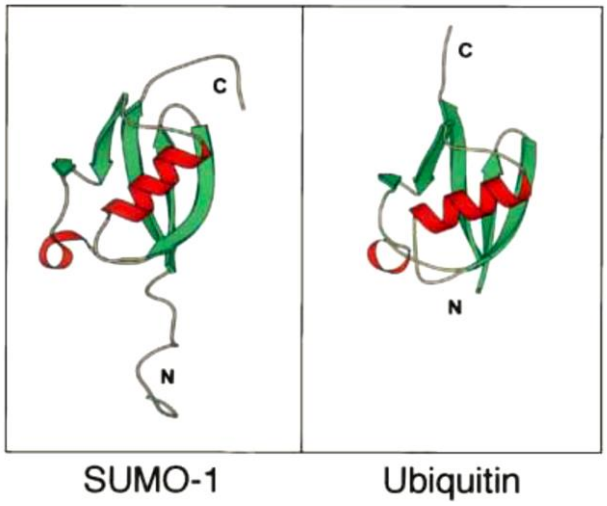


Figure 5: **Structural comparison of Human SUMO-1 and Ubiquitin proteins.** Ribbon diagram of the 3D structural similarity of SUMO-1 (left; (Bayer et al., 1998)) and Ubiquitin (right; (Vijay-Kumar et al., 1987)). Secondary structure elements, such as sheets (in green) and helices (in red) are presented. Unlike ubiquitin, the SUMO protein has an N-terminal extension.

Similar to ubiquitination, the sumoylation pathway involves a cascade of enzymatic activities involving E1-activating enzymes, a sole E2-conjugating enzyme, and in some cases, an E3-ligating

enzyme (Miteva et al., 2000) (**Table 1**). Generally, SUMO is synthesized as an inactive molecule. In the first step, a SUMO specific protease (Ubiquitin-like specific protease (Ulp) in yeast or SENtrin-specific protease (SENP) in humans) cleaves the C-terminus of the nascent SUMO peptide to expose a di-Gly motif at its C-terminus for conjugation (**Figure 6**) (Mukhopadhyay and Dasso, 2007). This step converts the precursor form of SUMO polypeptide to a mature form, which is then activated in an ATP-dependent reaction in which its C-terminal Gly residue forms a thioester conjugate with a Cys residue in the active site of the heterodimeric SUMO activating enzyme E1 (Aos1/Uba2in yeast and Sae1/Sae2 in mammals) (Johnson et al., 1997; Lois and Lima, 2005). Through an intermolecular thiol-transfer reaction, the activated SUMO is subsequently transferred to the E2 conjugating enzyme, Ubc9. From there, SUMO is conjugated to a specific Lys residue on a substrate protein via an isopeptide bond (Bernier-Villamor et al., 2002). Several SUMO E3 ligases, such as Siz1, Siz2 and Mms21 in yeast and several in mammalian cells, have been shown to stimulate the transfer of SUMO from Ubc9 to specific substrates (Johnson and Gupta, 2001; Zhao and Blobel, 2005).

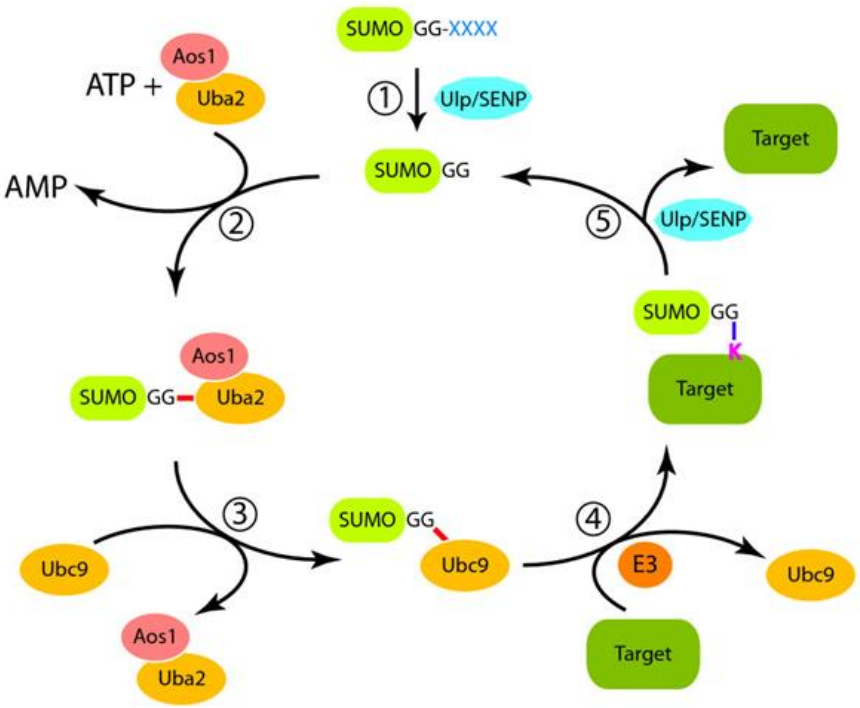


Figure 6: The Sumoylation pathway. Immature SUMO undergoes maturation through cleavage by a SUMO-specific protease to disclose its C-terminal Gly-Gly motif (Step 1). The mature SUMO protein is then activated by the E1 heterodimer in an ATP-dependent reaction (Step 2). The activated SUMO is then transferred to an E2 enzyme, UBC9 (Step 3). With the help of E3 ligases, Ubc9 then transfers the SUMO peptide to a specific Lys residue of the target protein (Step 4). Also, SUMO modifications can be removed from conjugated species by the action of SUMO proteases (Step 5).

(Adapted from Dasso, 2008)

Table 1**:** SUMO proteins and known enzymes of the sumoylation pathway in yeast and mammals

Proteins /Enzymes	Yeast *(S. cerevisiae)*	Mammals *(H. sapiens)*
SUMO	Smt3	SUMO1, SUMO2 and SUMO3
SUMO E1 (activating enzyme)	Aos1, Uba2	Sae1, Sae2
SUMO E2 (conjugating enzyme)	Ubc9	Ubc9
SUMO E3 (ligating enzyme)	Siz1, Siz2, Mms21 and Zip3	PIAS1, PIAS3, PIASxα, PIASxβ, PIASy, RanBP2, TOPORS, PC2, HDAC4 and Mms21
SUMO protease	Ulp1, Ulp2	SENP 1, SENP 2, SENP 3, SENP 5, SENP 6 and SENP 7.

SUMO modifications can be removed from substrates by SUMO-specific proteases, which have isopeptidase activity (Mukhopadhyay and Dasso, 2007). Several SUMO-specific proteases have been identified, including Ulp1 and Ulp2 in yeast, and the SENP family in humans (Li and Hochstrasser, 1999; Yeh et al., 2000). Therefore, sumoylation is a highly dynamic process in which its steady-state levels are tightly regulated by the complex interplay of the SUMO E1, E2, E3, and proteolytic enzymes. However, dysregulation of sumoylation is associated with a number of different diseases, including Huntington's, Alzheimer's, Parkinson's and various cancers (Steffan, 2004; Martin et al., 2007). For example, general sumoylation levels are significantly elevated in metastatic breast cancer cells, and both Ubc9 and SUMO protease expression levels are altered in a large number of tumors (Mo and Moschos, 2005; Cheng et al., 2006; Subramonian et al., 2014).

The consequences of protein sumoylation are diverse, and unlike ubiquitination, sumoylation has not been found to directly signal protein degradation. In some cases, SUMO modifications compete with ubiquitin for a particular Lys acceptor site, thereby preventing subsequent ubiquitination and eventual degradation by the proteasome (Desterro et al., 1998; Hoege et al., 2002). For instance, SUMO-modification of IkBa (inhibitor of nuclear factor kB alpha) blocks its ubiquitination at a specific Lys residue (K21), which eventually inhibits ubiquitin-dependent degradation by the proteasome (Desterro et al., 1998). Alternatively, SUMO-modification can lead to ubiquitination through recognition by SUMO-targeted ubiquitin E3 ligases (STUbLs), which recognize polysumoylated target proteins through their SUMO-interaction motifs (SIMs) (Prudden et al., 2007; Geoffroy and Hay, 2009). For instance, sumoylation of acute promyelocytic leukemia (PML) protein (specifically at K160 residues) triggers its recognition by the RNF4 STUbL, which then facilitates PML polyubiquitination and proteasome-dependent degradation (Lallemand-Breitenbach et al., 2008).

1.5 Sumoylation and Transcription:

Sumoylation has emerged as a fundamental regulatory cellular mechanism that targets a variety of proteins involved in an array of processes including cell cycle regulation, transcription, chromosome stability, and subcellular localization (Geiss-Friedlander and Melchior, 2007; Zhao, 2007). Although sumoylated proteins are found throughout the cell, the largest classes of SUMO targets are involved in transcription (Makhnevych et al., 2009; Cubeñas-Potts and Matunis, 2013). Fittingly, many transcriptional activators, repressors, co-regulators and GTFs have been identified as SUMO targets in both yeast and mammalian cells (Zhao, 2007; Cubeñas-Potts and Matunis, 2013; Raman et al., 2013). The effects of SUMO modification on transcription factor activity are

diverse. The sumoylation of some transcription factors, such as TCF-4 (T-cell factor-4) and Ikaros, is associated with an increase in transcription (Yamamoto et al. 2003; Gómez-del et al. 2005). However, in an overwhelming majority of cases, SUMO modification is associated with an inhibition in transcription, which can be accomplished by different SUMO-mediated mechanisms. For the transcription factor Elk-1, its sumoylation stimulates the recruitment of the histone deacetylase HDAC-2, which reduces histone acetylation and thereby inhibits transcription (Yang and Sharrocks, 2004). SUMO modifications can also inhibit transcription by regulating nuclear localization of some transcription factors, such as SATB2 and c-Myb (Morita et al., 2005). In yeast, sumoylation regulates the promoter occupancy of the transcriptional activator Gcn4 and the repressor Tup1, to reduce transcription of target genes (Rosonina et al., 2012; Ng et al., 2015).

1.6 Gcn4:

Gcn4, a yeast transcriptional activator and member of the AP-1 transcription factor family, regulates the expression of genes involved in amino acid or purine biosynthesis in response to amino acid deficiency, purine insufficiency, glucose limitation, high salinity or treatment with sulfometuron methyl (SM), which induces amino acid starvation (Struhl 1987; Yoshida et al. 1998; Goossens et al. 2001; Hinnebusch & Natarajan 2002). The Gcn4 (~32 kDa) protein is composed of 281 amino acids, containing two tandem N-terminal acidic activation domains (ADs) and a C-terminal basic leucine zipper DNA binding domain (DBD) (Hope and Struhl, 1987). Through its DBD, Gcn4 binds specifically to the consensus sequence ‘TGACTC’ of amino acid biosynthesis gene promoters, such as *ARG1*, as a homodimer (Ellenberger et al., 1992) (**Figure 7)**. Its AD then interacts with coactivator proteins thereby initiating a cascade of events leading to the formation

of the PIC and thus triggering transcription during amino acid deprivation (Ptashne & Gann 1997; Brzovic et al., 2011).

Although the Gcn4 protein plays an important role in many biological functions, its regulation in response to amino acid starvation has been studied in detail. Studies showed that under normal growth conditions, Gcn4 is an unstable protein due to its efficient proteasomal degradation mediated by phosphorylation and ubiquitination (Kornitzer et al., 1994; Lipford et al., 2005). Its half-life ranges from approximately two minutes under growth in rich medium to 10 minutes under amino acid starvation conditions (Shemer et al., 2002). In addition to phosphorylation and ubiquitination, Gcn4 is also modified by SUMO post-translational modifications (Rosonina et al., 2010).

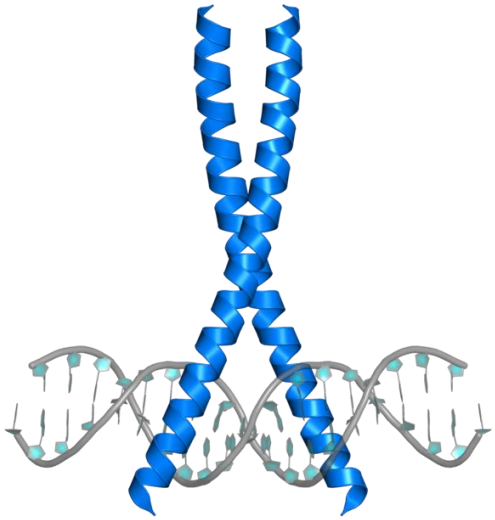


Figure 7. Structure of the Gcn4 bZIP domain bound to DNA. NMR structure of the Gcn4 bZIP domains (blue) that binds to the major groove of a specific DNA sequence (gray) as a homodimer. The dimer is formed through hydrophobic interactions between leucine residues in the two polypeptide chains (PDB ID: 1YSA).

1.7 Regulation of Gcn4:

Gcn4 is highly regulated. Two pathways are involved in Gcn4 degradation through ubiquitin-mediated proteolysis, each involving a different cyclin-dependent kinase (CDK), Pho85 or Cdk8 (Meimoun et al., 2000; Chi et al., 2001)(Irniger and Braus, 2003). Both kinases phosphorylate Gcn4 at one or more potential CDK phosphorylation sites (S17, T61, T105, T165 and S218) (Meimoun et al., 2000; Chi et al., 2001; Irniger and Braus, 2003) (**Figure 8**). In the presence of sufficient amino acids, Pho85 binds the cyclin Pcl5 and phosphorylates Gcn4 regardless of whether it is promoter-bound or unbound, whereas Cdk8, a component of the Mediator complex associated with RNAP II, phosphorylates promoter-bound Gcn4 independently of amino acid availability (**Figure 9)** (Meimoun et al. 2000; Chi et al. 2001).

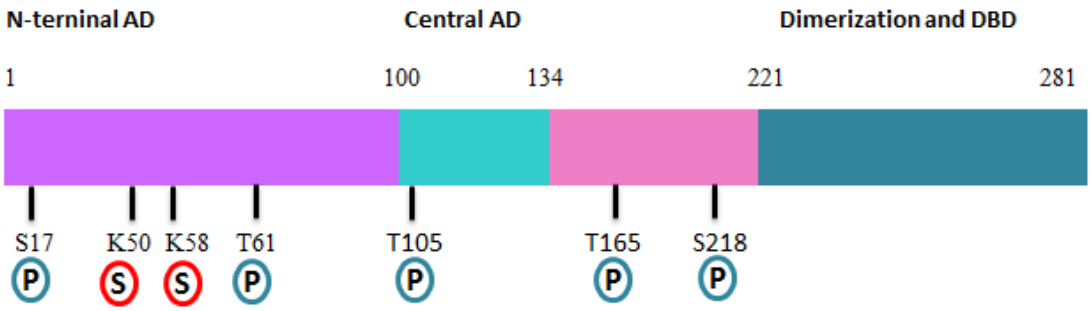


Figure 8: A schematic diagram of Gcn4 structure. Gcn4 is composed of three modular domains, two activation domains (ADs) and a bZIP dimerization and DNA-binding domain (DBD). It possesses five consensus CDK phosphorylation sites, (S17, T61, T105, T165 and S218) and two sumoylation sites (K 50, 58) that are indicated by encircled P and S respectively.

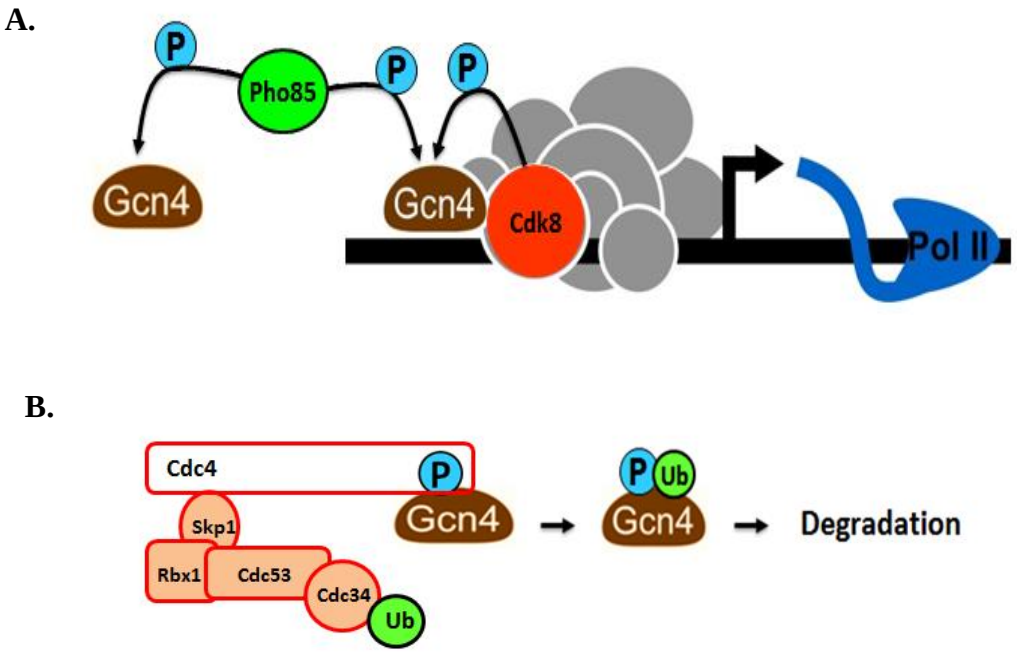


Figure 9: Regulation of promoter-bound or unbound Gcn4 by CDKs: Pho85 and Cdk8. ***(A)*** Both kinases target Gcn4 for phosphorylation, and ***(B)*** Phosphorylated Gcn4 is then specifically recognized by Cdc4, which further recruits Skp1, Rbx1, Cdc53 and the Ub-conjugating enzyme Cdc34. Eventually, Cdc34 transfers Ub to Lys residues in Gcn4 and triggers Ub-mediated proteasomal degradation.

In both pathways, phosphorylation targets Gcn4 for ubiquitination and subsequent degradation through the 26S proteasome (Lipford et al., 2005). The ubiquitination of Gcn4 is mediated by the specific multi-subunit ubiquitin ligase complex SCF, comprising Skp1, Cdc53 (or cullin) and F-box-containing Cdc4, with the additional association of the ubiquitin-conjugating enzyme Cdc34 (Meimoun et al. 2000; Chi et al. 2001). Cdc4 recognizes phosphorylated Gcn4 and recruits it into

the SCFCdc4 Ub– ligase complex, whereas Cdc34 transfers Ub to Lys residues in Gcn4 (Meimoun et al., 2000).

In addition to phosphorylation and ubiquitination, Gcn4 becomes sumoylated on two Lys residues **(K50, 58)** specifically after binding to target gene promoters (Rosonina et al., 2012). ChIP experiments performed in yeast strains expressing a mutant, non-sumoylatable form of Gcn4 showed that blocking Gcn4 sumoylation resulted in higher occupancy levels on target promoters suggesting that SUMO modifications of Gcn4 are important for its efficient clearance from target promoters (Rosonina et al., 2012).

1.8 Objectives:

We hypothesize that SUMO modification of Gcn4 takes place after it binds DNA, which then stimulates the removal of the activator from induced promoters by facilitating its Cdk8 and ubiquitin-mediated degradation after it has acted in transcriptional activation. To address this model, this project aimed to identify the determinants of Gcn4 sumoylation. Specifically, the project (1) examines how promoter-associated sumoylation of Gcn4 is coordinated with its phosphorylation and ubiquitination as part of a regulatory cascade of PTMs, and (2) provides further evidence that sumoylation of Gcn4 acts to enhance its removal from target promoters, after it has acted in transcriptional activation. To accomplish this, we determined how Gcn4 phosphorylation and ubiquitination are affected by blocking its sumoylation, and vice versa. Additionally, we examined the effect of blocking ongoing transcription on Gcn4 sumoylation to determine whether Gcn4 sumoylation is dependent on its ability to activate transcription. Finally, we generated a SUMO-Gcn4 fusion protein and examined the effects “permanently sumoylating” Gcn4 on its clearance from target promoters.

The results of this project shed light on the detailed mechanism by which Gcn4 is regulated on target promoters through a cascade of interdependent PTMs.

Chapter 2: Materials and Methods

2.1 Yeast Strains and Plasmids:

Yeast (*Saccharomyces cerevisiae*) strains used in this study are listed in **Appendix Table 1**. Strains were derived from either the S288C or W303 background strains, and all chromosomally Gcn4-6HA-tagged yeast strains were generated by homologous recombination as previously described (Rosonina et al., 2010). The presence of the 6HA-tagged was then confirmed by PCR and Western blot analysis with anti-HA antibody.

The pGcn4-6HA plasmid was generated by amplifying Gcn4 with 1 kb each of flanking upstream and downstream genomic sequence and subsequently cloning the PCR product into the *URA3*-marked CEN vector pRS316. A site-directed, PCR-based mutagenesis approach was employed to generate the mutagenic plasmids for Gcn4 activation mutant strains (Primers are listed in **Appendix Table 2**).

2.2 Yeast Media and Growth Conditions:

Yeast cultures (10 -25 mL) were grown at 30°C (unless otherwise specified) in either Synthetic Complete (SC; 0.17% YNB, 0.5% ammonium sulfate, and 2% glucose) media or SC dropout media based on the auxotrophic markers of the plasmids or strains used in experiments, to an optical density A_{595} (O.D.) of 0.6-0.8, then induced for 20 min (otherwise indicated) with SM (Sigma) at a final concentration of 0.5ug/ml.

For proteasome inhibition, the method of Liu et al was used (Liu et al., 2007). Strains were grown overnight in special SC medium (containing L-proline 0.1%) as sole nitrogen source instead of

ammonium sulfate). In the morning, samples were diluted to an A_{590} (O.D.) of 0.5 in the same medium plus SDS to a final concentration of 0.03%, and grown for three hours. Samples were then treated with the proteasome inhibitor MG132 (75μM) for 45 min followed by 20 min in SM (0.5 μg/mL) to induce Gcn4 expression.

2.3 Spot Assay:

Cells were grown in appropriate liquid medium overnight, and densities determined on the following morning. Approximately 10,000 cells of each strain were spotted side-by-side in the first position, and serial five-fold dilutions were spotted in the adjacent positions, on appropriate solid medium plates. All plates were incubated at 30˚C (unless otherwise stated) and images were recorded daily for up to three days.

2.4 Immunoprecipitation (IP):

For IP, all manipulations were performed at 4°C. Exponentially growing cells (yeast cultures were grown and treated as indicated for **Yeast Media and Growth Conditions**) were harvested by centrifugation and resuspended in IP buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl) plus 0.1 % Nonidet P-40 (NP40), 1X yeast protease inhibitor cocktail (BioShop), 0.1 mM DTT and 2.5mg/ml N-ethylmaleimide), followed by glass bead vortex homogenization for 30 min. Then the lysate was centrifuged twice at 3000*g* for 5 min, and 50μl of the lysate was 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 3 min; the remainder was incubated with washed protein G agarose beads (BioShop) and 1 μg of either rabbit or mouse

polyclonal anti-HA epitope tag primary antibody (Cedarlane) overnight at 4˚C . The next day, IPs were washed three times with ice-cold IP buffer plus 0.1% NP40, then samples were boiled in SDS sample buffer for 3 min at ~95 ^{0}C to release protein from the beads. For phosphatase treatment, lysates were incubated with 400 U lambda protein phosphatase (NEB) per 50 µL lysate for 15 min at 30°C prior to IP

2.5 Antibodies:

The antibodies for immunoblotting and immunoprecipitation were rabbit and mouse anit-HA (New England Biolabs), Smt3 (Santa Cruz), FK1/Ub (Cayman Chemical), Phospho-Ser/Thr/Tyr (BioLynx). As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-mouse IgG and anti-mouse IgM were used.

2.6 Western Blot:

Equal amounts of each sample were resolved on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes (BioShop) followed by incubation with blocking buffer (5% milk in 1x PBST [0.05% Tween-20 in 1% PBS (Fisher Scientific)] or TBST [(20mM Tris pH 7.5, 150mM NaCl, 0.05% tween -20)]) for 30 min at room temperature. The membranes were then probed with appropriate antibody [HA (1:3000; for detection of HA tagged Gcn4), Smt3 (1:3000; for detection of sumoylation), or FK1 (1:1000; for polyubiquitination chain detection)], for overnight at 4˚C. Following incubation with the primary antibodies, the membranes were washed 3 times in either 1x PBST or TBST for 5 min at room temperature. The blots were then incubated with the appropriate HRP conjugated secondary antibody (1:5000; Thermo Fisher Scientific) for 30 min at

room temperature. The blots were then washed 3 times again and the detection was done by chemiluminescence (ECL, Bio-Rad) using autoradiography or a MicroChemi chemiluminescence imager (DNR).

2.7 Chromatin immunoprecipitation (ChIP):

50 mL of SC medium (or selective, as appropriate) was inoculated with appropriate strain at 30˚C to an optical density A_{595} (O.D.) of 0.7-0.8. To induce Gcn4 expression, cultures were treated with SM for an appropriate time at a final concentration of 0.5ug/ml, followed by cross-linking with 1.1% formaldehyde for 20 min, before quenching with 450 mM of glycine for 5 min. Samples were pelleted by centrifugation and washed with ice-cold TBS (20mM Tris-HCl, pH 7.5 and 150mM NaCl), then in ChIP buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS). Pelleted samples were then resuspended in ChIP buffer and subjected to bead beating with glass beads followed by sonication to shear chromatin to fragments of ~500 bp length. Samples were then centrifuged for 5 min, and additional NaCl was added to the isolated supernatants to a final concentration of 212.5 mM. Small amounts of supernatants (40µL) were stored at -20˚C as Input samples, and the rest of the salt-adjusted supernatants were incubated for overnight at 4˚C with washed protein G agarose beads in addition to 1 µg of the appropriate antibody for IP. The following day, beads were then washed first in ChIP buffer with 275mM NaCl, then in ChIP buffer with 500mM NaCl, followed by an additional washing buffer (10mM Tris-HCl, pH 8, 0.25M LiCl, 1mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate) and finally with Tris-EDTA buffer (10mM Tris-HCl, pH 8 and 1mM EDTA). [Note: 4 min incubation at room temperature was followed between each washed]. Beads were then incubated with ChIP Elution buffer (50mM Tris-HCl, pH 7.5, 10mM EDTA and 1%

SDS) for 10min at 65 ^{0}C. Samples were then centrifuged at 8000 *g* and supernatants were treated with proteinase K (BioShop) at 42 ^{0}C for 1h, and then transferred to 65 ^{0}C for 4h to overnight to reverse cross-links. The following day, LiCl was added to each sample to a final concentration of 0.4M and DNA was recovered by phenol–chloroform extraction and ethanol precipitation. Using the same experimental condition, each experiment was performed at least three times and the average of quantitative PCR (qPCR) analyses are presented using the percent input method, with standard deviations shown as error bars.

For quantification, the signals obtained from the input samples were subtracted from the IPed samples, and referred to as normalized ChIP to input. Finally, the "Percent Input" value for each sample is calculated as follows: Percent Input = 100*2^(-ΔCt $^{[\text{normalized ChIP to input}]}$). Primer sequences used for qPCR are listed in Appendix **Table 3.**

2.8 RNA isolation and Reverse Transcription-PCR (RT-PCR):

To extract RNA, 10-mL yeast cultures were grown under appropriate conditions as described above. Cultures were treated with SM for 20 min (unless otherwise specified) at a final concentration of 0.5 µg/mL to induce Gcn4 expression. Samples were pelleted by centrifugation at 3000 *g* for 3 min at 4°C and washed twice with ice-cold AE buffer (50 mM sodium acetate, pH 5.2 and 10 mM EDTA, pH 8.0). Pelleted samples were then re-suspended in 400 µL of ice-cold AE buffer and 40 µL of 10% SDS was added followed by the addition of 440 µL of phenol (pH 5.2). Samples were then chilled in a dry ice/ethanol bath (where dry ice was crushed to powder and 95% ethanol was added to achieve a thick slurry mixture) for 5 min and then transferred to a 65°C water bath for 5 min followed by vortex mixing for 30s. After repeating the

freeze/thaw/vortex cycle, samples were centrifuged at 16,000 *g* for 7 min and aqueous layers were transferred to fresh micro-centrifuge tubes for standard phenol-chloroform extraction and ethanol precipitation of RNA.

For reverse-transcription, 12 µg samples of RNA were treated with DNase I (New England Biolabs) and approximately 1 µg of DNA-free-RNA was used for cDNA synthesis using the Super Script II reverse transcriptase reaction (Invitrogen) according to the manufacturer's instructions. For qualitative analysis, one-tenth of the transcripts were used for 24 cycles of standard PCR analysis and products were resolved on 2% agarose gels.

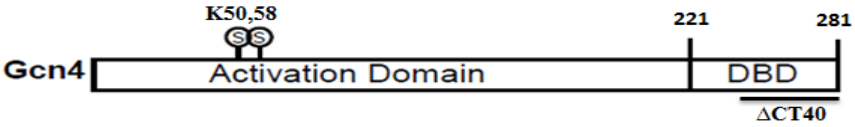
For quantification, qPCR was performed using SYBR Green mix (Froggabio) according to the manufacturer's instructions. Transcripts were normalized to 25S rRNA and calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All experiments were performed at least three times and the average values are presented with standard deviations shown as error bars. In addition, a two-tailed Student's t-test was applied with *P*-values less than 0.05 (where statistical comparisons were performed) indicated as asterisks between relevant samples. Primer sequences are listed in Appendix **Table 3**.

Chapter 3: Results

3.1 Effect of Gcn4 DNA-binding ability on its sumoylation:

Gcn4, a transcriptional activator, binds specifically to the consensus sequence 'TGACTC' of amino acid biosynthesis gene promoters as a homodimer (Ellenberger et al. 1992). It was previously demonstrated that Gcn4 is sumoylated at two specific Lys residues, K50 and K58, and that this modification depends on its ability to bind to target gene promoters (Rosonina et al., 2012). Therefore, we wished to further study the significance of DNA-binding on Gcn4 sumoylation. For this, a DNA binding domain (DBD) mutant Gcn4 strain was used which lacks the 40 C-terminal amino acids that are required for binding to DNA (Gcn4-ΔCT40) (Hope and Struhl, 1985; Rosonina et al., 2012). This Gcn4-ΔCT strain was then examined with wild type Gcn4 (Gcn4-WT) and SUMO mutant Gcn4 strain (Gcn4-K50,58R), in which two SUMO targeted Lys residues, K50 and K58, were substituted with Arg (Rosonina et al., 2012). Expression of Gcn4, fused with a C-terminal 6xHA epitope tag in these strains can be induced by treating with sulfometuron-methyl (SM), which triggers amino acid starvation in the cells (Rosonina et al., 2012). The tagged protein is then immunoprecipitated (IPed) with an HA antibody from lysates prepared in the presence of the SUMO protease inhibitor N-ethylmaleimide (NEM), followed by immunoblot analysis. Consistent with previous findings, we observed a band around ~60kDa for Gnc4 although the theoretical molecular weight of the Gcn4 protein with 6xHA epitope tag is calculated to be ~ 42 kDa. In addition, we also observed two prominent sumoylated forms of Gcn4 for Gcn4-WT in both HA and SUMO immunoblots following IP, which were not observed for the Gcn4-K50,58R and Gcn4-ΔCT mutants (asterisks in **Figure 10 B**; (Rosonina et al., 2012)). This suggests that Gcn4 is sumoylated specifically after binding to DNA.

A.



B.

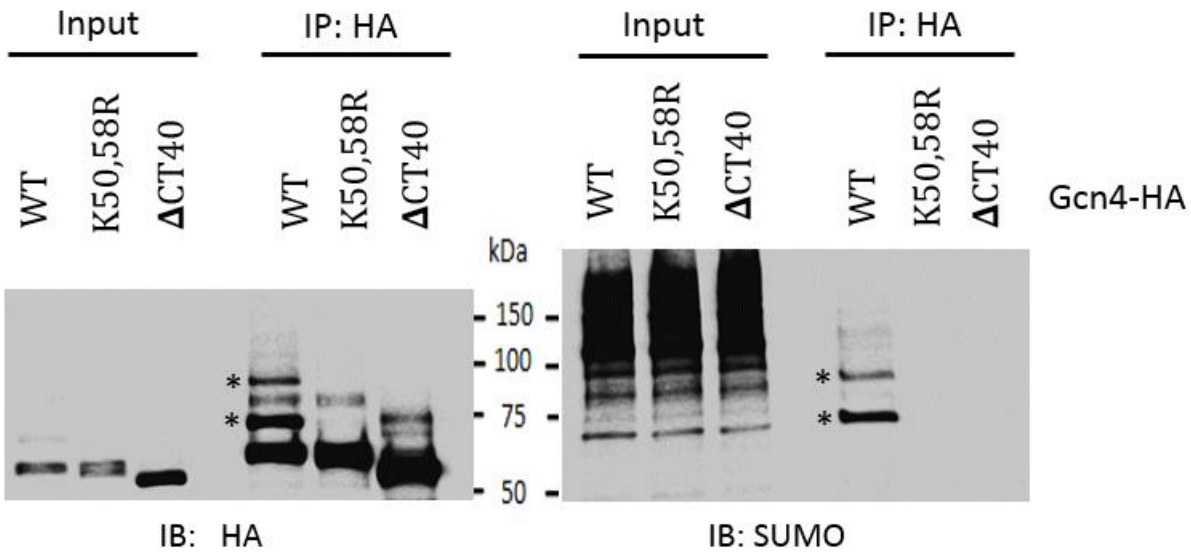


Figure 10: Effect of Gcn4 DNA-binding ability on its sumoylation. ***(A)*** The schematic diagram of Gcn4 representing probable sites of sumoylation (indicated by encircled S) and the location of the 40 C-terminal amino acids (ΔCT40) in the DBD necessary for DNA binding. As demonstrated previously, Gcn4-6HA migrates at approximately 60 kDa, and each SUMO modification adds approximately 12 kDa. ***(B)*** Gcn4-WT, Gcn4-K50,58R and Gcn4-ΔCT40 strains expressing 6xHA-tagged Gcn4 were used for HA IPs. Cells were treated with SM (0.5 µg/mL) for 20 min to induce Gcn4 expression. IPs were then analyzed by HA (1:3000) and SUMO (1:3000) immunoblots. Asterisk (*) indicates the sumoylated forms of Gcn4 visible in HA and SUMO immunoblots.

Further, to investigate whether directing Gcn4 to a heterologous UAS (upstream activator sequence), found upstream of the promoter, could also lead to its sumoylation, the HF7c yeast strain was transformed with a plasmid that express a fusion of the Gal4 DNA-binding domain (Gal4DB) with the ΔCT truncated form of Gcn4 (Gal4DB-Gcn4ΔCT40). IP-immunoblot analysis showed two prominent sumoylated forms in a SUMO IP, indicating that the fusion protein is indeed sumoylated (**Figure 11**). Although the presence of Gal4DB was necessary for sumoylation, we did not observe that Gal4DB by itself is sumoylated. Notably, when the Lys residues in the Gal4DB-Gcn4-ΔCT40, which corresponded to Gcn4 K50 and K58, were mutated (Gal4DB-Gcn4-K50,58R-ΔCT40), sumoylation of the fusion protein was ablated, suggesting these residues to be the specific sites for Gcn4 sumoylation, whenever it binds DNA (**Figure 11**). In addition, because the Gal4DB-Gcn4ΔCT40 fusion and derivatives were expressed constitutively from the *ADH1* promoter in normally growing HF7c cells without SM treatment Gcn4 sumoylation does not rely on amino acid starvation or on the addition of SM. Instead, the data indicates that DNA binding is a major prerequisite for Gcn4 sumoylation.

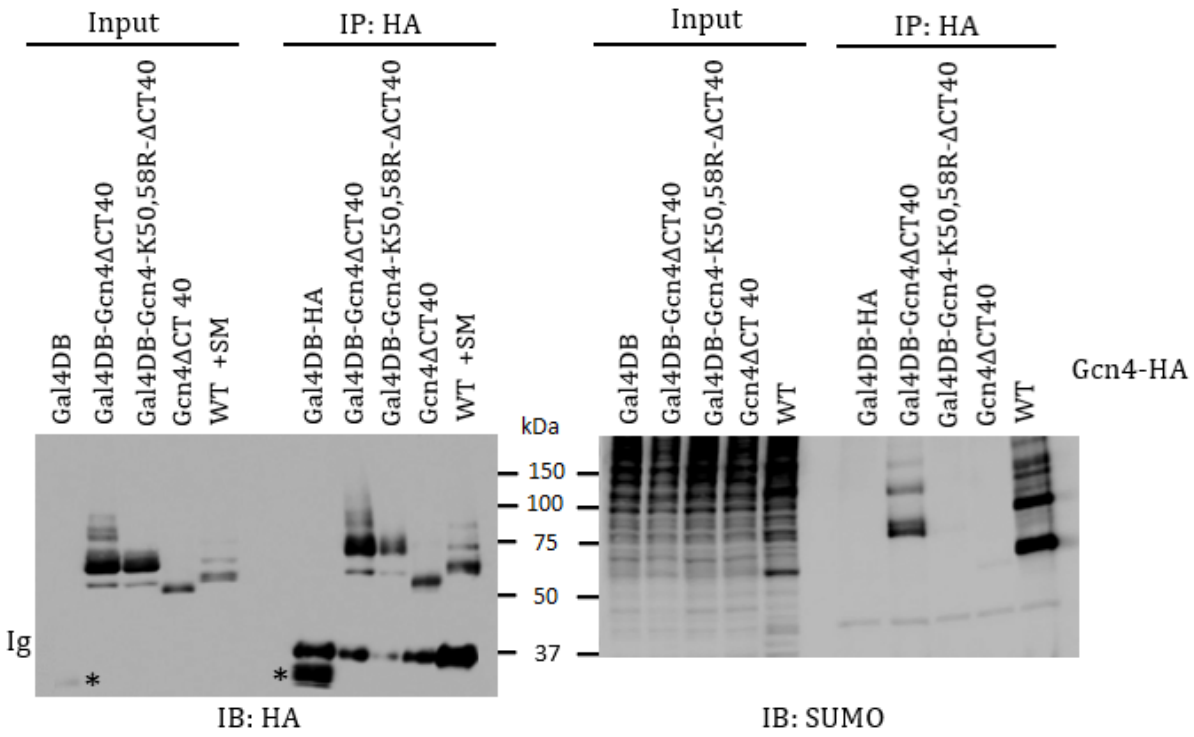


Figure 11: Effect of Gcn4 DNA-binding ability on its sumoylation. HF7c cells expressing Gal4DB and Gcn4ΔCT40 proteins as well as Gal4DB-Gcn4ΔCT40 and Gal4DB-Gcn4-K50,58R-ΔCT40 fusion proteins. Gcn4-WT strain expressed wild type Gcn4 protein. All these strains contain 6xHA epitope tags. Only Gcn4-WT strain was treated with SM (0.5 µg/mL) for 20 min to induce Gcn4 expression. Cell lysates were IPed with monoclonal anti-HA antibody. IPs were then analyzed by HA (1:3000) and SUMO (1:3000) immunoblots. Asterisk (*) indicates the Gal4DB visible on HA immunoblot.

3.2 Coordination of Gcn4 sumoylation and phosphorylation:

3.2.1 Effects of Gcn4 phosphorylation on its sumoylation

To elucidate how promoter-associated sumoylation of Gcn4 is associated with its phosphorylation by either Pho85 or Cdk8 as part of a regulatory cascade of PTMs, a phosphorylation-site mutant (Gcn4-3T2S*)* Gcn4 strain was generated by altering all five potential Cyclin dependent kinase (CDK) target Thr and Ser residues (3T2S) to Ala (Chi et al. 2001). This Gcn4-3T2S strain was then examined with Gcn4-K50,58R. When compared to a Gcn4-WT strain, both Gcn4-K50,58R and Gcn4-3T2S strains grew normally on amino acid starvation medium (contains SM) suggesting that Gcn4 sumoylation and phosphorylation are not essential to initiation of transcription of amino acid biosynthesis genes (**Appendix Figure 1)**.

We then examined the impact of impaired Gcn4 phosphorylation on its sumoylation using Gcn4-WT, Gcn4-K50,58R, and Gcn4-3T2S strains. IP-immunoblot analysis demonstrated that the phosphorylation mutant form of Gcn4 strain is subject to normal sumoylation **(Figure 12)**. This result indicates that Gcn4 phosphorylation is not necessary prior to its sumoylation.

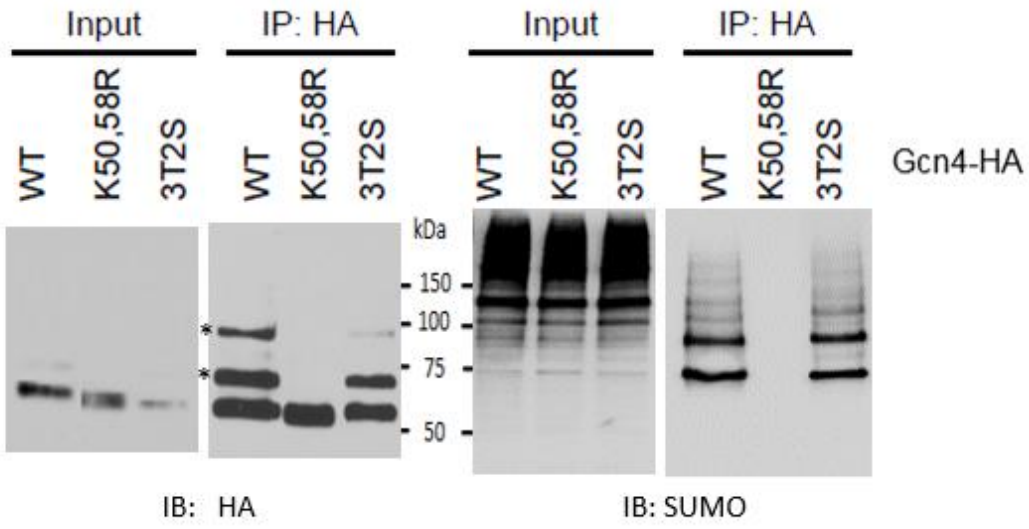


Figure 12: Effect of Gcn4 phosphorylation on its sumoylation. HA and SUMO immunoblot analysis of IPs from indicated yeast strains expressing 6xHA-tagged Gcn4. Cells were treated with SM (0.5 µg/mL) for 20 min to induce Gcn4 expression. IPs were then analyzed by HA (1:3000) and SUMO (1:3000) immunoblots. Asterisk (*) indicates the sumoylated form of Gcn4 visible in HA immunoblots.

3.2.2 Effect of Gcn4 sumoylation on its phosphorylation

In order to examine whether Gcn4 sumoylation affects its subsequent phosphorylation, we attempted to detect phosphorylated forms of IPed Gcn4 and Gcn4-K50,58R using a general phospho-Ser/Thr/Tyr antibody. However, no signal was detected, indicating that this approach would not succeed (data not shown). Instead, we used immunoblotting conditions that allowed us to resolve phosphorylated and unphosphorylated isoforms of Gcn4 on an HA immunoblot, without the need for phospho-specific antibodies. Such conditions had already been used to detect phosphorylated forms of in-vitro translated Gcn4 (Chi et al., 2001). Therefore, phosphatase analysis was performed with Gcn4-WT and Gcn4-K50,58R strains, where extracts were treated with Lambda protein phosphatase. Untreated Gcn4-WT and Gcn4-K50,58R extracts were also included as controls, and the phosphatase treated and untreated samples were analyzed side by side on an HA immunoblot. Treatment with phosphatase revealed that, sumoylated Gcn4 is also phosphorylated (compare lanes 1 and 2 in **Figure 13**), and, like Gcn4-WT, Gcn4-K50,58R is normally subject to different levels of phosphorylation (compare doublets in lanes 1 and 3 with lanes 2 and 4, respectively). However, the Gcn4-K50,58R sample showed an elevated abundance of the least phosphorylated form compared to Gcn4-WT (lowest band), suggesting that sumoylation can stimulate some level of Gcn4 phosphorylation (e.g. the increased abundance in this form might correspond to Gcn4 that would otherwise be sumoylated and phosphorylated; compare lanes 1 and 3 in **Figure 13)**. Similar results were observed when the analysis was performed in a strain lacking Pho85 (*pho85*Δ *gcn4- 6xHA* and *pho85*Δ *gcn4-6xHA-K50,58R),*which suggests that Cdk8 is responsible for generating the specific phosphorylated forms of Gcn4 detected in this assay (lanes 5-8 in **Figure 13**). Together, this analysis suggests that,

although sumoylation is not a requirement for Gcn4 phosphorylation, blocking Gcn4 sumoylation does alter its overall phosphorylation.

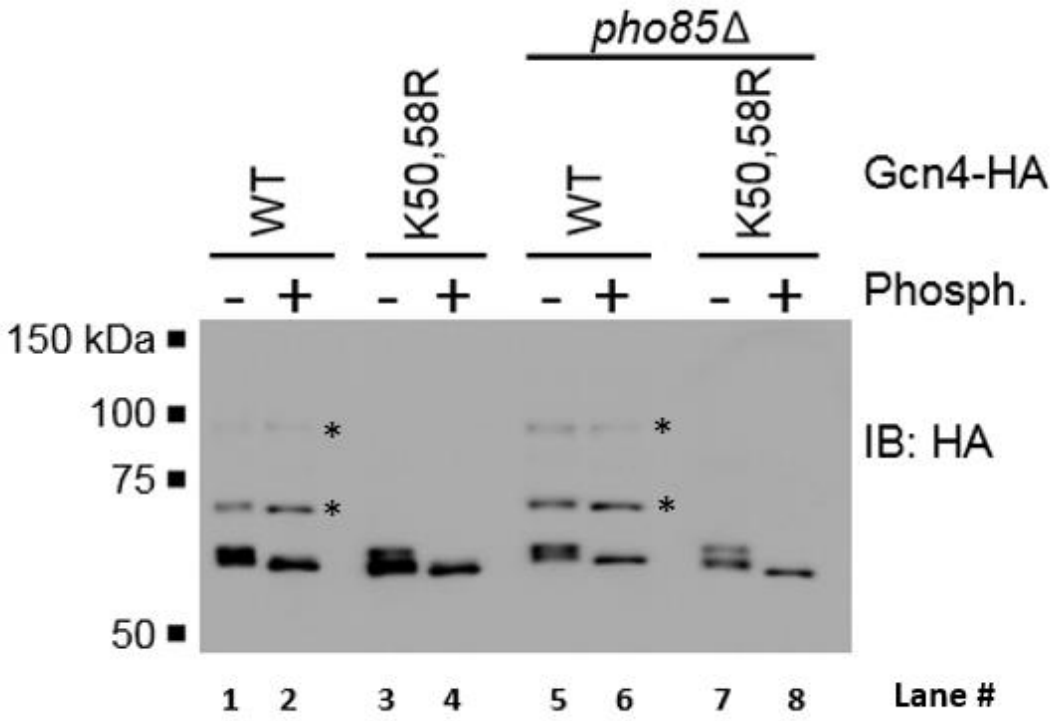


Figure 13: Effect of Gcn4 sumoylation on its phosphorylation. HA immunoblot analysis of extracts from Gcn4-WT and Gcn4-K50,58R strains expressing HA-tagged. Cells were treated with SM (0.5 µg/mL for 20 min) for Gcn4 induction. Indicated lysates were subsequently treated with Lambda phosphatase at 30 ^{0}C for 15 min, and were then analyzed by HA (1:3000) immunoblot.

3.3 Coordination of Gcn4 sumoylation and ubiquitination:

3.3.1 Effects of Gcn4 ubiquitination on its sumoylation

To study how promoter-associated sumoylation of Gcn4 is coordinated with its ubiquitination, we used a yeast strain in which Gcn4 ubiquitination is blocked by mutation of the Gcn4 ubiquitin conjugating enzyme Cdc34 (*cdc34-2*) (**Appendix Figure 2A**). This strain was previously used to demonstrate the role of Gcn4 ubiquitination on its degradation and transcriptional activity (Meimoun et al., 2000). The *cdc34-2* strain displayed moderate growth defects when grown under amino acid starvation conditions, although normal growth was observed under normal conditions **(Appendix Figure 2B)**. Gcn4-WT, Gcn4-K50,58R, and *cdc34-2* strains were analyzed by IP followed by immunoblotting, which demonstrated that Gcn4 that is not ubiquitinated is subject to virtually normal SUMO modification. Therefore, this result indicates that Gcn4 sumoylation is not dependent on its prior ubiquitination **(Figure 14)**.

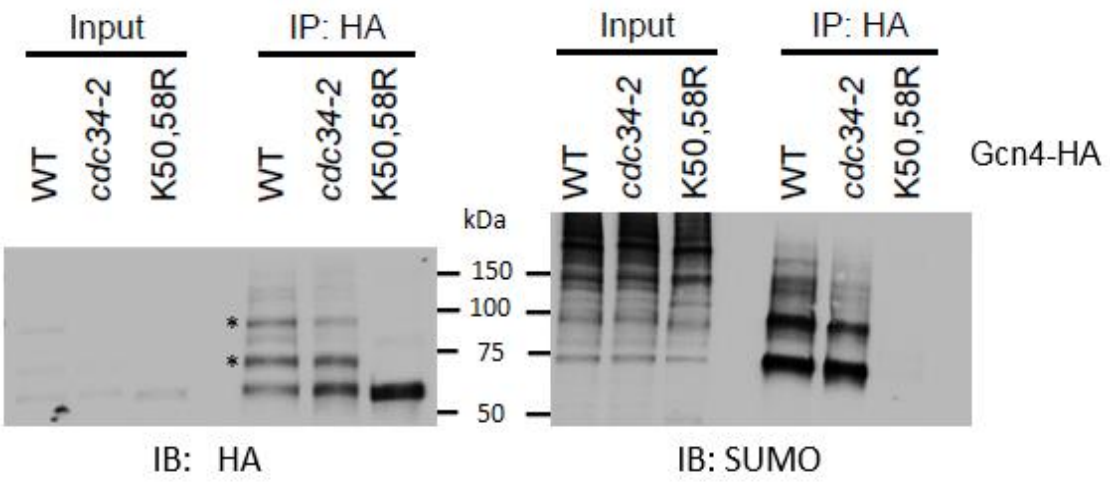


Figure 14: Effect of Gcn4 ubiquitination on its sumoylation. Analysis of HA IPs obtained from SM-induced cells expressing HA tagged Gcn4-WT, *cdc34-2* and Gcn4-K50,58R strains. IPs were analyzed by HA (1:3000) and SUMO (1:3000) immunoblots.

3.3.2 Effect of Gcn4 sumoylation on its ubiquitination

In order to study the effect of impaired Gcn4 sumoylation on its ubiquitination, Gcn4-WT, Gcn4-K50,58R and Gcn4-3T2S strains were treated with MG132, to inhibit proteolysis by the 26S proteasome, followed by SM to induce Gcn4 expression. As expected, immunoblot analysis with a ubiquitin antibody (FK1) did not display ubiquitinated forms of Gcn4 in the Gcn4-3T2S strain because Gcn4 phosphorylation is required for its ubiquitination (Lipford et al., 2005). However, a smear of ubiquitinated forms of Gcn4 was observed for both Gcn4-WT and Gcn4-K50,58R strains (**Figure 15; *right***). This result indicates that the bulk of Gcn4 ubiquitination is not dependent on its prior sumoylation.

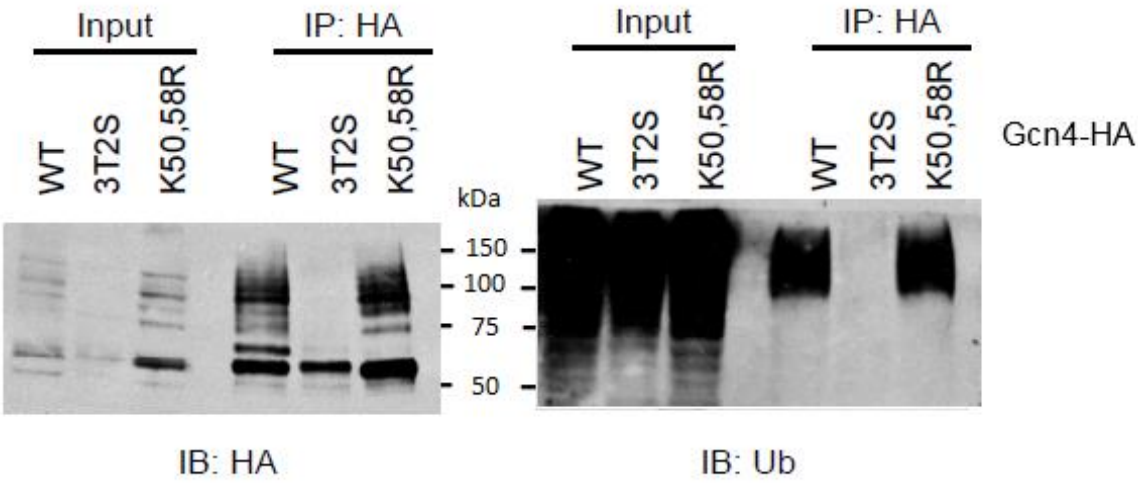


Figure 15: Effect of Gcn4 sumoylation on its ubiquitination. HA (1:3000) and Ub (1:1000) immunoblots analysis of IPs from Gcn4-WT, Gcn4-3T2S and Gcn4-K50,58R strains expressing HA-tagged treated with MG132 ((75µM for 45 min) followed by SM induction (0.5 µg/mL for 20 min).

However, Gcn4 is normally phosphorylated by two different CDKs, Pho85 and Cdk8, and prior phosphorylation is required for its efficient ubiquitination (Meimoun et al. 2000; Chi et al. 2001; Shemer et al. 2002). Therefore, we explored the relationship between sumoylation and specifically Cdk8-mediated ubiquitination of Gcn4 using Pho85-deleted Gcn4-WT and Gcn4-K50,58R strains. As controls, we also included Pho85-containing Gcn4-WT and Gcn4-K50,58R strains. As shown in **Figure 16A,** fewer modified forms of Gcn4 were detected in the *pho85*Δ Gcn4-WT and Gcn4-K50,58R cells compared to the Pho85-containingcells, reflecting the dependence on Pho85 for the bulk of Gcn4 ubiquitination. Nonetheless, Pho85-independent, MG132-stabilized forms of Gcn4 were detected in the Gcn4-WT strain, but not in the Gcn4-K50,58R strain (**Figure 16A** and **16B).** Together, these results demonstrate that there is no co-dependence of sumoylation and ubiquitination in Pho85-containing yeast, and that most Gcn4 modifications are dependent on the Pho85 pathway. However, our results point to a role for sumoylation in Pho85-independent ubiquitination of Gcn4, which is consistent with a model in which sumoylation triggers Cdk8 phosphorylation-mediated ubiquitination of Gcn4.

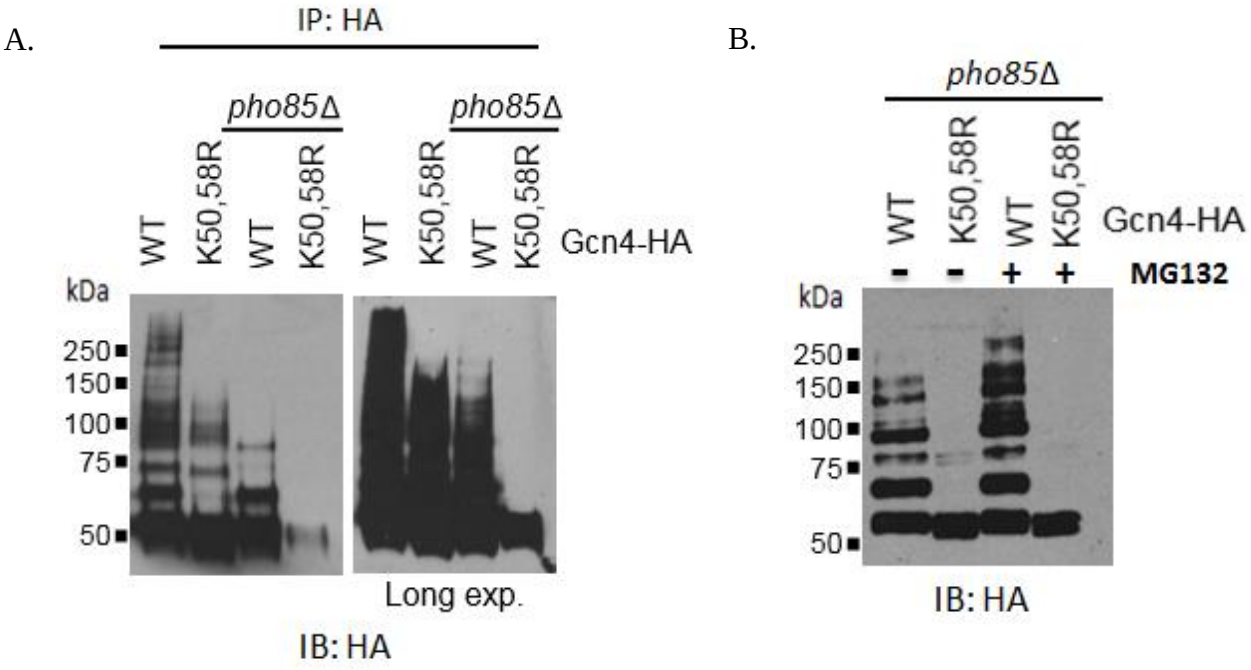


Figure 16: Effect of Gcn4 sumoylation on its ubiquitination. Analysis of MG132 ((75μM for 45 min) and SM treated (0.5 μg/mL for 20 min) Pho85 containing or deleted Gcn4-WT and Gcn4-K50,58R strains expressing 6xHA-tagged Gcn4. ***(A)*** IPs were analyzed by HA (1:3000) immunoblot. (L*eft panel*: light exposure; *Right panel*: long exposure of the same blot). ***(B)*** HA immunoblot (1:3000) analysis of MG132 treated and untreated extracts from pho85 deleted Gcn4-WT and Gcn4-K50,58R strains expressing HA-tagged.

3.4 Effect of Gcn4 activation on its sumoylation:

The activation domain (AD) of Gcn4 functions through a short conserved sequence motif, and seven hydrophobic residues (F97, F98, M107, L113, W120, L123 and F124) are reportedly essential for its function in activating transcription (Drysdale et al., 1995; Brzovic et al., 2011; Warfield et al., 2014). Using its AD, Gcn4 binds to coactivators which lead to the formation of the pre-initiation complex on target gene promoters, which enables transcription. To study whether promoter-associated sumoylation of Gcn4 is dependent on its ability to activate transcription, we first generated a strain in which the Trp 120 residue of the Gcn4 AD was substituted with Ala (W120A). Based on the results of previous mutational studies, we predicted that this mutant strain (Gcn4-W120A) would be deficient in target gene activation (Brzovic et al., 2011; Warfield et al., 2014). However, Gcn4-W120A was able to grow under amino acid starvation conditions, which indicate that the mutation is not activation deficient **(Figure 17).** A series of additional mutant strains were then generated, where each of the key hydrophobic residues were mutated in various combinations. However, all of these strains were also able to grow under amino acid starvation conditions **(Figure 17)**. Furthermore, RT-PCR was performed with these strains to analyze mRNA levels of Gcn4-dependent amino acid starvation induced genes *ARG1* and *CPA2* and a constitutively expressed gene, *PMA1*. As shown in **Figure 18A**, all of these mutants were transcriptionally active. This suggests that transcriptional activation by Gcn4 is complex and not restricted to a single region of an AD. Not surprisingly, IP-immunoblot analysis indicated that all of these mutant strains are targets of normal sumoylation **(Figure 18B)**. Further analysis will be required to examine a possible dependence of Gcn4 sumoylation on its ability to activate transcription.

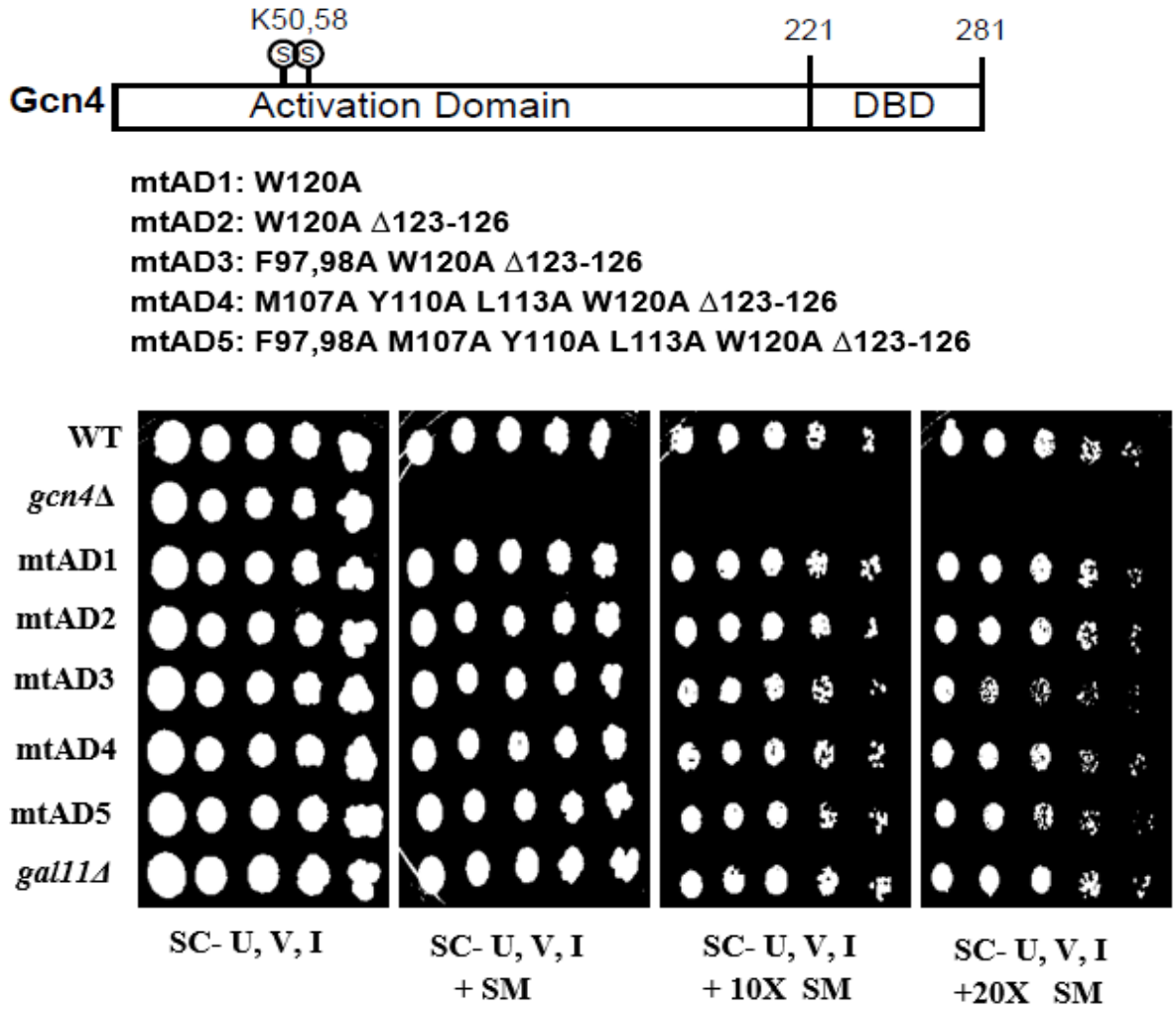
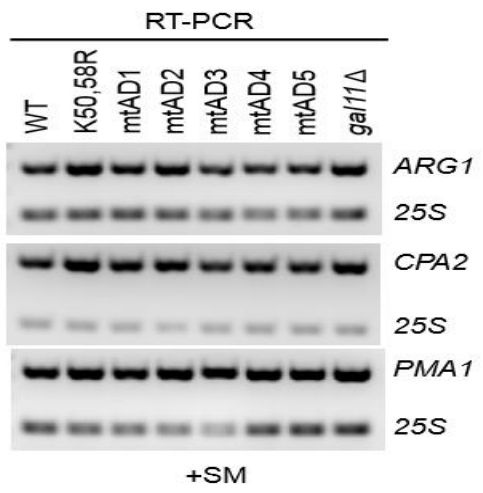


Figure 17: Effect of Gcn4 activation on its sumoylation. ***(A)*** The schematic diagram of Gcn4 representing probable sites of sumoylation (indicated by encircled S) and the activation domain (AD). ***(B)*** Spot assay comparing the growth of indicated strains. Cells were serially diluted and spotted on synthetic medium lacking Ura, Val and Ile (*Left*) and the same medium containing the indicated level of SM (1x is 0.5µg/mL), and incubated for 5 days at 30 ^{0}C.

A.



B.

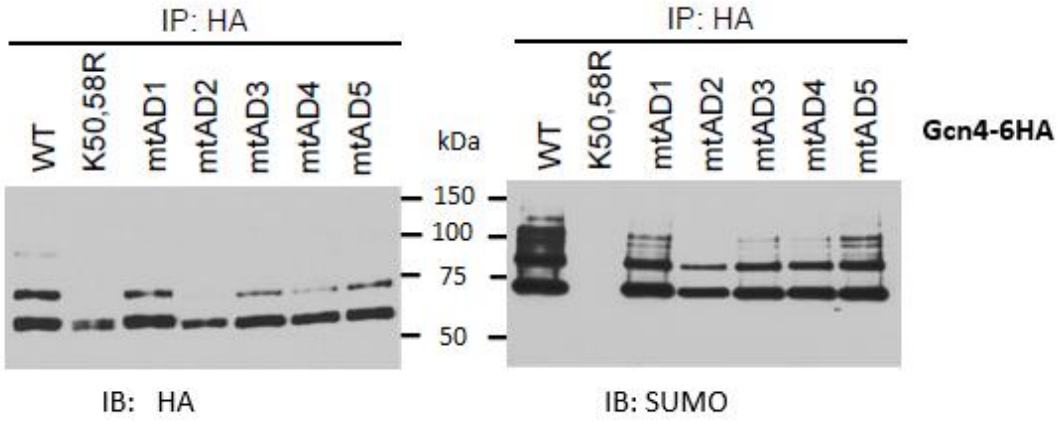


Figure 18: Effect of Gcn4 activation on its sumoylation. ***(A)*** RT-PCR analysis of SM induced genes (ARG1 and CPA2) and a control gene (PMA1). 25S refers to internal control 25S rRNA. ***(B)*** HA (1:3000) and SUMO (1:3000) immunoblots analysis of IPs from indicated yeast strains expressing 6xHA-tagged Gcn4. Cells were treated with SM (0.5 µg/mL for 20 min) to induce Gcn4 expression.

3.5 Effect of RNAP II recruitment on Gcn4 sumoylation

Gcn4 is a target for efficient sumoylation specifically after binding to target gene promoters, but it is not clear whether its sumoylation is dependent on ongoing transcription by RNAP II (Rosonina et al., 2012). Therefore, to examine the effect of ongoing transcription, specifically RNAP II recruitment, on Gcn4 sumoylation, Gcn4 was tagged with 6xHA in a strain that harbors a temperature sensitive allele of RNA polymerase II, *rpb1-1* (Nonet et al., 1987). Rpb1 is the largest subunit of RNAP II with a unique C-terminal domain (CTD) which plays crucial roles in transcription (Phatnani and Greenleaf, 2006). At elevated temperatures (~37°C), RNAP II is inactivated in this strain causing the *rpb1-1* Gcn4 strain to display growth defects at non-permissive temperatures (39°C) **(Figure 19A**). Gcn4-WT and *rpb1-1* strains were grown at normal (28˚C) or restrictive (39˚C) temperatures and treated with or without SM. mRNA levels of amino acid starvation induced genes (*ARG1*, *CPA2* induced by SM) and a constitutively expressed gene (*PMA1*; as a control) were then analyzed by RT-PCR. As expected, Gcn4 target gene expression was reduced in *rpb1-1* cells compared to Gcn4-WT at both the permissive and non-permissive temperatures (**Figure 19B**, *top & middle*). Using the same experimental conditions, IP-immunoblot analysis was performed with Gcn4-WT and *rpb1-1* cells. As shown in the figure 18C, *rpb1-1* cells displayed Gcn4 sumoylation even at non-permissive temperatures suggesting that ongoing transcription is not required for Gcn4 sumoylation (**Figure 19C**, right).

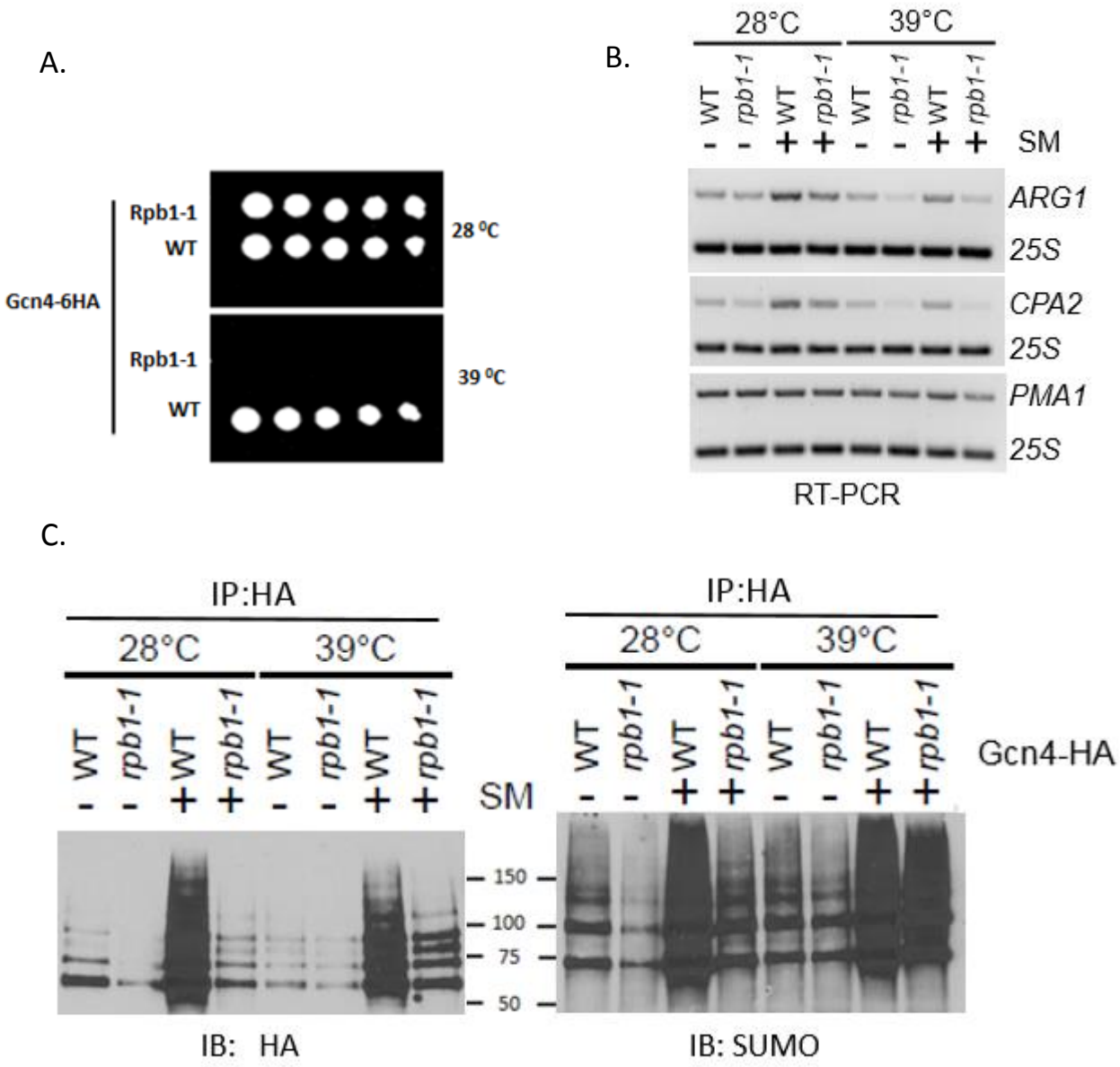


Figure 19: Effect of RNAP II recruitment on Gcn4 sumoylation. ***(A)*** Spot assay comparing growth of Gcn4-WT and *rpb1-1* strains on synthetic medium lacking uracil at the indicated temperature. ***(B)*** RT–PCR analysis of amino acid induced genes *ARG1*, *CPA2*, and a constitutively expressed gene *PMA1*. As an internal control, 25S rRNA was also analyzed. Gcn4-WT and *rpb1-1* Gcn4 strains were grown at 28˚C until they reached an exponential phase. They were then transferred to either 28˚C or 39˚C for 15 mins followed by SM treatment (indicated as "+") for 15 min. ***(C)*** HA and SUMO immunoblot analysis of IPs. Cells were grown and treated as mentioned in *(B)*.

To address the possibility that elevated temperature itself triggered Gcn4 sumoylation in the experiment described above, an additional method was used to block transcription and examine Gcn4 sumoylation. We obtained a previously reported Rbp1 Anchor Away strain, in which RNAPII can be conditionally and rapidly removed from nuclei in living yeast cells, by adding rapamycin to the growth medium (Haruki et al., 2008). By this system, Rpb1 is fused to the FRB domain of human mTOR in a yeast strain expressing RPL13A-FKBP12, a fusion of ribosomal protein RpL13A and human FKBP12, which binds FRB in the presence of rapamycin. Upon addition of rapamycin to the growth medium, FRB will bind to FKBP12 causing the Rpb1-FRB fusion to exit the nucleus with RPL13A, which will eventually block transcription without the need of elevated temperatures. For this analysis, we tagged Gcn4 with 6xHA in Rpb1-FRB strains (obtained from Kevin Struhl's lab) and the cells were grown in non-induced and induced conditions in the presence or absence of rapamycin as indicated in **Figure 20**. Immunoblotting with a SUMO antibody further supports our finding that Gcn4 is sumoylated in the absence of nuclear Rpb1 **(Figure 20, *right*).** Therefore, it can be concluded that on-going transcription is not necessary for promoter-bound Gcn4 sumoylation.

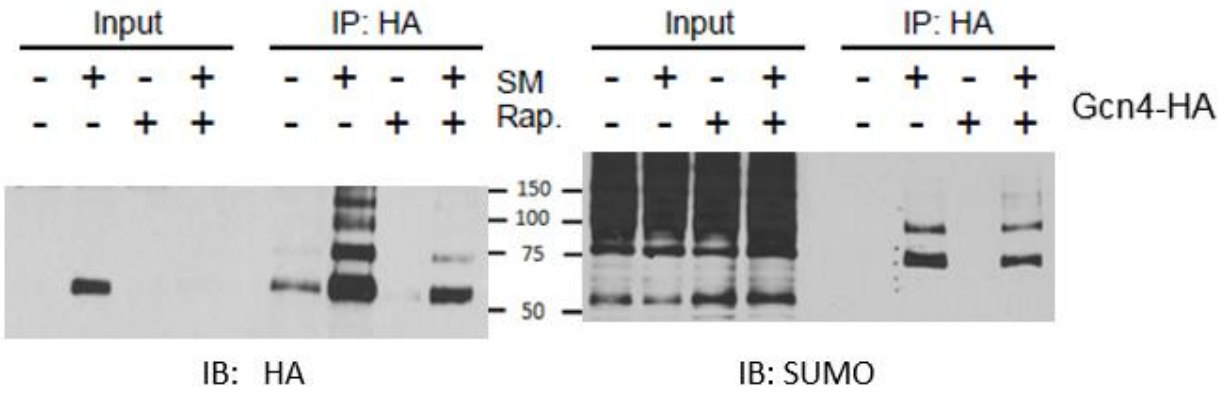


Figure 20: Effect of RNAP II recruitment on Gcn4 sumoylation. Analysis of HA and SUMO immunoblot using Rpb1-FRB fusion Gcn4 strain, where cells were treated with 1µg/mL rapamycin (indicated by "+" sign) for 20 min followed by 20 min treatment with SM (indicated by "+" sign) at a final concentration of 0.5ug/ml.

3.6 Effect of hyper-sumoylated Gcn4 on its promoter occupancy

As demonstrated above and previously, Gcn4 becomes sumoylated specifically after binding to target gene promoters, and this facilitates its removal from DNA (Rosonina et al., 2012). To further elucidate the significance of sumoylation on Gcn4 function, a "permanently sumoylated" form of Gcn4 fusion strain was generated (Gcn4-Smt3). In this fusion strain, the SUMO gene (*SMT3*) was inserted to the *GCN4* locus, such that an N-terminal SUMO fusion form of Gcn4 could be generated, leading it to be constitutively sumoylated. Although this continuously sumoylation form did not affect the strain's normal growth and viability **(Appendix Figure 3A),** it exhibited a significantly higher level of sumoylation compared to the Gcn4-WT (**Appendix Figure 3B**, right), allowing us to determine the effects of hyper-sumoylation of Gcn4.

To analyze the impact of higher levels of Gcn4 sumoylation on the expression of its target genes, an RT-qPCR analysis was performed. We found that Smt3-Gcn4 fusion cells generated significantly fewer Gcn4 activated *ARG1* and *CPA2* transcripts compared to Gcn4-WT, while no significant difference was observed for a constitutively expressed gene that is not regulated by Gcn4 such as *PMA1* **(Figure 21A)**. We then performed ChIP assays to compare the levels of Gcn4-WT and Smt3-Gcn4 cells on the promoter of the target gene *ARG1* over a time course of amino acid starvation. As seen in **Figure 21B,** significantly lower levels of Smt3-Gcn4 were detected on the *ARG1* promoter compared to Gcn4-WT (10 and 15 min post induction). These findings suggest that a high level of Gcn4 sumoylation is associated with lower levels of target gene activation and strongly supports our hypothesis that sumoylation stimulates the removal of Gcn4 from promoters.

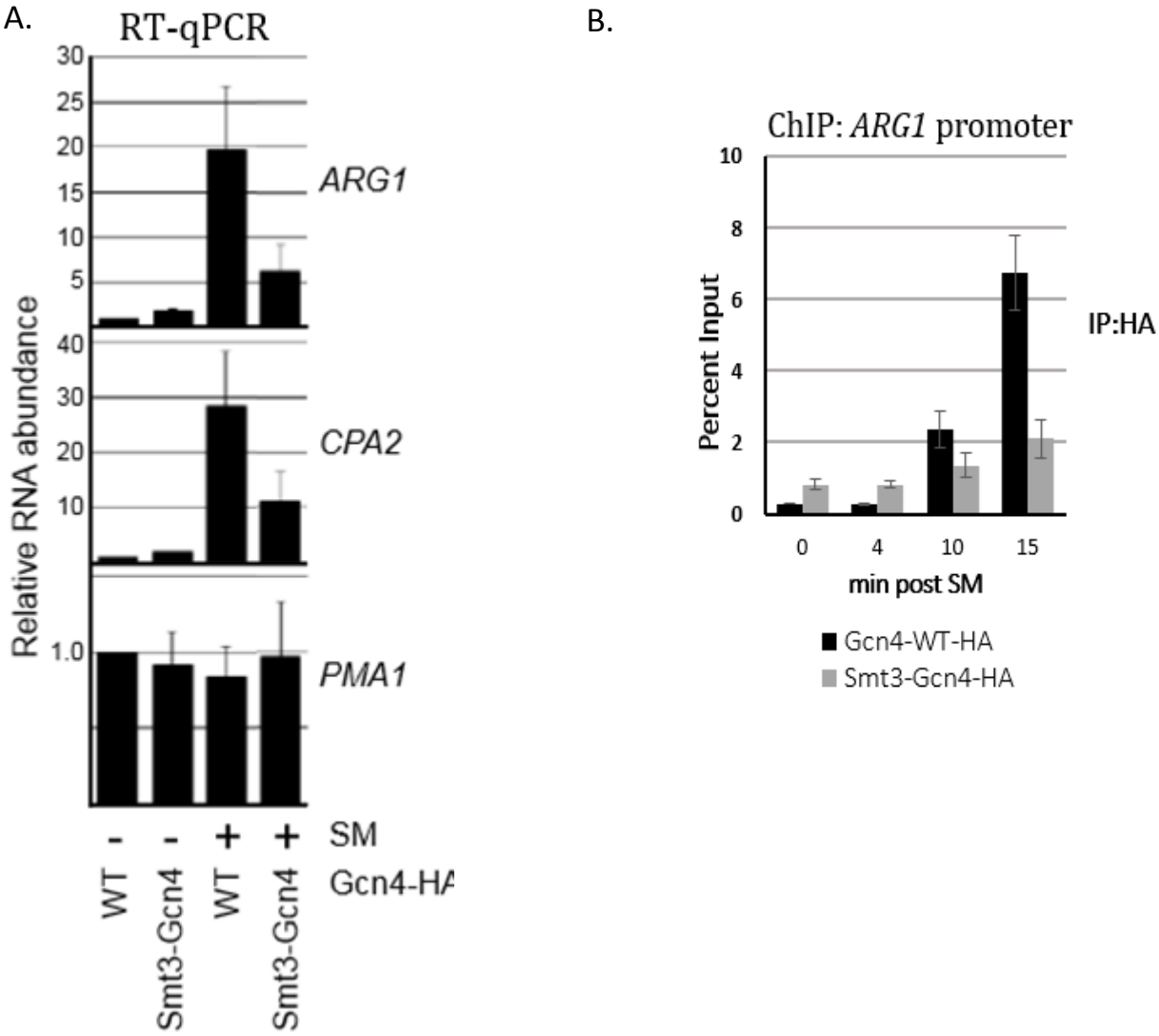


Figure 21: Effect of hyper-sumoylation of Gcn4 on its promoter occupancy. ***(A)*** mRNA levels of Gcn4-WT and Smt3-Gcn4 cells were determined by RT–PCR for amino acid starvation induced genes *ARG1*, *CPA2* and a control housekeeping gene, *PMA1*, with or without SM. Values were normalized to 25S rRNA. Data are represented as mean +/- SD of three independent experiments. ***(B)*** ChIP analysis of Gcn4-WT and Smt3-Gcn4 cells on *ARG1* promoter at indicated times after SM induction. Data are represented as mean +/- SD of three independent experiments.

We then wanted to study whether deletion of *CDK8* can reverse the effects of hyper-sumoylation of Gcn4 on expression of target genes to provide further evidence that Cdk8 is involved in removal of sumoylated Gcn4 from target promoters (Rosonina et al., 2012). For this, we deleted *CDK8* in the Smt3-Gcn4 fusion strain (*cdk8*ΔSmt3-Gcn4) and compared target gene induction and promoter occupancy with a Cdk8-containing strain. In otherwise wild-type cells, deletion of *CDK8* resulted in reduced expression of *ARG1* suggesting that Cdk8 normally plays a positive role in regulating RNAP II transcription (**Figure 22A**). However, we observed that the deletion of *CDK8* in cells expressing the Smt3-Gcn4 fusion protein at least partly reversed the effects of hyper-sumoylation of Gcn4 on expression of target genes. This was indicated by the significant increase in expression of *ARG1* compared to *CDK8* containing Smt3-Gcn4 fusion cells (**Figure 22B**). Consistent with this, significantly higher levels of Smt3-Gcn4 was detected on the *ARG1* promoter when Cdk8 was absent (10 and 15 min post induction; **Figure 23**). Taken together with our previous findings, these findings strongly support the idea that Gcn4 sumoylation triggers its removal from target DNA in a manner dependent on its subsequent phosphorylation by Cdk8.

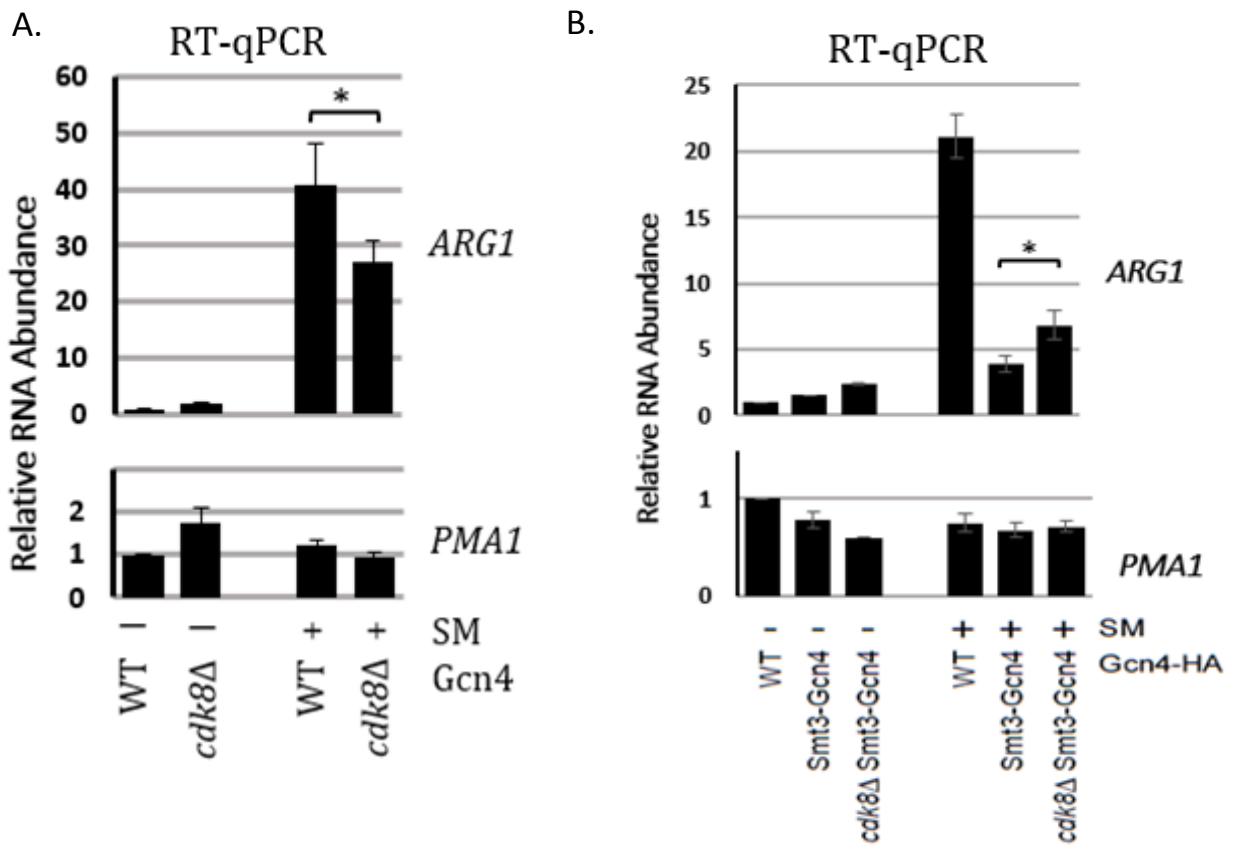


Figure 22: Effect of hyper-sumoylation of Gcn4 on its promoter occupancy. mRNA levels were determined by RT–PCR for amino acid starvation induced gene *ARG1*, and a control housekeeping gene, PMA1, with or without treatment with SM (0.5ug/ml for 20 min). Values were normalized to 25S rRNA. Data are represented as mean +/- SD of three independent experiments with * $p < 0.05$. ***(A)*** Analysis was performed with strains expressing Gcn4-WT and *cdk8*Δ Gcn4. ***(B)*** Analysis was performed with strains expressing Gcn4-WT, Smt3-Gcn4 and *cdk8*ΔSmt3- Gcn4.

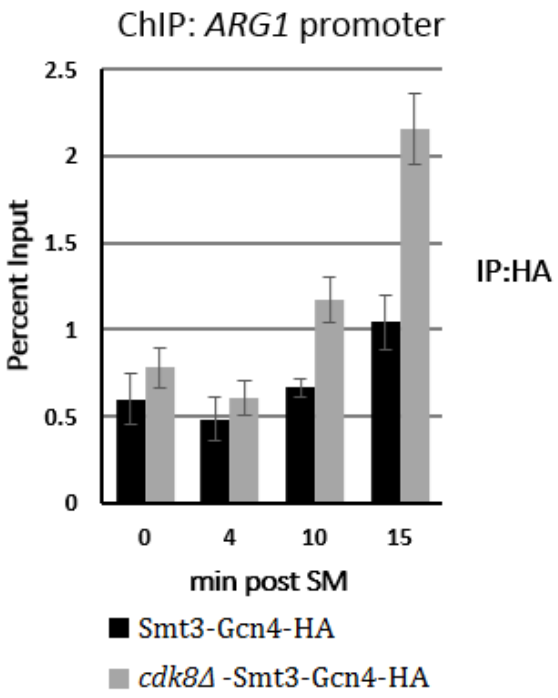


Figure 23: Effect of hyper-sumoylation of Gcn4 on its promoter occupancy. ChIP analysis of Smt3-Gcn4 and Cdk8Δ Smt3-Gcn4 fusion cells occupancy on ARG1 promoter at indicated times after SM induction. Data are represented as mean +/- SD of three independent experiments.

Chapter 4: Discussion

Post-translational modifications (PTMs) can have a profound impact on the function, subcellular localization and stability of a protein. There are numerous PTMs that a protein can undergo. Gcn4, a transcriptional activator of amino acid biosynthesis genes, is subjected to multiple protein PTMs, including phosphorylation, ubiquitination and sumoylation. Studies revealed that Gcn4 possesses two consensus sumoylation sites (K 50, 58) (Rosonina et al., 2012). In addition, it contains five potential sites (S17, T61, T105, T165 and S218) for phosphorylation, catalyzed by two specific CDKs: Pho85 and Cdk8. Pho85 phosphorylates Gcn4 regardless of whether it is promoter-bound or not, while Cdk8, a component of the Mediator complex, phosphorylates specifically promoter-bound Gcn4 (Chi et al. 2001; Meimoun et al. 2000). In both pathways, phosphorylation targets Gcn4 for ubiquitination and subsequent degradation through the 26S proteasome (Lipford et al., 2005). However, the crosstalk between different PTMs, especially between sumoylation, phosphorylation and ubiquitination, remains unclear. To address this, the purpose of this study was to understand how Gcn4 sumoylation is dependent on its binding to DNA, and how this modification is coordinated with its phosphorylation and ubiquitination as part of a regulatory cascade of PTMs.

To accomplish this, we first investigated whether Gcn4 sumoylation was affected by preventing binding to target gene promoters using a Gcn4 DNA-binding domain mutant yeast strain, and determining whether its sumoylation could be restored when fused to a heterologous DNA binding domain. In order to study whether Gcn4 phosphorylation and ubiquitination levels were affected by blocking its sumoylation, a yeast strain that expresses a sumoylation-deficient mutant form of Gcn4 (Gcn4-K50,58R) was used. Conversely, the effect of impaired Gcn4 phosphorylation and ubiquitination on its sumoylation was examined using phosphorylation-site mutant and

ubiquitination impaired Gcn4 strains. We then attempted to generate a series of transcriptional activation mutant Gcn4 strains to study whether Gcn4 sumoylation is affected by its ability to activate its target genes. However, none of the mutants displayed a detectable reduction in target gene activation. Further, we analyzed the effect of ongoing transcription, specifically RNAP II recruitment, on Gcn4 sumoylation using a temperature sensitive RNAP II mutant Gcn4 strain as well as utilizing an alternative approach, the 'Anchor away' technique (Haruki et al. 2008). Finally, using a "permanently sumoylated" form of Gcn4 fusion strain, we further demonstrated that sumoylation acts to facilitate Gcn4 removal from target promoters, after it has acted in transcriptional activation.

Gcn4 binds to target gene promoters as a homodimer, and this specific DNA binding activity resides in the 56 C-terminal amino acids that facilitates both dimerization and promoter recognition, a region that folds independently of the rest of the protein (Hope & Struhl 1986; Struhl 1987; Ellenberger et al. 1992). Employing immunoprecipitation assays, we showed that although Gcn4 becomes sumoylated specifically after binding to target gene promoter, its efficient phosphorylation by either Pho85 or Cdk8 is not a prerequisite for this PTM. This was indicated by the lack of a significant difference in the level of sumoylation between Gcn4-WT and phosphorylation impaired Gcn4 protein. We also observed that SUMO-mutant Gcn4 is phosphorylated by both CDKs, but SUMO-deficient Gcn4 displays an increase in the least phosphorylated form of Gcn4 compared to Gcn4-WT. We observed this effect in both Pho85-containing yeast, as well as in a Pho85-deleted background where promoter-bound Gcn4 can only be phosphorylated by Cdk8. Therefore, it could be concluded that even though most modifications are dependent on the Pho85 pathway, some Cdk8-mediated Gcn4 phosphorylation (and subsequent modifications) are likely stimulated by its sumoylation. Consistent with this finding, we also

observed that the level of Gcn4 ubiquitination detected in *pho85Δ* cells disappears in cells expressing SUMO-deficient Gcn4, suggesting that Cdk8-mediated ubiquitination of Gcn4 is stimulated by its sumoylation. In addition, we did not observe Gcn4 ubiquitination in the phosphorylation mutant Gcn4 strain, which further supports prior studies that phosphorylation is required for ubiquitin-mediated Gcn4 degradation (Meimoun et al. 2000; Shemer et al. 2002).

Mutagenesis studies previously demonstrated that the Gcn4 activation domain functions through a short conserved sequence motif, and that seven hydrophobic residues are essential for its transcriptional activation function. When these residues are mutated in combination, it can perturb activation (Drysdale et al., 1995; Brzovic et al., 2011; Warfield et al., 2014). Therefore, we attempted to generate a transcriptional activation mutant Gcn4 strain by altering these hydrophobic residues without affecting Gcn4 sumoylation and DNA binding ability in order to analyze the effect of transcriptional activation on Gcn4 sumoylation. Unfortunately, although we were able to generate the mutant strains, none of them displayed a deficiency in Gcn4 activation, as determined by induction of Gcn4 target genes, and growth on amino acid starvation medium.

However, by using a temperature sensitive RNAP II mutant strain and employing immunoprecipitation assays, we showed that on-going transcription, specifically RNAP II recruitment, is not essential for promoter-bound Gcn4 sumoylation. In addition, to avoid ambiguities regarding the effect of high temperature (37˚C) on Gcn4 sumoylation, we used the Rpb1-FRB fusion strain, in which RNAPII can be conditionally removed from nuclei upon addition of rapamycin, which further supports our finding that transcription is not required for Gcn4 sumoylation.

To further explore the role of Gcn4 sumoylation, we generated a SUMO fusion version of Gcn4 and, employing RT-PCR and ChIP assays, we observed that hyper-sumoylation of Gcn4 is associated with less target gene activation and reduced occupancy of Gcn4 on target promoters. Furthermore, the Smt3-Gcn4 fusion strains displayed significantly higher levels of ubiquitination than Gcn4-WT (Appendix Figure 4). However, the increased ubiquitination detected on the Smt3-Gcn4 fusion was abolished by deletion of *CDK8*, which further support the involvement of Cdk8 in clearing sumoylated Gcn4 from target promoters by stimulating its ubiquitination and degradation (**Appendix Figure 4**). Consistent with our findings, we also observed that deletion of *CDK8* can partly reverse the effects of hyper-sumoylation of Gcn4 on expression and occupancy of target genes.

Taken together, the results of our experiments led us to propose a model depicted in **Figure 24**. According to the model, Gcn4 becomes sumoylated after binding to the target gene promoter. Indeed, our study demonstrates that DNA binding appears to be the only critical determinant of Gcn4 sumoylation. Although it is still not clear whether sumoylation is dependent on the efficient recruitment of general transcription factors (i.e. activation), it is evident that ongoing transcription, specifically RNAP II recruitment, is not necessary for sumoylation. Further, SUMO modification of Gcn4 stimulates Cdk8-dependent phosphorylation, which then targets Gcn4 for ubiquitination and subsequent degradation through the 26S proteasome. The promoter can then be shut down or additional, unbound Gcn4 molecules can then bind to it, permitting subsequent rounds of transcription.

Although some questions still remain unanswered in this model, the investigation of the coordination of PTMs on promoter-bound Gcn4 shed significant light on the multiple intricate mechanisms by which the cell regulates an important transcription factor.

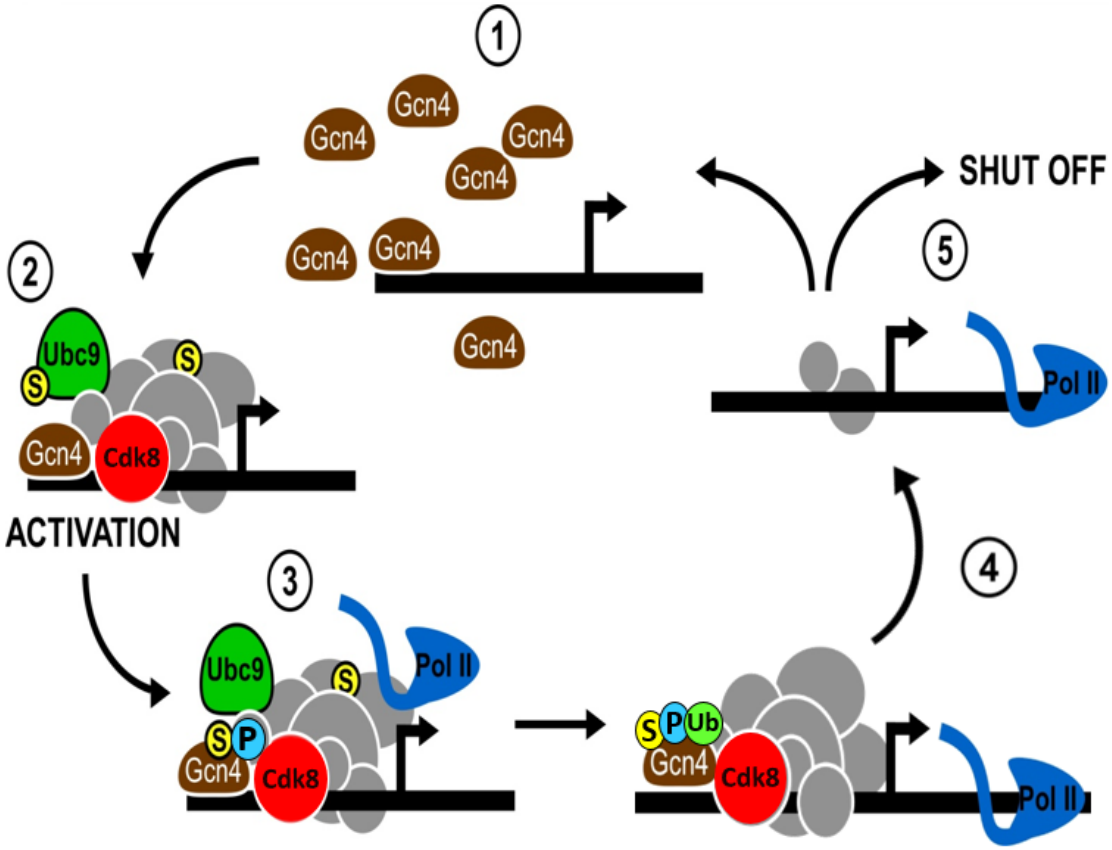


Figure 24: A proposed model for regulation of promoter-bound Gcn4 by Cdk8-phosphorylation dependent, ubiquitin-mediated degradation. [1] Unmodified Gcn4 binds to the promoter. [2] Promoter-bound Gcn4 may (or might not) recruit transcription complexes including Cdk8, Ubc9 (the SUMO E2 conjugation enzyme) and SUMO to activate target promoters; Ubc9 then sumoylates Gcn4, and [3] this modification specifically activates the Cdk8 phosphorylation-mediated pathway, [4,5] which targets Gcn4 for ubiquitination and subsequent degradation through the 26S proteasome.

Future Directions:

Future investigations as a continuation of this work will focus on the sumoylation of individual Lys residues to determine whether sumoylation of either Lys 50 or 58 alone is sufficient to affect promoter-associated Gcn4 phosphorylation and ubiquitination. For this, strains expressing Gcn4 mutated at either Lys 50 or 58 (to non-sumoylatable Arg) will be tested by using IP-immunoblot and time-course ChIP analyses.

In addition, it would be interesting to investigate whether sumoylation influences Gcn4 occupancy time at a target promoter by using competition ChIP, which has previously been used to measure turnover rates of promoter-associated transcription factors Rap1 and TBP (Werven et al., 2009: Lickwar et al., 2012). By this method, a yeast strain will be generated that harbors constitutively expressed HA-tagged Gcn4 and inducible Flag-tagged Gcn4 that is either WT or SUMO mutant. Once induced, the ability of the Flag-tagged SUMO mutant to exchange with WT HA-Gcn4 on a target gene promoter will be determined over a time-course by ChIP with Flag and HA antibodies. Slower exchanges rates in the SUMO mutant compared to WT Gcn4 will indicate that sumoylation acts to reduce Gcn4 residence time on target promoters. Using the same method, the role of ubiquitination in controlling Gcn4 occupancy time at a target promoter should also be analyzed.

Regardless of the existing data, it is still unknown whether Cdk8-dependent phosphorylation and ubiquitination of Gcn4 exclusively depends on its ability to bind to gene promoters. To test for this, strain expressing Gcn4-6HA that is unable to bind to DNA, in a *pho85Δ* background, where Cdk8 is the major Gcn4 kinase, will be examined using IP-immunoblot assays after phosphatase analysis, which we already employed to resolve phosphorylated and unphosphorylated isoforms of Gcn4-6HA on an HA immunoblot.

Finally, the influence of transcriptional activation on Gcn4 sumoylation should be investigated further by obtaining an activation impaired Gcn4 strain. This is an especially challenging approach as the objective is to obtain a strain which is impaired in recruiting transcription factors while preserving other characteristics like 3D structure, folding, DNA binding and PTMs like sumoylation. Although various studies prepared differernt Gcn4 activation mutant strains, they were mostly dissimilar to our approach as they were not concerned about keeping the aforementioned characteristics intact (Drysdale et al., 1995; Brzovic et al., 2011; Warfield et al., 2014). For instance, Drysdale et al. (1995) deleted amino acid residues 18-169 to obtain an activation mutant strain without considering the effects of blocking residues important for other functions. This includes residues K50 and 58, both known sites for sumoylation.

Some preliminary attempts were conducted with different combinations of mutations (already mentioned in the results section), albeit with unsuccessful results. Future attempts will focus on obtaining a mutant strain by deleting/substituting various amino acid residues in different combinations so as not to impede other functions. A successful activation mutant should ideally not have the residues necessary for co-activator binding as well as activation. This mutant strain should then be tested by spot assay to see if it really is activation deficient. Upon successful confirmation, ChIP assay should then be performed to determine whether this AD mutant Gcn4 strain is still capable of binding to DNA. If it is capable to binding to DNA, it will then be tested by using IP-immunoblot with Smt3 antibody to determine whether it gets sumoylated.

These future research goals will provide more insight into the role of sumoylation in regulation of promoter-bound Gcn4, and as a result a better understanding of the crosstalk between different PTMs that are with sumoylation and relationships between them.

Reference:

Audic, Y., Hartley, R.S., 2004. Post-transcriptional regulation in cancer. *Biol. Cell* **96:** 479–98.

Basehoar, A., Zanton, S., Pugh, B., 2004. Identification and distinct regulation of yeast TATA box-containing genes. *Cell* **116:** 699–709.

Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., Becker, J., 1998. Structure determination of the small ubiquitin-related modifier SUMO-1. J. *Mol. Biol.* **280:** 275–86.

Bernier-Villamor, V., Sampson, D.A., Matunis, M.J., Lima, C.D., 2002. Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell* **108:** 345–56.

Biddick, R., Young, E.T., 2005. Yeast mediator and its role in transcriptional regulation. *C. R. Biol.* **328:** 773–82.

Borggrefe, T., Davis, R., Erdjument-Bromage, H., Tempst, P., Kornberg, R.D., 2002. A complex of the Srb8, -9, -10, and -11 transcriptional regulatory proteins from yeast. *J. Biol. Chem.* **277:** 44202–7.

Brzovic, P.S., Heikaus, C.C., Kisselev, L., Vernon, R., Herbig, E., Pacheco, D., Warfield, L., Littlefield, P., Baker, D., Klevit, R.E., Hahn, S., 2011. The acidic transcription activator Gcn4 binds the mediator subunit Gal11/Med15 using a simple protein interface forming a fuzzy complex. *Mol. Cell* **44:** 942–53.

Buchman, A., Kimmerly, W., 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8:** 210-25.

Buratowski, S., Hahn, S., Guarente, L., Sharp, P.A., 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56:** 549–61.

Bushnell, D.A., Westover, K.D., Davis, R.E., Kornberg, R.D., 2004. Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms. *Science* **303:** 983–88.

Cantin, G.T., Stevens, J.L., Berk, A.J., 2003. Activation domain-mediator interactions promote transcription preinitiation complex assembly on promoter DNA. *Proc. Natl. Acad. Sci. U. S. A.* **100:** 12003–8.

Cheng, J., Bawa, T., Lee, P., Gong, L., Yeh, E.T.H., 2006. Role of desumoylation in the development of prostate cancer. *Neoplasia* **8:** 667–76.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R. a., Annan, R.S., Carr, S. a., Deshaies, R.J., 2001. Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev.* **15:** 1078–92.

Chowdary, D.R., Dermody, J.J., Jha, K.K., Ozer, H.L., 1994. Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Mol. Cell. Biol.* **14:** 1997–2003.

Conaway, R.C., Sato, S., Tomomori-Sato, C., Yao, T., Conaway, J.W., 2005. The mammalian Mediator complex and its role in transcriptional regulation. *Trends Biochem. Sci.* **30:** 250–55.

Cubeñas-Potts, C., Matunis, M., 2013. SUMO: a multifaceted modifier of chromatin structure and function. *Dev. Cell.* **24:** 1-12.

Dasso, M., 2008. Emerging roles of the SUMO pathway in mitosis. *Cell Div.* **3:** 5.

Deng, W., Roberts, S.G.E., 2007. TFIIB and the regulation of transcription by RNA polymerase II. *Chromosoma* **116:** 417–29.

Desterro, J.M.., Rodriguez, M.S., Hay, R.T., 1998. SUMO-1 Modification of IκBα Inhibits NF-κB Activation. *Mol. Cell* **2:** 233–39.

Donner, A.J., Szostek, S., Hoover, J., Espinosa, J., 2007. CDK8 is a stimulus-specific positive coregulator of p53 target genes. *Mol. Cell.* **27:** 121-33.

Donner, A.J., Ebmeier, C.C., Taatjes, D.J., Espinosa, J.M., 2010. CDK8 is a positive regulator of transcriptional elongation within the serum response network. *Nat. Struct. Mol. Biol.* **17:** 194–201.

Drysdale, C.M., Dueñas, E., Jackson, B.M., Reusser, U., Braus, G.H., Hinnebusch, a G., 1995. The transcriptional activator GCN4 contains multiple activation domains that are critically

dependent on hydrophobic amino acids. *Mol. Cell. Biol.* **15:** 1220–33.

Egly, J.M., Coin, F., 2011. A history of TFIIH: two decades of molecular biology on a pivotal transcription/repair factor. *DNA Repair (Amst).* **10:** 714–21.

Ellenberger, T.E., Brandl, C.J., Struhl, K., Harrison, S.C., 1992. The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein-DNA complex. *Cell* **71:** 1223–37.

Flores, O., Lu, H., Killeen, M., Greenblatt, J., Burton, Z.F., Reinberg, D., 1991b. The small subunit of transcription factor IIF recruits RNA polymerase II into the preinitiation complex. *Proc. Natl. Acad. Sci. U. S. A.* **88:** 9999–10003.

Galbraith, M.D., Donner, A.J., Espinosa, J.M., 2010. CDK8: a positive regulator of transcription. *Transcription* **1:** 4–12.

Geiss-Friedlander, R., Melchior, F., 2007. Concepts in sumoylation: a decade on. *Nat. Rev. Mol. Cell Biol.* **8:** 947–56.

Geoffroy, M.C., Hay, R.T., 2009. An additional role for SUMO in ubiquitin-mediated proteolysis. Nat. Rev. *Mol. Cell Biol.* **10:** 564–68.

Gerber, H.P., Seipel, K., Georgiev, O., Höfferer, M., Hug, M., Rusconi, S., Schaffner, W., 1994. Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science* **263:** 808–11.

Gómez-del Arco, P., Koipally, J., Georgopoulos, K., 2005. Ikaros SUMOylation: switching out of repression. *Mol. Cell. Biol.* **25:** 2688–97.

Goossens, A., Dever, T.E., Pascual-Ahuir, A., Serrano, R., 2001. The protein kinase Gcn2p mediates sodium toxicity in yeast. *J. Biol. Chem.* **276:** 30753–60.

Hainaut, P., Hollstein, M., 2000. p53 and human cancer: the first ten thousand mutations. Adv. *Cancer Res.* **77:** 81–137.

Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S., Young, R.A., 1998. Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol. Cell* **2:** 43–53.

Hinnebusch, A.G., Natarajan, K., 2002. Gcn4p , a Master Regulator of Gene Expression , Is Controlled at Multiple Levels by Diverse Signals of Starvation and Stress. *Eukaryot Cell* **1:** 22-32.

Hirst, M., Kobor, M., Kuriakose, N., 1999. GAL4 is regulated by the RNA polymerase II holoenzyme–associated cyclin-dependent protein kinase SRB10/CDK8. *Mol. Cell* **3:** 673-78.

Hoege, C., Pfander, B., Moldovan, G.-L., Pyrowolakis, G., Jentsch, S., 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419:** 135–41.

Hope, I.A., Struhl, K., 1985. GCN4 protein, synthesized in vitro, binds HIS3 regulatory sequences: implications for general control of amino acid biosynthetic genes in yeast. *Cell* **43:** 177–188.

Hope, I., Struhl, K., 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46:** 885-94.

Hope, I., Struhl, K., 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. *EMBO J.* **6:** 2781–84.

Hoy, M. Van, Leuther, K., Kodadek, T., Johnston, S., 1993. The acidic activation domains of the GCN4 and GAL4 proteins are not α helical but form β sheets. *Cell* **26:** 587-94.

Irniger, S., Braus, G.H., 2003. Controlling transcription by destruction: the regulation of yeast Gcn4p stability. *Curr. Genet.* **44:** 8–18.

Johnson, E.S., Gupta, A.A., 2001. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **106:** 735–44.

Johnson, E.S., Schwienhorst, I., Dohmen, R.J., Blobel, G., 1997. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J.* **16:** 5509–19.

Juven-Gershon, T., Kadonaga, J.T., 2010. Regulation of gene expression via the core promoter and the basal transcriptional machinery. *Dev. Biol.* **339:** 225–29.

Klein, C., Struhl, K., 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains in vivo. *Science* **266:** 280–82.

Kobayashi, N., Boyer, T.G., Berk, A.J., 1995. A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. *Mol. Cell. Biol.* **15:** 6465–73.

Kornitzer, D., Raboy, B., Kulka, R.G., Fink, G.R., 1994. Regulated degradation of the transcription factor Gcn4. *EMBO J.* **13:** 6021–30.

Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., de Thé, H., 2008. Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat. Cell Biol.* **10:** 547–55.

Lee, S., Hahn, S., 1995. Model for binding of transcription factor TFIIB to the TBP-DNA complex. *Nature* **376:** 609–12.

Li, S.J., Hochstrasser, M., 1999. A new protease required for cell-cycle progression in yeast. *Nature* **398:** 246–51.

Lickwar, C.R., Mueller, F., Hanlon, S.E., McNally, J.G., Lieb, J.D., 2012. Genome-wide protein-DNA binding dynamics suggest a molecular clutch for transcription factor function. *Nature* **484:** 251–55.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:** 402–08.

Lipford, J.R., Smith, G.T., Chi, Y., Deshaies, R.J., 2005. A putative stimulatory role for activator turnover in gene expression. *Nature* **438:** 113–116.

Liu, C., Apodaca, J., Davis, L., Rao, H., 2007. Proteasome inhibition in wild-type yeast Saccharomyces cerevisiae cells. *Biotechniques* **42:** 158–162.

Lois, L.M., Lima, C.D., 2005. Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *EMBO J.* **24:** 439–51.

Luscombe, N.M., Austin, S.E., Berman, H.M., Thornton, J.M., 2000. An overview of the structures of protein-DNA complexes. *Genome Biol.* **1:** REVIEWS001.

Makhnevych, T., Sydorskyy, Y., Xin, X., 2009. Global map of SUMO function revealed by protein-protein interaction and genetic networks. *Mol. Cell.* **16:** 124-35.

Malumbres, M., Harlow, E., Hunt, T., Hunter, T., 2009. Cyclin-dependent kinases: a family portrait. *Nat. cell Biol.* **11:** 1275-76.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. *Science* **298:** 1912-34.

Marmorstein, R., Carey, M., Ptashne, M., Harrison, S.C., 1992. DNA recognition by GAL4: structure of a protein-DNA complex. *Nature* **356:** 408–14.

Martin, S., Wilkinson, K.A., Nishimune, A., Henley, J.M., 2007. Emerging extranuclear roles of protein SUMOylation in neuronal function and dysfunction. *Nat. Rev. Neurosci.* **8:** 948–59.

Martinez, E., 2002. Multi-protein complexes in eukaryotic gene transcription. *Plant Mol. Biol*. **50:** 925-47.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R., Kornitzer, D., 2000. Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. *Mol. Biol. Cell* **11:** 915–27.

Meluh, P.B., Koshland, D., 1995. Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol. Biol. Cell* **6:** 793–807.

Meyer, K.D., Donner, A.J., Knuesel, M.T., York, A.G., Espinosa, J.M., Taatjes, D.J., 2008. Cooperative activity of cdk8 and GCN5L within Mediator directs tandem phosphoacetylation of histone H3. *EMBO J.* **27:** 1447–57.

Miteva, M., Keusekotten, K., Hofmann, K., Praefcke, G.J.K., Dohmen, R.J., 2000. Sumoylation as a Signal for Polyubiquitylation and Proteasomal Degradation. *Subcell. Biochen.* **54:** 195-214.

Mo, Y.Y., Moschos, S.J., 2005. Targeting Ubc9 for cancer therapy. *Expert Opin. Ther. Targets* **9:** 1203–16.

Morita, Y., Kanei-Ishii, C., Nomura, T., Ishii, S., 2005. TRAF7 sequesters c-Myb to the

cytoplasm by stimulating its sumoylation. *Mol. Biol. Cell* **16:** 5433–44.

Morse, R., 2007. Transcription factor access to promoter elements. *J. Cell Biochem.* **102:** 560-70.

Mukhopadhyay, D., Dasso, M., 2007. Modification in reverse: the SUMO proteases. Trends *Biochem. Sci.* **32:** 286–95.

Nemet, J., Jelicic, B., Rubelj, I., Sopta, M., 2014. The two faces of Cdk8, a positive/negative regulator of transcription. *Biochimie* **97:** 22–27.

Ng, C.H., Akhter, A., Yurko, N., Burgener, J.M., Rosonina, E., Manley, J.L., 2015. Sumoylation controls the timing of Tup1-mediated transcriptional deactivation. *Nat. Commun.* **6:** 6610.

Nikolov, D.B., Chen, H., Halay, E.D., Usheva, A.A., Hisatake, K., Lee, D.K., Roeder, R.G., Burley, S.K., 1995. Crystal structure of a TFIIB–TBP–TATA-element ternary complex. *Nature* **377:** 119–128.

Phatnani, H.P., Greenleaf, A.L., 2006. Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.* **20:** 2922–36.

Prudden, J., Pebernard, S., Raffa, G., Slavin, D.A., Perry, J.J.P., Tainer, J.A., McGowan, C.H., Boddy, M.N., 2007. SUMO-targeted ubiquitin ligases in genome stability. *EMBO J.* **26:** 4089–101.

Ptashne, M., 1988. How eukaryotic transcriptional activators work. *Nature*. **335:** 683-89.

Ptashne, M., Gann, A., 1997. Transcriptional activation by recruitment. *Nature* **386:** 569–77.

Raithatha, S., Su, T.C., Lourenco, P., Goto, S., Sadowski, I., 2012. Cdk8 regulates stability of the transcription factor Phd1 to control pseudohyphal differentiation of Saccharomyces cerevisiae. *Mol. Cell. Biol.* **32:** 664–74.

Raman, N., Nayak, A., Muller, S., 2013. The SUMO system: a master organizer of nuclear protein assemblies. *Chromosoma* **122:** 475-85.

Rechsteiner, M., 1988. Regulation of enzyme levels by proteolysis: the role of pest regions. *Adv. Enzyme Regul.* **27:** 135–51.

Rhee, H.S., Pugh, B.F., 2012. Genome-wide structure and organization of eukaryotic pre-initiation complexes. *Nature*. **483:** 295-301.

Rodriguez, M.S., Dargemont, C., Hay, R.T., 2001. SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J. Biol. Chem.* **276:** 12654–59.

Rogers, S., Wells, R., Rechsteiner, M., 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234:** 364–68.

Rosonina, E., Duncan, S.M., Manley, J.L., 2010. SUMO functions in constitutive transcription and during activation of inducible genes in yeast. *Genes Dev.* **24:** 1242–52. doi:10.1101/gad.1917910

Rosonina, E., Duncan, S.M., Manley, J.L., 2012. Sumoylation of transcription factor Gcn4 facilitates its Srb10-mediated clearance from promoters in yeast. *Genes Dev.* **26:** 350–55.

Saitoh, H., 2000. Functional Heterogeneity of Small Ubiquitin-related Protein Modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* **275:** 6252–58.

Salghetti, S.E., Kim, S.Y., Tansey, W.P., 1999. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *EMBO J.* **18:** 717–26.

Salghetti, S.E., Muratani, M., Wijnen, H., Futcher, B., Tansey, W.P., 2000. Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl. Acad. Sci.* **97:** 3118–23.

Shemer, R., Meimoun, A., Holtzman, T., Kornitzer, D., 2002. Regulation of the Transcription Factor Gcn4 by Pho85 Cyclin Pcl5. *Mol. Cell. Biol.* **22:** 5395–404.

Smale, S.T., Kadonaga, J.T., 2003. The RNA polymerase II core promoter. *Annu. Rev. Biochem.* **72:** 449-79.

Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y.Z., Cattaneo, E. and Pandolfi, P.P., 2004. SUMO Modification of Huntingtin and Huntington's Disease Pathology. *Science* **304:** 100–4.

Struhl, K., 1987. Promoters, activator proteins, and the mechanism of transcriptional initiation in

yeast. *Cell*. **49:** 295-97.

Struhl, K., 1993. Yeast transcription factors. *Curr. Opin. Cell Biol*. **5:** 513-20.

Subramonian, D., Raghunayakula, S., Olsen, J. V, Beningo, K.A., Paschen, W., Zhang, X.-D., 2014. Analysis of changes in SUMO-2/3 modification during breast cancer progression and metastasis. *J. Proteome Res.* **13:** 3905–18.

Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H., Hay, R.T., 2001. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem.* **276:** 35368–74.

Thomas, M.C., Chiang, C.-M., 2015. The general transcription machinery and general cofactors. Crit. *Rev. Biochem. Mol. Biol.* **41:** 105–78.

Treier, M., Staszewski, L.M., Bohmann, D., 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell* **78:** 787–98.

Van Dyke, M.W., Sawadogo, M., Roeder, R.G., 1989. Stability of transcription complexes on class II genes. *Mol. Cell. Biol.* **9:** 342–44.

Van Werven, F.J., van Teeffelen, H.A.A.M., Holstege, F.C.P., Timmers, H.T.M., 2009. Distinct promoter dynamics of the basal transcription factor TBP across the yeast genome. *Nat. Struct. Mol. Biol*. **16:** 1043–48.

Vijay-Kumar, S., Bugg, C.E., Cook, W.J., 1987. Structure of ubiquitin refined at 1.8 A resolution. *J. Mol. Biol.* **194:** 531–44.

Vincent, O., Kuchin, S., Hong, S.P., Townley, R., Vyas, V.K., Carlson, M., 2001. Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **21:** 5790–96.

Wang, G., Cantin, G.T., Stevens, J.L., Berk, A.J., 2001. Characterization of mediator complexes from HeLa cell nuclear extract. *Mol. Cell. Biol.* **21:** 4604–13.

Wang, Y., Dasso, M., 2009. SUMOylation and deSUMOylation at a glance. *J. Cell Sci.* **122:** 4249–52.

Warfield, L., Tuttle, L.M., Pacheco, D., Klevit, R.E., Hahn, S., 2014. A sequence-specific

transcription activator motif and powerful synthetic variants that bind Mediator using a fuzzy protein interface. *Proc. Natl. Acad. Sci. U. S. A.* **111:** 3506–13.

Yamamoto, H., Ihara, M., Matsuura, Y., Kikuchi, A., 2003. Sumoylation is involved in beta-catenin-dependent activation of Tcf-4. *EMBO J.* **22:** 2047–59.

Yang, C., Bolotin, E., Jiang, T., Sladek, F., Martinez, E., 2007. Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters. *Gene* **389:** 52-65.

Yang, S.H., Sharrocks, A.D., 2004. SUMO promotes HDAC-mediated transcriptional repression. *Mol. Cell* **13:** 611-17.

Yeh, E.T., Gong, L., Kamitani, T., 2000. Ubiquitin-like proteins: new wines in new bottles. *Gene* **248,** 1–14.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., Mori, K., 1998. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins Involvement of basic leucine zipper transcription factors. *J. Biol.Chem.***271:** 33741-49.

Zawel, L., Kumar, K.P., Reinberg, D., 1995. Recycling of the general transcription factors during RNA polymerase II transcription. *Genes Dev.* **9:** 1479–90.

Zhao, X., Blobel, G., 2005. A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc. Natl. Acad. Sci. U. S. A.* **102:** 4777–82.

Zhao, J., 2007. Sumoylation regulates diverse biological processes. *Cell. Mol. life Sci.* **64:** 3017-33.

Appendix:

Table 1: *S. cerevisiae* strains used in this study

Strains	Genotype
ERYM 055	Wild type without any tag
ERYM 079	HF7c
ERYM 613	Wild type Gcn4 with 6 HA tag
ERYM 631	*gcn4Δ::kanMX [p GCN4-6HA /CEN URA3]*
ERYM 663	*gcn4Δ::kanMX [pGCN4-6HA/CEN URA3]*
ERYM 664F	*gcn4Δ::kanMX [pGCN4- 6 HA -K50,58R /CEN URA3]*
ERYM 665	*gcn4Δ::NATr pho85Δ::kanMX [pGCN4-6HA/CEN URA3]*
ERYM 666	*gcn4Δ::NATr pho85Δ::kanMX [pGCN4-K50,58R-6HA/CEN URA3]*
ERYM 667	*gcn4Δ::NATr srb10Δ::kanMX [pGCN4-6HA/CEN URA3]*
ERYM 671	*gcn4Δ::NATr srb10Δ::kanMX pho85Δ::LEU2 [pGCN4-6HA / CEN URA3]*
ERYM 709	*gcn4Δ::kanMX [pGCN4-6HA-ΔCterminal40/CEN URA3]*
YAA 001	*cdc4-1 GCN4-6HA::kl TRP1*
YAA 002	*cdc34-2 GCN4-6HA::kl TRP1*
YAA 003	*gcn4Δ::kanMX [pGCN4-3T2S-6HA / CEN URA3]*
YAA 010	*gcn4Δ::kanMX [pGCN4-6HA/URA3 CEN]*
YAA 011	*gcn4Δ::kanMX rpb1-1 [pGCN4-6HA/ CEN URA3]*
YAA 013	*pGcn4-W120A / CEN URA3*
YAA 029	*cdc34-2 GCN4-6HA [Prs316 / CEN URA]*
YAA 030H	*SMT3-GCN4-6HA::Kl TRP1*
YAA 031	*gcn4Δ::kanMX [p-Gcn4-W120A, Δ(129-126) /CEN URA3]*
YAA 032	*pGcn4-6HA RPB1-FRB Anchor Away strain*
YAA 033	*gcn4Δ::kanMX [pGcn4-F97,98R, W120A, Δ(123-126)-6HA / CEN URA3]*
YAA 034	*SMT3-GCN4-6HA::Kl TRP1 cdk8Δ::KanMX6*
YAA 035	*gcn4Δ::kanMX [pGcn4-M107A Y110A L113A W120A Δ(123-126)-6HA/CEN URA3]*
YAA 036	*gcn4Δ::kanMX [pGcn4- F97,98A M107A Y110A L113A W120A]*
YAA 038	*gcn4Δ::kanMX [pGCN4-flag /CEN URA3] RPB1-FRB Anchor Away strain]*
YAA 039	*gcn4Δ::kanMX [pGCN4-6HA / CEN URA3] gal11Δ::KanMX6]*
YER 026	HF7c *[pGalDB-Gcn4ΔCT-6HA]*

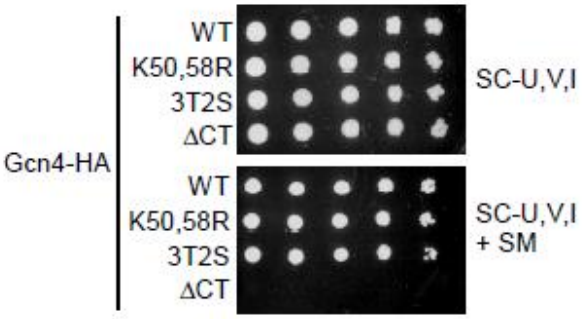
Strains	Genotype
YER 027	HF7c *[pGalDB-6HA]*
YER 028	HF7c *[pGalDB-Gcn4ΔCT-K50,58R-6HA]*
YER 029	HF7c *[pGcn4ΔCT-6HA]*

Table 2: Primers used for activation mutant Gcn4 strains

Mutant Strains	Direction	Sequence (from 5' to 3')
W120A	Sense	CCTAGAAGACAACTCTAAAGAAGCGACATCCTTGTTTGAC
	Anti-sense	TTCTTTAGAGTTGTCTTCTAGGTTTTCATACTCAAAC
W120A Δ123-126	Sense	TCTAAAGAAGCGACATCCATTCCAGTTACCACTGACG
	Anti-sense	GGATGTCGCTTCTTTAGAGTTGTCTTCTAGGTTTTCATACTC
F97,98R W120A Δ123-126	Sense	GCTGTAGTGGAATCTGCCGCGTCGTCAAGCACTGAT
	Anti-sense	AGATTCCACTACAGCGTCATCTAGCTCCGG
M107A Y110A W120A Δ123-126	Sense	TCAAGCACTGATTCAACTCCAGCGTTTGAGGCCGAAAACGCG GAAGACAACTCTAAA
	Anti-sense	TGGAGTTGAATCAGTGCTTGACGAAAAGAAAGATTCCACTAC
M107A Y110A F97,98R W120A Δ123-126	Sense	TCAAGCACTGATTCAACTCCAGCGTTTGAGGCCGAAAACGCG GAAGACAACTCTAAA
	Anti-sense	TGGAGTTGAATCAGTGCTTGACGAAAAGAAAGATTCCACTAC

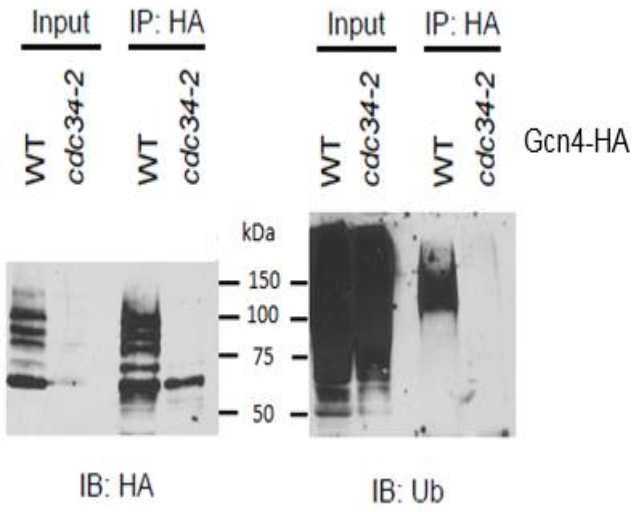
Table 3: Primers used for qPCR (ChIP and RT-PCR)

Target Gene	Region	Primer Sequence (from 5' to 3')	Direction
ARG1	Promoter	GACGGCTCTCCAGTCATTTAT	Sense
		TTCCATACGGCACCGTTAAT	Anti-sense
ARG1	ORF	ACGGTACTGTCAGGGTTAGA	Sense
		GGTGGTATCGGTAGGTAAGAAAC	Anti-sense
CPA2	ORF	GCTGCTGAAAGGGTCAAATAC	Sense
		AAACCTGAGCCTAACCCACCCAAA	Anti-sense
PMA1	ORF	CTGGTCCATTCTGGTCTTCTATC	Sense
		TCAGACCACCAACCGAATAAG	Anti-sense
25S	ORF	TCTAGCATTCAAGGTCCCATTC	Sense
		CCCTTAGGACATCTGCGTTATC	Anti-sense



Appendix Figure 1: Characterization of SUMO-mutant and phosphorylation impaired Gcn4. Spot assay for comparison of growth of the indicated strains. Strains were serially diluted and spotted on synthetic medium lacking Ura, Val and Ile (*top*) and the same medium containing 0.5 μg/mL SM (*bottom*), and incubated for 3 days at 30 ˚C.

A.



B.

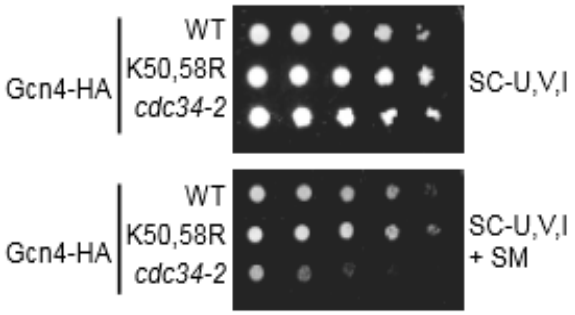


Figure 2: Characterization of ubiquitin mutant Gcn4. ***(A)*** Immunoblots analysis of IPs from Gcn4-WT and ubiquitin-impaired Gcn4 cells treated with MG132 ((75µM for 45 min) followed by SM induction (0.5 µg/mL for 20 min). IPs were then analyzed by anti-HA (1:1000) and anti-poly Ub (1:1000) antibodies. ***(B)*** Spot assay for comparison of growth of the indicated strains. Cells were serially diluted and spotted on synthetic medium lacking Ura, Val and Ile (*top*) and the same medium containing 0.5µg/mL SM (*bottom*), and incubated for 3 days at 30 ^{0}C.

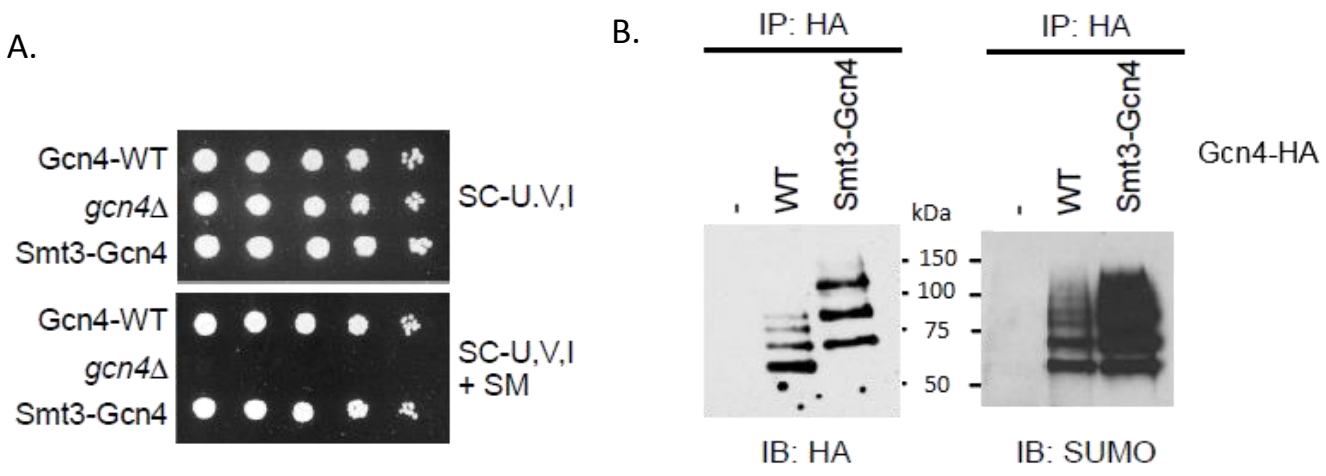


Figure 3: Characterization of hyper-sumoylated Smt3-Gcn4 fusion strain. *(A)* Spot assay for comparison of growth of the indicated strains. Strains were serially diluted and spotted on synthetic medium lacking Ura, Val and Ile (*top*) and the same medium containing 0.5µg/mL SM (*bottom*), and incubated for 3 days at 30 ^{0}C. *(B)* HA and SUMO immunoblots analysis of IPs from indicated yeast strains. Cells were treated with SM (0.5 µg/mL for 20 min) to induce Gcn4 expression.

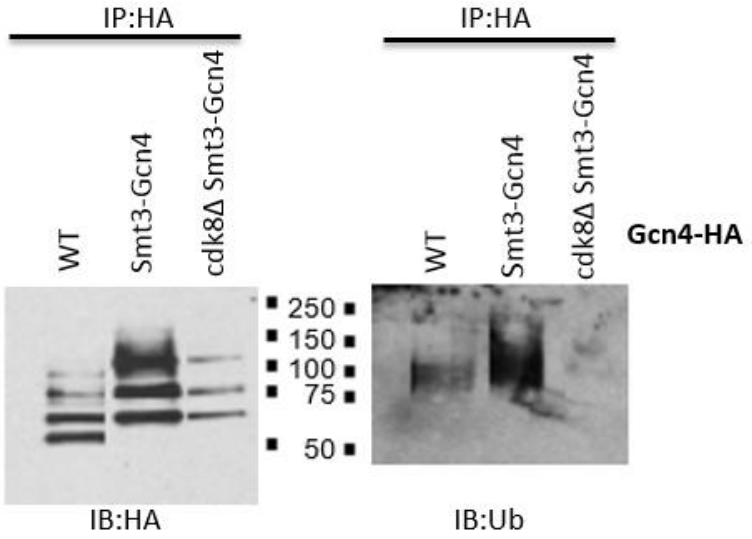


Figure 4: Characterization of hyper-sumoylated Smt3-Gcn4 fusion strain. *A)* Immunoblots analysis of IP's from Gcn4-WT, SUMO-fusion and *cdk8* deleted SUMO-fusion Gcn4 strains treated with MG132 ((75μM for 45 min) and SM (0.5 μg/mL for 20 min) IPs were then analyzed by anti-HA (1:1000) and anti- poly Ub (1:1000) antibodies.