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Regulation of SAGA by the N-terminus of Spt7 in *Saccharomyces cerevisiae*

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biochemistry

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REGULATION OF SAGA BY THE N-TERMINUS OF SPT7 IN *SACCHAROMYCES
CEREVISIAE*

(Thesis format: Monograph)

By

Dominik Dobransky

Graduate program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Spt7 is a 1,332 residue protein critical for maintaining structural integrity of the SAGA complex. I demonstrated that the extreme N-terminus of Spt7 plays an important role in SAGA function. Deletion of the first 73 (Spt7₇₃₋₁₃₃₂) and 121 (Spt7₁₂₁₋₁₃₃₂) N-terminal residues resulted in slow growth, decreased transcriptional activation at *PHO5* and *INO1*, and a partial decrease in acetylation at lysine 18 of histone H3 at *PHO5*. The Spt7₁₂₁₋₁₃₃₂ mutant did not affect Spt7's association with Gcn5 or Tra1, or its localization within the cell. Mutation of the first four positively charged residues to glutamine severely reduced PtdInsP binding by Spt7. I mapped this binding to the first two positively charged residues, arginine 4 (R4) and lysine 8 (K8). Overall, the N-terminus of Spt7 is important for the function of SAGA, likely in part through its ability to bind PtdInsPs.

Key words: Transcriptional regulation, SAGA complex, Spt7, Gcn5, acetylation, Phosphatidylinositol phosphate, Tra1, Tti2

Co-authorship Statement

Work from Genereaux *et al* (2012) is included in this thesis. I performed all of the fluorescence microscopy shown in **Figure 3.12**, **3.13**, and **3.14**, with the assistance of Dr. Karagiannis, who kindly allowed me to use his microscope. In addition, I analyzed the visual data and calculated the ratio of nuclear to cytoplasmic eGFP signal for Tra1-F3744A in the context of wild-type Tti2 or the Tti2-F328S suppressor, as illustrated in **Table 3.1**. Generation and preparation of the *Saccharomyces cerevisiae* strains used for microscopic analysis was done by Dr. Brandl. The growth assay shown in **Figure 1.5** was also done by Dr. Brandl.

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It was of course a pleasure to share and discuss my work with my co-supervisors, Dr. Ball and Dr. Litchfield, as they always provided me with great feedback and encouragement; thank you both! Another professor who helped me tremendously in my work was Dr. Karagiannis, who taught me the subtle ways of fluorescence microscopy, and allowed me to use his microscope to achieve the visual results presented in this thesis. Next, I would like to thank Julie Genereaux for all of her amazing support in the lab. Without her I would undoubtedly be very lost at times. In addition, I would also like to extend a big thank you to Steven Hoke, Stephanie Kvas, Lance DaSilva, Tom Kolaczyk, Simon Lam, Samantha Pillon and Aaron Simkovich, as well as all other past and present members of the Biochemistry Department for making my two years a great experience: you know who you are!

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List of Abbreviations

Ada	Alteration/Deficiency in Activation
ASTRA	Assembly of Tel, Rvb, and Atm-like kinase
CHD	Chromatin organization modifier, Helicase, and DNA-binding domains
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DUB	Deubiquitylation
EDTA	Ethylenediaminetetraacetic acid
Fab	Forms Aploid and Binucleate cells
FAT	FRAP, ATM, TRRAP
FATC	FRAP, ATM, TRRAP, C-terminus
Gcn	General Control Nonderepressible
GFP	Green Fluorescence Protein
GTF	General Transcription Factor
HAT	Histone Acetyltransferase
HEAT	Huntintin, Elongation factor 3, A subunit of protein phosphatase 2A and Tor1
INO	INOsitol requiring
ISW	Imitation SWitch subfamily
MDa	Megadalton
mRNA	Messenger RNA
NuA4	Nucleosomal Acetyltransferase of histone H4
PAGE	PolyAcrylamide Gel Electrophoresis
PIC	Pre-Initiation Complex
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
PtdInsP	Phosphatidylinositol Phosphate
RFP	Red Fluorescence Protein

RNA	Ribonucleic Acid
RNA Pol II	RNA Polymerase II
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SALSA	SAGA Altered, Spt8 Absent
SC	Synthetic Complete
SDS	Sodium Dodecyl Sulfate
SLIK	SAGA-Like
SNF	Sucrose NonFermenting
SPT	SuPpressor of Ty
STAGA	Spt3-Taf9-Ada-Gcn5-acetyltransferase
SWI	SWItching deficient
TAF	TATA-binding protein associated factor
TBP	TATA-binding protein
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween 20
TFII	Transcription Factor II
TRAAP	TRansformation/tRanscription domain-Associated Protein
Tti	Two Tel2-Interacting protein
TTT	Tel2-Tti1-Tti2
Vps	Vacuolar Protein Sorting
YPD	Yeast Peptone Dextrose

Chapter 1: Introduction

SAGA (Spt-Ada-Gcn5-Acetyltransferase) regulates approximately 10% of yeast genes, most of which are involved in stress response. Although the SAGA complex has been studied extensively, the exact role of many of its subunits is not known. Spt7 is a 1,332 amino acid residue protein that acts as a core component of the SAGA complex in *Saccharomyces cerevisiae*. Along with Spt20 and Ada1, Spt7 is required for the structural integrity of the complex (Sterner *et al.* 1999). An Spt7 deletion results in very slow growth, severe phenotypes under conditions of stress, as well as transcriptional defects at SAGA-targeted genes. The human form of Spt7, STAF65 γ , is 414 amino acid residues long, and is homologous to the 543 C-terminal end of yeast Spt7. For the most part, previous studies have focused on elucidating the function of Spt7 via examination of its C-terminal region, which contains a histone-fold motif, as well as a site which controls its interaction with another SAGA component, Spt8. In addition, the C-terminus contains a cleavage site, which produces an alternate version of Spt7, Spt7^{SLIK}, found in the SAGA-Like (SLIK) complex. Prior to undertaking this study, the lab of Fred Winston found that deletion of the extreme N-terminus of Spt7 (upstream of residue 851) resulted in partial function of the protein, similar in phenotype to strains carrying C-terminally truncated Spt7. I found that deletion of the N-terminus (up to residue 73) was consistent with this decreased growth phenotype demonstrated by Wu and Winston (2002). This led us to suggest a role for the N-terminus in Spt7 function. In addition, we observed that every fourth residue for the first 28 N-terminal residues is either a lysine or arginine. Since phosphatidylinositol phosphate

(PtdInsP) binding motifs often consist of a pattern of positively charged residues, we predicted that SAGA may be partly regulated by the binding of PtdInsPs by Spt7.

Besides furthering our understanding of Spt7's functioning in yeast, I was also involved in studying the role of Tra1, SAGA's largest subunit. My main goal was to determine the function of the FATC domain via the nature of suppression of the Tra1-F3744A mutant by an allele of Tti2 harbouring a phenylalanine to serine mutation at residue 328 (Tti2-F328S). I contributed to this research by performing fluorescence microscopy to track the localization of eGFP-tagged Tra1-F3744A, and whether any mislocalization due to the mutation would be rescued by the presence of the suppressor.

1.1 Transcriptional regulation

Eukaryotic transcription is a highly regulated process that involves numerous protein factors acting in a co-ordinated manner. These factors often function as part of large multi-subunit complexes acting in response to a wide variety of environmental signals. In general, transcription requires a core promoter as the platform for the assembly of the pre-initiation complex (PIC) consisting of several general transcription factors (GTFs) along with RNA polymerase II (pol II). The GTFs include TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, as well as TFIIH, and are recruited to the promoter via the TATA-binding protein (TBP) which is part of the TFIID (transcription factor II D) complex. Specifically, TFIIA and TFIIB bind TFIID/TBP in a sequential manner after TFIID is docked at target DNA sites (containing TATA consensus sequences) during formation of the PIC (Buratowski *et al.* 1989). TBP is also found individually within the nucleus, and can be bound directly by the

SAGA complex to facilitate its successful recruitment to the promoter and thus facilitate transcription of SAGA-targeted genes (Sterner *et al.* 1999). The assembly of the PIC alone on the core promoter is sufficient for basal transcription (reviewed by Kronberg 2001; Hahn and Young, 2011). However, gene specific regulation requires activators or repressors which bind to specific sites on DNA to facilitate or prevent transcription, respectively. Furthermore, there are a number of transcriptional co-regulators that bind to, and regulate the mechanism of action of various transcription factors on target DNA (**Figure 1.1**) (reviewed by Thomas and Chiang, 2006).

Except for sites encoding some housekeeping genes, most DNA in the cell is tightly wound around two molecules of each of the conserved histone proteins (H2A, H2B, H3, and H4). The structures formed by DNA and histone proteins, nucleosomes, are further compacted into a highly condensed chromatin structure during periods of transcriptional inactivity. This highly compacted DNA must be unwound and spatially accessible for the binding of GTFs, activators, coactivator complexes and RNA polymerase II in order for transcription to commence. This is achieved by the combined action of chromatin remodelers, which utilize ATP to physically slide (Fazzio and Tsukiyama, 2003), or displace (Boeger *et al.* 2004) histone octamers from target DNA. Four different families are found in yeast, including Swi/Snf, Isw, Chd, and Ino80; their mechanisms of action differ such that the Swi/Snf class of remodelers affect nucleosome stability, and the Isw class facilitates nucleosome sliding (reviewed by Rando and Winston, 2012).

Chromatin modifying complexes also play a key role in regulating transcriptional dynamics. There are at least eight known types of chromatin modifications: acetylation,

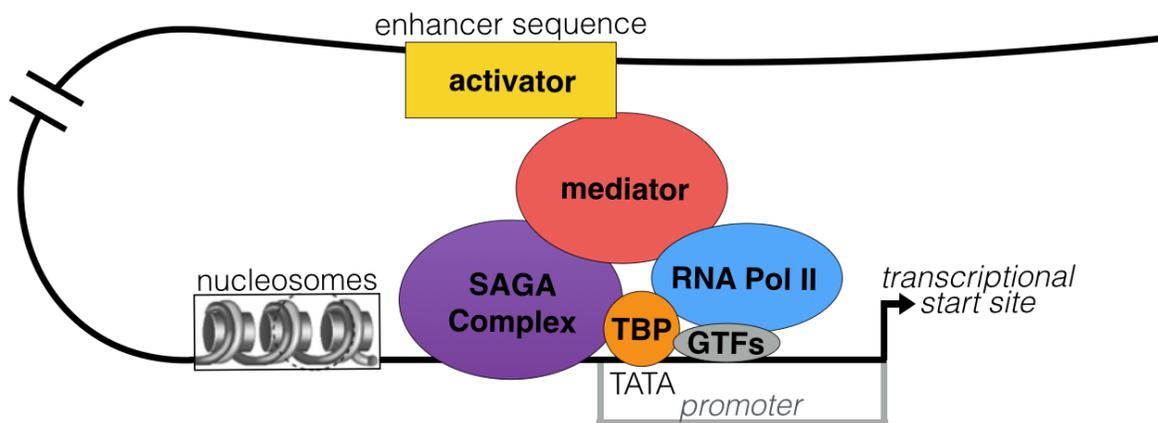


Figure 1.1: The intricate interplay of activators, chromatin remodelers, general transcription factors, and coactivator complexes drives RNA polymerase II transcription. Gap in DNA represents any number of base pairs (Figure adapted from Kvas, 2012). Illustration of nucleosomes courtesy of Elsevier Inc. Kelley's Textbook of Rheumatology)

methylation, ubiquitylation, phosphorylation, sumoylation, ADP ribosylation, deimination and proline isomerization. Acetylation, the most common form of modification, is driven by acetyltransferases that add acetyl groups to lysine residues on histone tails. These N-terminal tails constitute approximately 30% of the mass of histones; however they are not visible when subjected to crystallography due to their highly unstructured nature. The positive charges on the lysine residues are thought to neutralize electrostatic interactions between the negative phosphate backbone of the DNA and the positively charged histone proteins. Thus, acetylation coincides with transcriptional activation via its disruption of compact chromatin structure (Hebbes *et al.* 1988). Methylation, on the other hand, largely drives chromatin to its transcriptionally repressive state, although this is dependent on the exact residue being modified (reviewed by Zhang and Reinberg, 2001; Krebs, 2007).

Ubiquitylation acts to activate transcription, and may also facilitate gene silencing (Henry *et al.* 2003). One specific example showcasing the interdependence of histone modifications was illustrated by Sun and Allis (2002), where they showed that Rad6-mediated ubiquitylation of histone H2B in yeast facilitates methylation of lysine 4 (K4) on histone H3, and thus leads to transcriptional repression. Phosphorylation, another highly studied histone modification, typically leads to transcriptional activation. One such specific example can be illustrated by the known action of the Snf1 kinase, which phosphorylates serine 10 (Ser10) on histone H3 at *INO1*; this phosphorylation signals the recruitment of the SAGA complex to the *INO1* promoter, which subsequently leads to acetylation of K14 on histone H3 by the SAGA acetyltransferase subunit, Gcn5 (Lo *et al.* 2005). This cascade of histone modifications at *INO1* is also complemented by the action of the Ino80 and Swi-Snf

remodelling complexes, which act to coordinate transcriptional activation in an activator dependent manner (Ford *et al.* 2008). Such examples described here offer only a glimpse into the complexity and high level of coordination required to drive transcription forward in a eukaryotic cell that is constantly subjected to changing environmental cues.

1.2 SAGA complex

The SAGA complex is a 2.0MDa transcriptional coactivator composed of 19 subunits (**Table 1.1**). First identified for its histone acetylase activity by Grant *et al* (1997), SAGA has since been found to regulate expression of approximately ten percent of the yeast genome; the majority of these genes are involved in stress response (Huisinga and Pugh, 2004). The remaining ninety percent of RNA polymerase II-mediated transcription is regulated by TFIID, which plays more of a housekeeping role and acts to coordinate down-regulation of housekeeping genes under conditions of stress (Huisinga *et al.* 2004). Wu *et al* (2004) determined the 3D structure of SAGA using electron microscopy, via which they found that the complex consists of five distinct modules (**Figure 1.2a**). This was confirmed later by Lee *et al* (2011) who found the same organization through a combination of mass spectrometry and computational methods (**Figure 1.2b**). The different components that make up these five modules are described below.

The first set of proteins to mention are the TATA Binding Protein Associated Factors (TAFs): TAF5, TAF6, TAF9, TAF10 and TAF12. Besides being present in SAGA, TAFs also make up a portion of TFIID. TAFs are highly conserved proteins and contribute to the backbone of SAGA. Interestingly, TAF5, 6, and 10 share similar spatial orientation between

Table 1.1: List of all identified SAGA components

SAGA Subunits	
Ada1	Spt20
Ada2	Sus1
Ngg1/Ada3	Taf5
Gcn5	Taf6
Sgf11	Taf9
Sgf29	Taf10
Sgf73	Taf12
Spt3	Tra1
Spt7	Ubp8
Spt8	

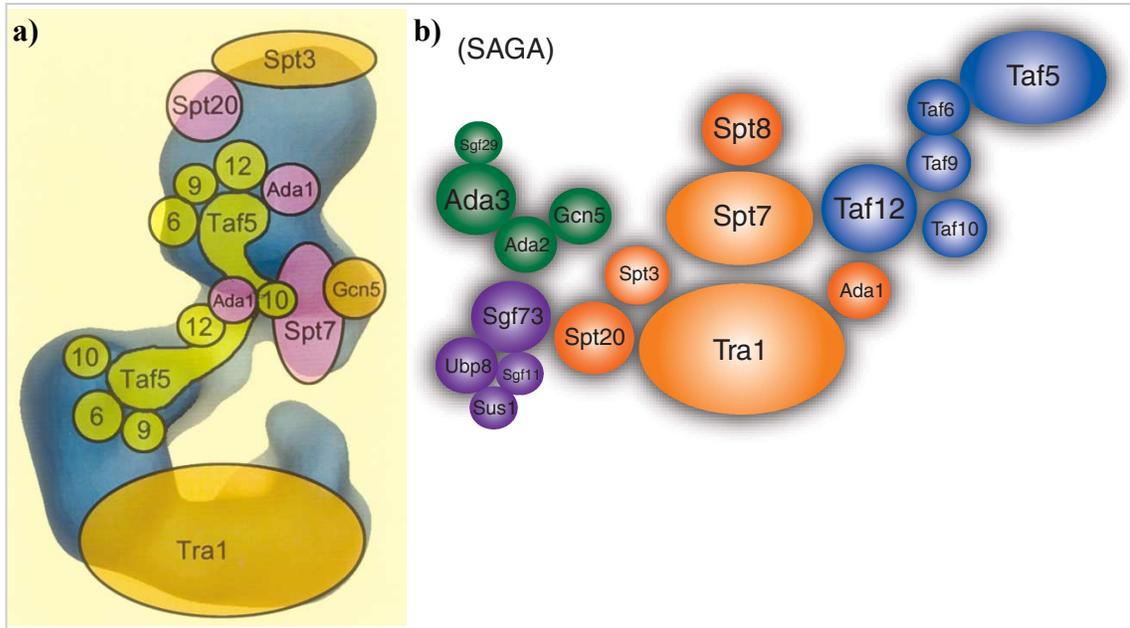


Figure 1.2: Schematic representation of the SAGA complex and the two dimensional spatial distribution of subunits. **a)** Determined by electron microscopy. Reprinted from Wu *et al* (2004) with permission from Elsevier: licence ID 3454440423263 (Copyright Clearance Centre). **b)** Determined by combinatorial depletion analysis/mass spectrometry and computational methods. Reprinted from Lee *et al* (2011) under the *Creative Commons Attribution License*.

SAGA and TFIID, whose functions overlap at some RNA Pol II transcribed genes (Huisinga *et al.* 2004).

The largest subunit of SAGA is Tra1, which makes up the next class of proteins and occupies its own module within the complex. Tra1 is an essential gene and a member of the PIKK (phosphoinositide-3-kinase-related kinase) family (Saleh *et al.* 1998). It binds to acidic activators, such as Gcn4 and Gal4, providing one way of targeting the SAGA complex to specific promoters (Brown *et al.* 2001; Bhaumik *et al.* 2004; Reeves and Hahn, 2005). Apart from SAGA, Tra1 is also present in the NuA4 and ASTRA complexes, as identified by Allard *et al.* (1999) and Shevchenko *et al.* (2008), respectively. Tra1 will be discussed later in more detail.

The Ada (alteration/deficiency in activation) group of proteins form the next family of proteins in the SAGA complex, consisting of Ada1 through 5. The Adas were originally identified via their suppression of toxicity as a result of GAL4-VP16 overexpression in yeast (Berger *et al.* 1992). The Ada4 subunit, known as Gcn5 (general control nonrepressed), was observed in 1996 by Brownell *et al.* to possess HAT activity; this was initially presumed through identifying the homologous, and catalytically active p55 subunit of histone acetyltransferase A (HAT A) found in *Tetrahymena thermophila* (Brownell and Allis, 1995). Gcn5 contains a bromodomain, a region within transcription factors that binds acetylated lysines and that helps coordinate transcriptional activation (Winston and Allis, 1999). Interestingly, the Gcn5 bromodomain is necessary for the stabilization of the Swi/Snf remodelling complex on the promoter after Gcn5-mediated histone acetylation (Syntichaki *et al.* 2000). Gcn5, along with Ada2, Ngg1 (Ada3) and Sgf29, make up the

ADA module in SAGA, which is also present as its own entity in the nucleus (Grant *et al.* 1997; Lee *et al.* 2011). This finding was complemented by Wu *et al.* in 2002, who looked at phenotypes of different SAGA component deletions. They found that a *gcn5 Δ spt7 Δ* phenotype is notably worse than either deletion alone, signifying that Gcn5 still exhibits catalytic activity outside of its association with the SAGA complex. Within the ADA module, Ada2 enhances acetylation by Gcn5, while Ngg1 confers lysine specificity (Balasubramanian *et al.* 2002). Ada1 plays a non-catalytic role by partially maintaining structural integrity of the complex (Wu and Winston, 2002). Lastly, Ada5, also known as Spt20, is implicated in structural maintenance of SAGA, as well as TBP binding (Sterner *et al.* 1999). It is important to note that neither Ada1 nor Ada5 are part of the ADA complex, as they occupy a different module within SAGA that is responsible for the maintenance of structural integrity (Wu and Winston, 2002; Lee *et al.* 2011).

Aside from acting as a histone-acetylase, SAGA also possesses deubiquitylation activity via its deubiquitylation (DUB) module. This module consists of Ubp8, Sgf11, Sgf73, and Sus1. Of these, Ubp8 is the catalytic subunit; in the context of SAGA, it catalyzes the removal of monoubiquitin from K123 of histone H2B (Daniel *et al.* 2004; Lee *et al.* 2005). Deubiquitylation by Ubp8 results in K4 di- and tri-methylation at histone H3, a mark of active transcription (Daniel *et al.* 2004). In addition, it was found that Ubp8 is required for the incorporation of Sgf11 and Sus1 into SAGA. Conversely, the association of Ubp8 with SAGA is dependent on Sgf11 (Shukla *et al.* 2006). Interestingly, Sus1 links SAGA to mRNA export via its interaction with the Sac3-Thp1 complex, which is a part of the mRNA export machinery (Rodriguez-Navarro *et al.* 2004).

The Spt (SuPpressor of Ty) class of molecules within SAGA consist of Spt3, Spt7, Spt8 and Spt20 (Ada5). Initially, Spt proteins were identified as suppressors of the Ty transposable element insertion mutations in the *LYS2* and *HIS4* genes (reviewed by Winston and Carlson, 1992). Their functions vary within the SAGA complex. As the main focus of this study, Spt7 will be discussed in greater detail in the following section. In brief, its major role elucidated thus far is in maintaining structural integrity of the SAGA complex. Together, Spt3 and Spt8 make up the binding surface through which SAGA interacts with TBP to drive promoter-specific regulation, with the potential to facilitate both transcriptional activation as well as repression (Yu *et al.* 2003; Bhaumik *et al.* 2004; Warfield *et al.* 2004; Mohibullah and Hahn, 2008). However, the presence of Spt3 is independent of Spt8, and vice versa, and deletions in either gene result in only minor phenotypes (Sterner *et al.* 1999). On this note, it was found that Spt7 controls the association of Spt8 with SAGA, while Ada1 and Spt20, which also act to maintain complex integrity, control that of Spt3 (Wu and Winston, 2002).

1.3 Spt7

The bulk of the work described in this thesis was focused on further elucidating the role of Spt7 in the context of SAGA by studying its extreme N-terminal region. As mentioned, Spt7 is a core component of the multi-subunit SAGA complex. The 1,332 residue protein is critical for maintaining structural integrity of the complex alongside the other two core SAGA components Ada1 and Spt20. A deletion of any of these core subunits results in severe phenotypes in yeast and a structurally compromised complex lacking

specific components. In addition, Spt7 was found to regulate the levels of both Ada1 and Spt20, thus likely playing a role in the process through which SAGA is assembled into a fully functional coactivator (Wu and Winston, 2002). The importance of Spt7 is further exemplified by the fact that an Spt7 deletion alters the expression of approximately 10% of yeast genes. Wu and Winston (2002) also suggest that since Spt7 is vital for controlling the levels of several SAGA subunits, SAGA levels may be regulated via the regulation of Spt7 itself. Yeast strains lacking Spt7 exhibit severe phenotypes, including the inability to grow on medium lacking inositol, medium containing galactose, medium lacking lysine, as well as medium containing ethanol (Gansheroff *et al.* 1995; Wu and Winston, 2002; Lee *et al.* 2011). Such variety of phenotypic effects would coincide with compromising SAGA's ability to function properly in response to a constantly changing cellular environment.

Apart from being an integral subunit of SAGA, a second C-terminally truncated form of Spt7, Spt7^{SLIK}, is found in the SLIK (SAGA-like) complex. This C-terminal truncation results from processing which occurs between residues 1125 and 1150 (**Figure 1.3a**) (Sterner *et al.* 2002; Wu and Winston, 2002). This processing coincides with a loss of the Spt8 subunit, as suggested by the alternate name for the SLIK complex; SALSA (SAGA Altered, Spt8 Absent). The lab of Fred Winston (2002) mapped the region responsible for binding Spt8 in SAGA (residues 1151-1180), the deletion of which coincides with a loss of binding to Spt8. It should also be mentioned that truncation of full-length Spt7 at the C-terminus was found to facilitate formation of the SLIK complex, which coincided with the derepression of transcription at the *HIS3* promoter under activating conditions. *HIS3* is normally repressed by the SAGA complex; this example provides a glimpse into the

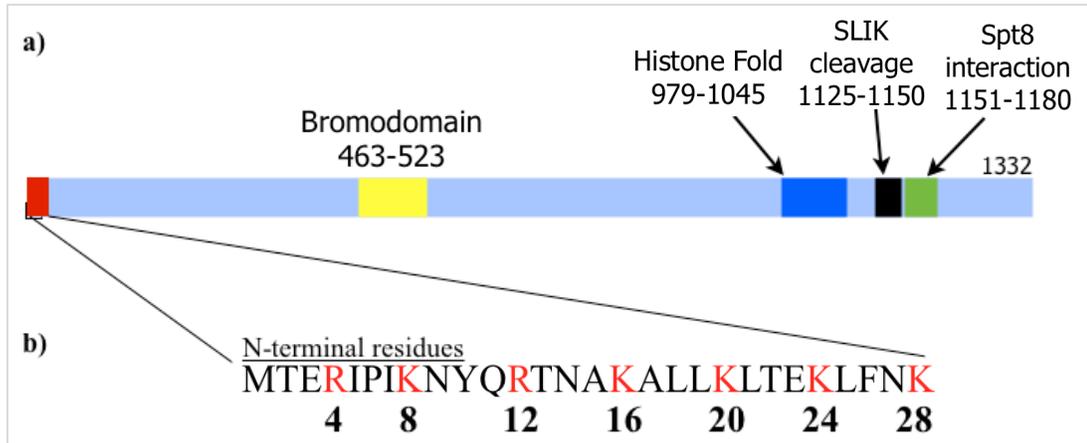


Figure 1.3: a) Schematic diagram of *Saccharomyces cerevisiae* Spt7. b) Every fourth residue of the first 28 residues of *S. cerevisiae* Spt7 is either a lysine (K) or arginine (R).

interplay between SAGA and SLIK, which aids in effectively mediating varied transcriptional cues as a result of environmental changes (Sterner *et al.* 2002). Besides lacking Spt8, SLIK uniquely contains Rtg2, a member of the retrograde response pathway in yeast. This finding potentially connects SLIK with the retrograde response pathway, although a direct link has yet to be established (Pray-Grant *et al.* 2002). Other postulated roles of SLIK include its involvement in the general amino acid control pathway, as strains subjected to amino acid starvation have been found to contain increased levels of SLIK (Belotserkovskaya *et al.* 2000). Interestingly, it was found that impairment of both the SAGA and SLIK complexes in combination showed an additive phenotype in comparison to either deletion alone. This was shown through deletion of subunits (Spt7, Rtg2, Spt8, and Rtg2/Spt8) unique to each complex, followed by phenotypic analysis under varying growth conditions (Pray-Grant *et al.* 2002). A third, still smaller form of Spt7 also exists, and is conveniently termed Spt7^{form3}. Its function and context within which it is found is not known. Interestingly enough, it is as stable as the other two forms of Spt7, suggesting that it too carries a functional role. There is of course the possibility that Spt7^{form3} may represent an intermediate stage in the degradation process of Spt7, as suggested by Hoke *et al.* (2007).

Spt7 contains a histone fold motif (residues 979-1045), through which it interacts with Taf10 (Gangloff *et al.* 2001). Spt7 also contains a bromodomain (residues 463 to 523), a motif which binds acetylated lysines as part of many transcription factors (Winston and Allis, 1999) (**Figure 1.3a**). A deletion in the bromodomain of Spt7, however, causes no detectable phenotype, and is thought to be redundant with that of Gcn5, which does in fact result in minor transcriptional effects upon deletion (Syntichaki *et al.* 2000). In addition,

Spt7 is highly acetylated at its lysine residues, especially around the bromodomain region, the functional consequences of which are unknown (Mischerikow *et al.* 2009). To date, the vast majority of research on Spt7 has been focused on its C-terminus. In addition to containing the site of cleavage which generates Spt7^{SLIK} and interacts with Spt8, the C-terminus of Spt7 has also been attributed to maintaining complex integrity via the finding that expressing the C-terminal 459 amino acid residues of Spt7 complements numerous *spt7* deletion phenotypes (Wu and Winston, 2002). Interestingly, the human counterpart of yeast Spt7, STAF65 γ , only contains the 414 amino acids homologous to the 543 residue C-terminal region of yeast Spt7. This suggests a unique function for the N-terminus of Spt7 in yeast, as well as the possibility that a separate protein has adapted this role in mammalian cells (Wu and Winston, 2002). Deletion of STAF65 γ causes many severe phenotypes, including destabilization of STAGA (Spt3-Taf9-Ada-Gcn5-acetyltransferase; the human counterpart of SAGA), deregulation of Myc-dependent genes and a loss of Spt3-dependent association of STAGA and core Mediator (Liu *et al.* 2008).

Overall, the role of the N-terminal portion of Spt7 has been largely unstudied. An N-terminal truncation lacking the first 873 amino-terminal residues of Spt7 was tested for growth defects in *S. cerevisiae* under several conditions by Wu and Winston (2002). The results showed a decrease in growth comparable in magnitude to that of cells carrying another Spt7 mutant carrying the missing 873 N-terminal residue region under several conditions (YPD, SC, SC -inositol). Because the paper was focused on the C-terminal portion of Spt7, further research to expand on the biochemical consequences of the N-terminal truncation was not pursued (Wu and Winston, 2002). Continuing research into the

role(s) of yeast Spt7 by studying its N-terminus will undoubtedly further our understanding of how this protein functions to regulate SAGA.

Prior to beginning the work presented in this study, our lab discovered a potentially novel motif at the extreme N-terminus of Spt7. This motif contains a positively charged amino acid (either an arginine or lysine) at every fourth residue spanning the first 28 residues (**Figure 1.3b**). It is widely accepted that positively charged residues are highly prevalent in lipid binding motifs (Gozani *et al.* 2003; Rosenhouse-Dantsker and Logothetis, 2007). Also, it was previously found by our lab that the Ada2 subunit of SAGA binds phosphatidylserine (PS), hinting at the possibility for regulation of the SAGA complex through lipid binding (Hoke *et al.* 2008a). The discovery of this potentially novel lipid binding domain points to a yet unstudied role of Spt7, and thus a new way in which SAGA may be partially regulated. For this reason, my research also involved studying this potential lipid-binding property of the N-terminus of Spt7.

1.4 Phosphatidylinositol phosphates

Lipids play a major role in all aspects of cellular functioning. Phosphatidylinositols (PtdIns), which act as key signalling molecules, are comprised of non-polar fatty acid tails attached to a polar inositol head group (Reviewed by Strahl and Thorner, 2005). Phosphorylation of the head group by numerous kinases forms the different species of phosphatidylinositol phosphates (PtdInsPs). In yeast, the PtdInsP species include PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P₂ and PtdIns(4,5)P₂. Each of these is generated by a specific kinase, except for PtdIns(4)P, which can be converted from PtdIns by three

different enzymes: Lsb6, Stt4 and/or Pik1 (Audhya *et al.* 2000; Han *et al.* 2001). PtdIns(4)P is subsequently converted to PtdIns(4,5)P₂ by Mss4 (Audhya *et al.* 2002). PtdIns(3)P is enzymatically converted from PtdIns by the Vsp34 kinase (Schu *et al.* 1993); the subsequent addition of a phosphate group by Fab1 to the fifth position of the inositol ring generates PtdIns(3,5)P₂ (Cooke *et al.* 1998). These two enzymes, Vps34 and Fab1, are of direct relevance to my work.

Each of these lipids plays a unique role in the cell. According to a model of compartmentalization as reviewed by Strahl and Thorner (2007), PtdIns(4)P is targeted to the Golgi and secretory vesicles leaving the Golgi. PtdIns(4,5)P₂ is mostly associated with the plasma membrane. PtdIns(3)P localizes to the endosomal membranes and is also found in multivesicular bodies (MVBs). PtdIns(3,5)P₂ localizes to vacuolar membranes. Although PtdInsPs are found in the nucleus, evidence regarding the exact species present there as well as the nature of their roles in contributing to transcriptional regulation remains limited (Martelli *et al.* 2001; Cocco *et al.* 2001; Shisheva, 2013). One study found phosphoinositide signalling pathways to be associated with nuclear speckles containing pre-mRNA processing factors (Boronenkov *et al.* 1998). It is also valuable to note that PtdIns species are quickly metabolized in response to environmental changes, and thus act as optimal candidates in helping the cell mount an immediate response to a constantly changing environmental state (Dove *et al.* 1997; Perera *et al.* 2004). Since SAGA mostly regulates genes involved in stress response (Huisinga *et al.* 2004), a potential interplay between PtdInsPs and SAGA would provide one efficient way for the cell to adapt to constantly varying cellular conditions. In fact, an example of such interplay has already

been demonstrated by Han and Emr (2011), who showed that in the context of a Gal4-compromised pathway, Tup1-repressed chromatin can be reverted to a transcriptionally active state by the presence of PtdIns(3,5)P₂ at the late endosome and vacuolar membranes. The Tup1-Cyc8 co-repressor complex is recruited by the lipid along with Cti6, resulting in their fusion and switch to the Cti6-Cyc8-Tup1 coactivator complex. The coactivator is then shuttled into the nucleus and subsequently interacts with SAGA to drive transcription at the *GALI* promoter (Han and Emr, 2011). A separate study demonstrated that the localization of Pik1, the phosphatidylinositol 4-kinase, was dependent on the presence of nuclear PtdIns(4)P (Strahl *et al.* 2005). Such evidence links the dynamic role of PtdIns molecules to nuclear processes, presenting an exciting avenue of research. In this light, a portion of my thesis was focused on discovering the implication of Spt7's potential lipid binding ability, and the implications associated with such function.

1.5 Tra1

The final aspect of my thesis was focused on helping determine the mechanism of suppression of a FATC domain mutant of Tra1 (Tra1-F3744A) by a Tti2 allele harbouring a phenylalanine to serine mutation at position 328 (Tti2-F328S). Tra1 is an essential 433kDa protein which directly binds to acidic activators such as Gal4 and Gcn4 to facilitate transcriptional activation by targeting SAGA to specific promoters (Brown *et al.* 2001; Qiu *et al.* 2005). As previously mentioned, it is also part of the NuA4 and ASTRA complexes (Saleh *et al.* 1998; Allard *et al.* 1999; Shevchenko *et al.* 2008). It is part of the PIKK (phosphoinositide-three-kinase-related kinase) family of proteins, although it does not

possess kinase activity (McMahon *et al.* 1998; Saleh *et al.* 1998; Abraham, 2004). This is due to the lack of ATP binding residues required for enzymatic function (Saleh *et al.* 1998; Bosotti *et al.* 2000; Mutiu *et al.* 2007).

It has been a long term objective of the Brandl lab to determine the role of Tra1 in the cell. Such knowledge would not only be useful for further understanding of transcriptional regulation and protein dynamics in yeast, but would also be highly relevant in shedding light on Tra1's human homolog, TRRAP (TRansformation/TRanscription domain-Associated Protein). TRRAP is important in that it regulates the transcription of genes involved in oncogenesis by direct interaction with transcription factors such as Myc and E2F (McMahon *et al.* 1998). Tra1's multiple domains include 40-50 N-terminal HEAT domain repeats, a 300 residue PI3K domain further downstream, followed by a 500 residue FAT (FRAP, ATM, TRAP) region as shown in **Figure 1.4**. At the very extreme C-terminus is the FATC (FRAP, ATM, TRAP, C-terminus) domain, which is thought to regulate the catalytic activity of the PI3K domain. One such instance has been illustrated in the context of mTOR, where the FATC domain of the protein was shown to loop back and contact the PI3K domain (Takahashi *et al.* 2000; Sturgill and Hall, 2009; Yang *et al.* 2013). Another interesting example where the C-terminus loops back to interact with the rest of the protein can be found in mammalian DNA-PKc, where the C-terminus interacts with its N-terminal region upon DNA binding to regulate kinase activity (Rivera-Calzada *et al.* 2005). Unlike Spt7, Tra1 is not necessary for maintaining structural integrity of the SAGA complex, as shown by Helminger *et al.* (2011) in the context of *Schizosaccharomyces pombe*; a *tral* knockout mutant is lethal in *Saccharomyces cerevisiae*.

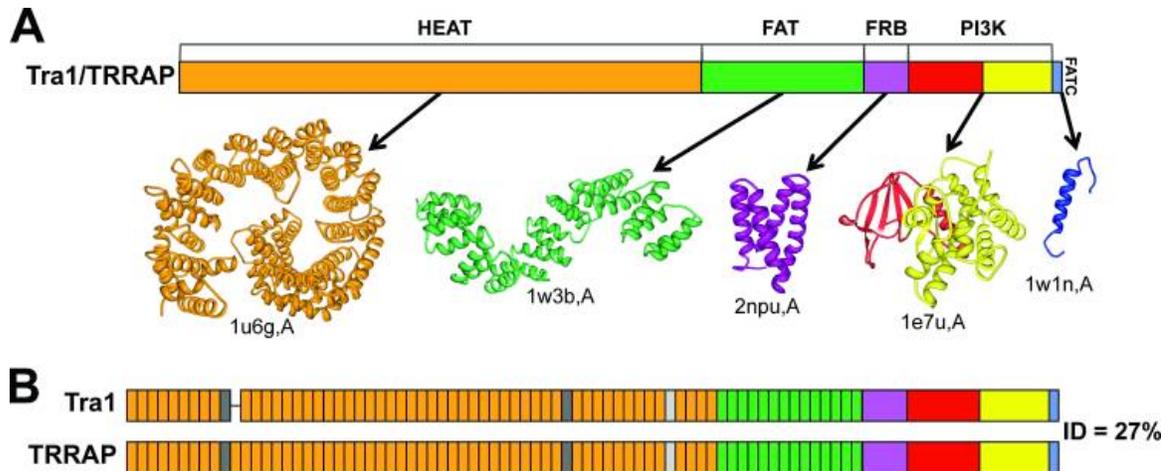


Figure 1.4: **a)** Modular structure of Tra1/TRRAP. The Protein Databank Code is shown below each modular structure **b)** Alignment of domains between Tra1 and TRRAP. Dark grey rectangles represent regions of disorder, and light grey sections represent non-repeating regions. ID, identity. Reprinted from Knutson and Hahn (2011) with the permission of the American Society of Microbiology.

A lack of kinase activity by Tra1 raises the question as to the role of the FATC domain. Given that the domains of PIKK proteins are highly conserved, the FATC region of Tra1 is likely important in another aspect of its function. At the time of my studies, the precise position of the FATC domain was unknown, as the crystal structure of the C-terminal domains of mTOR has only recently been determined (Yang *et al.* 2013). In their publication, Yang *et al.* (2013) show that the FATC domain folds back to interact with the activation loop of the PI3K domain. Prior to this discovery however, multiple potential roles had been proposed for the FATC domain. For example, several studies have implicated the C-terminus of Tra1 in interaction with other proteins. Park and colleagues (2001) showed that upon deletion of the C-terminus of the human homologue TRRAP, Gcn5 binding was lost and HAT activity was abolished. Likewise, studies in other PIKK family members have found the C-terminus to be important in facilitating interaction with other proteins. The FATC domain of Mec1 is needed to interact with Rfa1 and Rfa2 (replication protein A components), driving its recruitment to sites of DNA damage (Nakada *et al.* 2005). Previous studies examining the role of the FATC domain of Tra1 have found that mutations in this region result in slow growth and in cases, inviability of the cell. For example, Hoke *et al.* (2010) showed that an addition of a single glycine residue to the C-terminal end of the domain resulted in loss of cellular viability. Other mutations in the FATC region, such as a change from a leucine to alanine at position 3733 (Tra1-L3733A) results in slow growth and changes in transcription under stressful conditions (Kvas, 2012). This particular L3733A mutation was rescued by a loss of nonsense mediated decay

(NMD), a process which identifies premature stop codons on mRNA in the cell (Muhleman *et al.* 2008).

Our lab has generated mutants of the FATC domain of Tra1 in an effort to understand its function (Hoke *et al.* 2010). Several mutants of interest were found, one of which contained a phenylalanine to alanine change at the terminal FATC residue 3744 (Tra1-F3744A). This mutant showed several effects, including slow growth under various conditions, transcriptional defects, mild effects on SAGA-targeted acetylation, and protein stability (Genereaux *et al.* 2012). Such results suggest a broad importance of the FATC domain on Tra1 function. However, the exact role was yet to be determined. In an effort to uncover this role, our lab set out to identify suppressor mutations that rescue the growth defects associated with Tra1-F3744A. Following next-generation sequencing using the ABI-Solid4 platform, it was discovered that a phenylalanine to serine mutation in residue 328 of Tti2 (Tti2-F328S) suppressed *tral-F3744A*. The level of suppression of Tti2-F328S was slightly better than that of another Tti2 allele harbouring an isoleucine to phenylalanine mutation at position 336 (Tti2-F336I), and was thus pursued further (**Figure 1.5**). Interestingly, Tra1 and Tti2 are both components of the putative ASTRA complex (ASsembly of Tel, Rvb, and Atm-like kinase), making the suppression of Tra1-F3744A by Tti2-F328S that much more intriguing (Shevchenko *et al.* 2008).

1.6 Tti2

Tti2 (Tel two interacting protein 2) is also part of the triple-T complex, alongside Tti1 and Tel2 (Hurov *et al.* 2010). It is thought that the complex aids in the biogenesis and

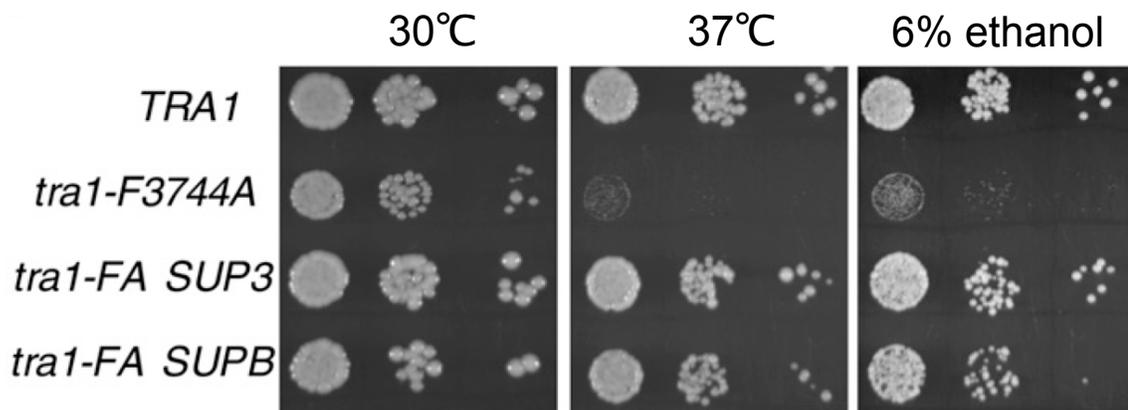


Figure 1.5: Suppression of Tra1-F3744A by Tti2-F328S (SUP3) and Tti2-I336F (SUPB). Yeast strains carrying wild-type Tra1 (CY4353), Tra1-F3744A (CY4350), Tra1-F3744A/Tti2-F328S (CY5667), and Tra1-F3744A/Tti2-I336F (CY5842) containing YCplac33-YHR100C were serially diluted and spotted onto YPD at 30°C, 37°C, and YPD containing 6% ethanol at 30°C (Genereaux *et al.* 2012). Reprinted from Genereaux *et al* (2012) with permission from the Genetics Society of America: licence ID 3456110806992 (Copyright Clearance Centre).

maturation of PIKKs, based on evidence that it binds newly synthesized proteins and thus acts in the maintenance of protein stability (Takai *et al.* 2007; Horejsi *et al.* 2010). To aid in determining the mechanism of suppression of Tra1-F3744A by Tti2-F328S, I set out to examine the effect of Tra1-F3744A on the protein localization within yeast cells using fluorescence microscopy. My aim was also to analyze the localization of both wild-type and Tti2-F328S, but most importantly whether the suppressor mutation in Tti2 would rescue any mislocalization observed with the Tra1-F3744A mutant. Such analysis would help in pinpointing the nature of suppression by the Tti2-F328S allele, and thus provide a narrower understanding of the role of the FATC domain in the context of Tra1, as well as SAGA function.

1.7 Thesis Overview

The N-terminus of Spt7 has been largely unstudied. Based on our initial observations of decreased growth of strains carrying N-terminally truncated Spt7 mutants, as well as our finding of a potential novel lipid binding domain at the extreme N-terminus, **I hypothesized that the N-terminus of Spt7 is crucial in the regulation of the SAGA complex, and that this regulation is partly achieved via Spt7's lipid binding function.**

To test my hypothesis, I examined the effects of a 1-121 amino terminal truncation mutation (Spt7₁₂₁₋₁₃₃₂) on protein expression, growth, transcriptional activation, histone acetylation at lysine 18 of histone H3 (H3K18) of *PHO5*, interaction with the Tra1 and Gcn5 SAGA subunits, as well as localization using eGFP-tagged versions of wild-type and truncated Spt7 mutants under normal growth and stress conditions. I found that the N-

terminal truncation affected expression, as well as growth levels and transcriptional activation at SAGA-targeted promoters. In addition, the Spt7₁₂₁₋₁₃₃₂ mutant caused a partial decrease in acetylation at H3K18. However, interaction of Spt7 with both Tra1 and Gcn5 remained unaltered in context of the Spt7₁₂₁₋₁₃₃₂ truncation protein. Likewise, neither the Spt7₇₃₋₁₃₃₂ nor the Spt7₁₂₁₋₁₃₃₂ mutant caused mislocalization away from the nucleus under normal growth condition or conditions of stress.

Interestingly, Hoke *et al.* (2008a) found that Spt7 bound lipid. I mapped the region of binding to a putative N-terminal lipid binding domain characterized by the presence of a pattern of repeating positively charged residues. The 1-73 amino terminal fragment bound to PtdIns(3)P and PtdIns(3,5)P₂, which was abrogated as a result of mutating the first four positively charged residues to glutamines (R₄QK₈QR₁₂QK₁₆Q). The double mutant R₄QK₈Q also resulted in a loss of binding to both lipid species, while binding by the R₁₂QK₁₆Q mutant was unchanged. These mutants, especially Spt7-R₄QK₈Q, showed minor effects on growth under numerous conditions, suggesting a functional importance to the lipid binding of Spt7. Localization of eGFP-wild-type versus eGFP-Spt7₇₃₋₁₃₃₂ and eGFP-Spt7₁₂₁₋₁₃₃₂ mutants was also tested in strains lacking PtdIns(3)P (*vps34* null strain) and PtdIns(3,5)P₂ (*fab1* null strain) to determine whether the absence of these specific lipid species would affect localization of Spt7. Interestingly, the cellular distribution of eGFP-Spt7 differed in the two strain backgrounds.

I also analyzed how Tra1 localization is affected as a result of the F3744A mutation, and more importantly what effect the Tti2-F328S suppressor has on the cellular distribution of Tra1 to help determine how the FATC domain of Tra1 regulates its function. My part in

determining the nature of suppression of the Tra1-F3744A FATC mutant by Tti2-F328S involved performing fluorescence microscopy. I first tracked the localization of eGFP-tagged wild-type Tti2 and Tti2-F328S, both of which showed nuclear as well as cytoplasmic signal; localization to foci at the vacuolar membrane was more pronounced in context of growth in SC medium containing 6% ethanol. eGFP-Tra1-F3744A showed partial mislocalization to cytoplasmic foci. The precise nature of these foci could not be determined with confidence, as a general overlap with numerous RFP-tagged membrane components was observed. However, the presence of Tti2-F328S lessened the mislocalization effect of Tra1-F3744A, suggesting that this mutation may partly reverse Tra1-F3744A's increased affinity for cytoplasmic membrane components.

Overall, the goal of my thesis was to further our understanding of how Spt7, namely its N-terminal region, regulates the functioning of the SAGA complex in *Saccharomyces cerevisiae*. In addition, I sought to aid in determining how Tti2 regulates Tra1 function via studying Tti2 and Tra1 through the use of fluorescence microscopy. Future research needs to be done to determine precisely how Tti2 acts to regulate the FATC domain of Tra1, and what implication this regulation has on structure/function of the protein. Similarly, the importance of the extreme N-terminus of Spt7 on SAGA function is intriguing. Learning more about how this lipid binding affects Spt7, as well as the regulation of SAGA, could potentially uncover new angles through which we look at, and approach the complex topic of protein complex assembly and/or transcriptional regulation in the cell.

Chapter 2: Materials and Methods

2.1 *Saccharomyces cerevisiae* strains

All *S. cerevisiae* strains used in this study are listed in Table S1 of the Appendix. CY4367, CY4359, CY4384, and CY4385 were engineered by transforming the *spt7* knockout strain FY1093 (*MATa spt7-402::LEU2 his4-917-lys2-173R2 leu2-1 ura3-52 trp1-63 ade8*) with DNA constructs CB2157 (single-copy Spt7_{WT}), CB2154 (single-copy Spt7₁₂₁₋₁₃₃₂), CB2162 (multi-copy Spt7_{WT}), and CB2161 (multi-copy Spt7₁₂₁₋₁₃₃₂), respectively. These strains were used to test for protein expression levels. In addition, FY1093, CY4385, CY4367, as well as CY5616 were used to test for growth phenotypes and acetylation at lysine 18 of histone H3; CY5616 was made by transforming FY1093 with CB2216 (multi-copy Spt7₇₃₋₁₃₃₂). These strains were selected for via growth in medium lacking leucine (*spt7* disruption marker) and tryptophan (plasmid maintenance).

BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was transformed with DD37 (multicopy myc⁹-Spt7₁₋₁₂₀ fragment) to generate the CY6161 strain, which was used to test for growth phenotypes; growth was done in minimal medium lacking uracil to ensure plasmid maintenance. Strains carrying LacZ-constructs tested in this study were made by transforming DNA constructs CB1503 (LacZ-*PHO5*), CB1476 (LacZ-*INO1*), CB2112 (LacZ-SRE element) and CB1533 (LacZ-*RPL53A*) into CY4465 to generate CY5633, CY5634, CY5834 and CY6071. Note that CY4465 is isogenic to FY1093, except that the *spt7* knockout *leu2* marker was switched to *ura3*.

Strains CY6132 and CY6139 were used in the Tra1-Spt7 immunoprecipitation assay. These were made by transforming CY5871 with CB1779/CB2288 (single-copy flag³-Tra1/

single copy *myc*⁹-Spt7_{WT}) and CB1779/CB2293 (single-copy *flag*³-Tra1/multi-copy *myc*⁹-Spt7₁₂₁₋₁₃₃₂), respectively. Plasmid maintenance was selected for via growth in minimal medium lacking leucine (*spt7* knockout marker), tryptophan (*flag*³-Tra1 maintenance) and uracil (*myc*⁹-Spt7 maintenance). CY5871 represents a *spt7* knockout in consortium background, and was engineered by crossing FY1093 with BY4741/BY4742, dissected by Dr. Brandl and selected for *trp*⁻ spore colonies. To generate control strains CY6110 and CY6108 for the Tra1-Spt7 pull-down assay, CB1779 and CB2288 constructs were separately transformed into CY5871, respectively.

To test for Gcn5-Spt7 interaction, CY6099 and CY6308 strains were generated by transforming DD31/DD33 (single-copy *flag*³-Spt7_{WT}/multi-copy *myc*⁹-Gcn5) and DD39/DD33 (multi-copy *flag*³-Spt7₁₂₁₋₁₃₃₂/multi-copy *myc*⁷-Gcn5), respectively.

In order to test for localization of Spt7 using fluorescence microscopy, yeast strains CY6079, CY5922, and CY5921 were made by transforming BY4741 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) with CB1966 (eGFP-Spt7_{WT}), DD29 (eGFP-Spt7₇₃₋₁₃₃₂) and DD28 (eGFP-Spt7₁₂₁₋₁₃₃₂), respectively.

To test for lipid-binding of N-terminal fragments of Spt7, *Escherichia coli* Rosetta strain (Novagen, Inc.) was transformed with N-terminal 1-73 residue fragment mutants DD13 (GST-Spt7-R₄QK₈Q), DD14 (GST-Spt7-R₁₂QK₁₆Q), and DD7 (GST-Spt7-R₄QK₈QR₁₂QK₁₆Q). Rosetta strain was also transformed with DD6 to express the wild-type Spt7₁₋₇₃ fragment. To test for growth of these mutants, CY4465 was transformed with full-length equivalents of these N-terminal mutations DD19 (full-length Spt7-R₄QK₈Q), DD20 (full-length Spt7-R₁₂QK₁₆Q) and CB2279 (full-length Spt7-R₄QK₈QR₁₂QK₁₆Q) to

generate CY5798, CY5795 and CY5797 respectively. CB2157 was used as the Spt7_{WT} carrying construct to generate CY5796.

The *fab1* knockout strains CY6085, CY6087, and CY6086 carrying eGFP-Spt7_{WT} (CB1966), eGFP-Spt7₇₃₋₁₃₃₂ (DD29), and eGFP-Spt7₁₂₁₋₁₃₃₂ (DD28) were made by crossing the CY5871 strain with a *fab1* deletion strain (Open Biosystems). Diploid strains were sporulated and dissected by Dr. Brandl, and selected for tetrads that grew in the presence of G418 (*fab1*Δ marker) and minimal medium lacking leucine (*spt7*Δ marker). The *vps34* deletion strains carrying eGFP-wild-type Spt7 (CY6089), eGFP-Spt7₇₃₋₁₃₃₂ (CY6091), and eGFP-Spt7₁₂₁₋₁₃₃₂ (CY6090) were generated in identical fashion. BY4741 strains CY5854 and CY6062 containing eGFP-wild-type-Tti2 and eGFP-Tti2-F328S were made by Dr. Brandl to test for localization of Tti2.

Diploid yeast strains containing eGFP-Tra1/Tra1 Tti2/Tti2 (CY6029), eGFP-Tra1-F3744A/Tra1 Tti2/Tti2 (CY6025), and eGFP-Tra1-F3744A/Tra1 Tti2-F328S/Tti2 (CY6063) were also generated by Dr. Brandl via mating of BY4741 with a strain containing integrated GFP-tagged Tra1 (wild-type or F3744A mutant). Strains containing RFP-tagged membrane components in the EY0987 background (*MATα his3D1 lys2D0 ura3D0*; Huh *et al.* 2003), kindly provided by Peter Arvidson, were made by crossing with CY6018 strain carrying eGFP-Tra1-F3744A/Tti2.

2.2 DNA constructs

All DNA constructs and oligonucleotides used in this study are listed in Table S2 and S3 of the appendix, respectively. LacZ-*PHO5* (CB1503) and LacZ-*INO1* (CB1476)

constructs were from Mutiu *et al.* 2007. The LacZ-SRE element containing construct (CB2112) was made from a *HIS3* promoter regulated by two SRE (stress responsive) elements in place of the Gcn4 binding site, as described by Hoke *et al.* (2010). The *RPL35A* promoter was made by PCR with oligonucleotides 3203-1 and 3203-2, and cloned into CB1503 using *Bam*HI/*Hind*III to generate the LacZ-*RPL35A* DNA construct, CB1533. All LacZ-promoter fusions were expressed on the LEU2 centromeric plasmid YCp87 (Brandl *et al.* 1993).

CB2157 (single-copy Spt7_{WT}) was made by cloning full-length Spt7 *Not*I/*Eco*RI fragment into YCplac22 single-copy vector carrying *trp*⁺ marker and a myc⁹ tag. CB2154 (single-copy Spt7₁₂₁₋₁₃₃₂) was expressed from the same vector, but cloned into full-length Spt7 carrying plasmid using *Not*I/*Nco*I fragment starting at the N-terminal amino acid residue 121. This fragment was generated via PCR using primers 5741-1 and 5741-2. CB2162 (multi-copy Spt7_{WT}) was made by subcloning *Not*I/*Eco*RI full-length Spt7 into YEplac112 multi-copy vector also carrying *trp*⁺ marker and a myc⁹ tag. CB2161 (multi-copy Spt7₁₂₁₋₁₃₃₂) was cloned into the same plasmid carrying wild-type Spt7 via *Not*I/*Nco*I to delete the base pairs coding for the first 121 amino terminal residues.

The eGFP-Spt7₇₃₋₁₃₃₂ and eGFP-Spt7₁₂₁₋₁₃₃₂ constructs were engineered by cloning pieces of CB2215 and CB2161, respectively, into YCplac33 expressing eGFP from a *DEDI* promoter using *Not*I/*Sst*I; eGFP was generated via PCR using oligonucleotides 5077-1 and 5077-2. CB2288 (multi-copy Spt7_{WT}) and CB2293 (multi-copy Spt7₁₂₁₋₁₃₃₂) constructs used in the Tra1-Spt7 pulldowns were expressed on YEplac195 multi-copy *ura*⁺ myc⁹-carrying

plasmid. Cloning into this plasmid was performed using *NotI/EcoRI* fragments from CB2157 (Spt7_{WT}) and CB2161 (Spt7₁₂₁₋₁₃₃₂).

The flag³-Tra1 (CB1779) was cloned using *NotI/SstI* fragment of Tra1 and ligated into YCplac22 *trp*⁺ single-copy flag³-expressing vector. To determine Gcn5-Spt7 interaction, this same vector (YCplac22) was used to generate DD31 (single-copy Spt7_{WT}) via subcloning of the *NotI/EcoRI* piece of CB2157. DD39 was cloned (Spt7₁₂₁₋₁₃₃₂) via triple ligation (*PstI/NotI* and *NotI/EcoRI*) into YEplac112 *trp*⁺ flag³-multicopy expressing plasmid. DD33 (multi-copy Gcn5) was cloned *NotI/SstI* into YEplac195 *ura*⁺ *myc*⁹-multicopy vector.

The GST-Spt7-R₄QK₈Q (DD13), R₁₂QK₁₆Q (DD14), and R₄QK₈QR₁₂QK₁₆Q (CB2279) 1-73 N-terminal mutant fragments were expressed on pGEX-KGT vector (kindly provided by Eric Ball), and cloned using *BamHI/EcoRI*. Spt7-R₄QK₈Q mutant was generated via PCR using primers 5939-1 and 5799, while the R₁₂QK₁₆Q mutant was made by primers 5939-2 and 5799. The quadruple mutant R₄QK₈QR₁₂QK₁₆Q was generated by PCR using primers 5914-1/5914-2, and triple ligated into CB2157 using *NotI/EcoRI* and *NcoI/EcoRI*. DD19 and DD20 represent full length Spt7 carrying R₄QK₈Q, and R₁₂QK₁₆Q mutations, respectively. Template for PCR was a 1441bp intermediate molecule of Spt7 cut *HindIII/HindIII* out of CB2157 (Spt7_{WT}). After mutagenic amplification of this piece using oligonucleotides 6001-1/5741-2 (R₄QK₈Q), and 6001-2/5741-2 (R₁₂QK₁₆Q), pieces were cloned into CB2157 using *NotI/NcoI*.

To generate the full-length Spt7-R₄QK₈QR₁₂QK₁₆Q carrying mutant, DD7 (1-73 amino terminal coding Spt7-R₄QK₈QR₁₂QK₁₆Q fragment) was sub cloned into DD18 (1441bp

portion of CB2157) via *HindIII/HindIII*, followed by subsequent cloning of this fragment into full length Spt7 (CB2157) using triple ligation *NotI/NcoI - NcoI/EcoRI*. eGFP-tagged Tti2, Tti2-F328S, Tra1 and Tra1-F3744A constructs were engineered as described in Genereaux *et al* (2012). RFP-tagged membrane constituents were generated as described in Huh *et al* (2003).

2.3 Growth assays

Yeast strains were grown to stationary phase in either synthetic dropout media (composed of yeast nitrogen base, glucose, and the appropriate amino acid dropout mixture for strain/plasmid selection) or YPD (a combination of yeast extract, peptone, and glucose to provide a rich growth medium). Four 10-fold dilutions were made, and 4 μ l of each was spotted onto the various media: YPD, medium lacking inositol, YPD containing 4% and 6% ethanol, galactose, 1.2M NaCl, and low phosphate medium. All cultures were grown at 30°C, with the exception of cells grown on YPD medium at 37°C to test for temperature sensitivity.

2.4 β -galactosidase assays

LacZ assays were performed using the *spt7* knockout strain FY1093 carrying the following LacZ-promoter fusions on plasmids: LacZ-*PHO5* (CB1503), LacZ-*INO1* (CB1467), LacZ-SRE/*HIS3* (CB2112), and LacZ-*RPL35A* as the positive control (CB1533). These strains were transformed with the various Spt7 constructs, including wild-type Spt7, Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂. All strains were grown to saturation in synthetic

dropout (SD) medium, washed 3 times with 3ml of sterile water, and grown in appropriate medium for 16 hours. The strain carrying the LacZ-*PHO5* construct on a *LEU2* centromeric plasmid was grown in YPD depleted of phosphate. The strain carrying LacZ-*INO1* was grown in medium lacking inositol. The strain carrying LacZ-SRE/*HIS3* was grown in 6% ethanol and was made from *HIS3* promoter regulated by two SRE (stress responsive) elements in place of the Gcn4 binding site as described by Hoke *et al.* (2010). β -galactosidase assays were performed as previously described by Hoke *et al.* (2010). Activity for each promoter was measured in triplicate.

2.5 Chromatin immunoprecipitation

ChiP assays were performed using anti-H3 (Abcam, Inc ab1791) and anti-AcH3K18 (ab1191), as described by Mutiu *et al.* (2007). Since acetylation at the *PHO5* promoter was being measured, strains CY4367, CY5616, CY4385, and FY1093 were grown in YPD depleted of phosphate. Normalization of input chromatin was done by PCR using oligonucleotides flanking the *PHO5* gene; 6095-1 and 4987-2. Levels of immunoprecipitated *PHO5* promoter were determined using AlphaImager 3400 software (Alpha Innotech, San Leandro, CA) after separation of PCR products (in triplicate) by electrophoresis and ethidium bromine staining. The ratios of the acetylated H3K18 to total histone H3 were calculated as a percentage of the wild-type strain. The same assay was repeated using *PGK1* oligonucleotides 2927-1 and 2927-2, in which case the strains were grown in YPD.

2.6 Western blotting

Yeast extracts were prepared from CY4367 (*cen myc⁹-Spt7_{WT}*), CY4359 (*cen myc⁹-Spt7₁₂₁₋₁₃₃₂*), CY4384 (*2 μ myc⁹-Spt7_{WT}*) and CY4385 (*2 μ myc⁹-Spt7₁₂₁₋₁₃₃₂*) by glass bead lysis (Brandl *et al.* 1993) to test for expression. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad) to ensure consistent loading between samples. Following separation by SDS-PAGE, Western blots were performed using PVDF membranes (Roche) as previously described by Mutiu *et al.* (2007). All flag-tagged proteins were detected using anti-flag (M2; Sigma-Aldrich) primary antibody at a dilution of 1:4000. All myc-tagged proteins were detected using anti-myc (9E10; Sigma Aldrich) primary antibody at the same dilution. Blots were subsequently probed with secondary anti-mouse antibody (IgG HRP conjugate; Promega) at a dilution of 1:10,000. Scanning of films was performed using the AlphaImager 3400 software (Alpha Innotech).

2.7 Immunoprecipitation

After growth of CY6132 (*cen flag³-Tra1 + cen myc⁹-Spt7_{WT}*) and CY6139 (*cen flag³-tra1 + 2 μ myc⁹-Spt7₁₂₁₋₁₃₃₂*) *S. cerevisiae* strains to OD₆₀₀ ~2.0, extracts were lysed via grinding in liquid nitrogen (Saleh *et al.* 1997). Tra1-Spt7 immunoprecipitation was performed using anti-flag M2 magnetic beads (Sigma-Aldrich), as described by DaSilva *et al.* (2013), using 2.5mg of protein extract. To elute, 60 μ l of 2X SDS loading buffer was added to each tube followed by boiling at 95°C for two minutes. Eluent was then transferred to clean Eppendorf tubes, and 20 μ l of each was loaded onto 5% and 12.5% polyacrylamide gels to blot for flag³-Tra1 and myc⁹-Spt7, respectively. Prior to performing

the assay, a Western blot was done to normalize the amount of wild-type Spt7 to that of Spt7₁₂₁₋₁₃₃₂.

2.8 Gel filtration chromatography

Yeast strains CY6099 (*cen* flag³-Spt7_{WT} + 2 μ myc⁹-Gcn5) and CY6308 (2 μ flag³-Spt7₁₂₁₋₁₃₃₂ + 2 μ myc⁷-Gcn5) were grown up to OD₆₀₀ ~2.0, and lysed by grinding in liquid nitrogen (Saleh *et al.* 1997). Extract was prepared in IPP150 buffer (10mM Tris-HCl pH8.0, 150mM NaCl) and 2mg of extract was applied over FPLC Superose 6 HR10/30 column (Amersham Biosciences) at a flow rate of 0.3 ml/min, as described by DaSilva *et al* (2013). Following washing with elution buffer (0.05M 1M NaPhosphate solution, 0.15M NaCl), selected fractions were resuspended in SDS loading buffer and separated on an 8% SDS-polyacrylamide gel. A Western blot was performed using anti-myc antibody (9E10; Sigma Aldrich) to detect for the presence of myc⁹-gcn5 (CY6099) and myc⁷-Gcn5 (CY6308), which were loaded as alternating fractions.

2.9 Fluorescence microscopy

Yeast cells expressing eGFP and/or RFP fusions were grown in synthetic complete medium then diluted 1:4 into synthetic complete medium with or without 8% ethanol. Growth in ethanol was for 18 hours. Prior to visualization, cells were concentrated 10-fold and 4',6-diamidino-2-phenylindole (DAPI) was added to 0.02 mg/mL. Fluorescent images were obtained using a Zeiss Axioskop 2 microscope (at an overall magnification of 630x) driven by ImageJ 1.41 software (National Institutes of Health) and a Scion CFW

Monochrome CCD Firewire Camera (Scion Corporation, Frederick Maryland) using DAPI, RFP and GFP filter sets. Quantification of GFP-signal intensity was performed using ImageJ software (version 1.45). The *freehand selections* tool was used to trace each whole cell and nucleus separately. The *measure* function was used to output the signal intensity per unit area for each selection. To correct for background noise, the average intensity of three background selections adjacent to each cell was subtracted from the signal intensity measured for any cell given. The corrected nuclear intensity was then divided by the corrected whole cell intensity to give a nuclear to cell intensity ratio. The mean from 20 cells was calculated plus/minus a standard deviation (Genereaux *et al.* 2012).

2.10 Lipid overlay assays

Expression of GST-tagged N-terminal Spt7 (Spt7₁₋₄₃₉, Spt7₁₋₁₂₁, Spt7₁₋₇₃) peptides, as well as the Spt7-R₄QK₈Q, Spt7-R₁₂QK₁₆Q, and Spt7-R₄QK₈QR₁₂QK₁₆Q mutants was induced via addition and overnight growth in the presence of 0.5mM IPTG at 15°C, followed by purification using a GST-column and elution with 25mM glutathione (pH 8.0).. Lipid-binding assay was performed as described by Hoke *et al* (2008a), except that 200µg of protein was loaded onto PIP-strips (Echelon biosciences). Lipid-binding was detected by blotting with anti-GST antibody (Upstate) at a dilution of 1:4000 and secondary anti-mouse antibody conjugated to horse radish peroxidase (Promega) at 1:10000.

Chapter 3: Results

3.1 Lack of the N-terminal 121 residues of Spt7 results in decreased protein expression

The Winston lab previously found that an Spt7 truncation mutant lacking the first 873 N-terminal residues decreased cellular growth under condition of stress, including medium lacking inositol. I therefore set out to compare the growth of wild-type Spt7 to two N-terminally truncated mutants (Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂) under different conditions. Prior to testing for growth defects, it was necessary to determine if the N-terminal truncation mutants were expressed at comparable levels to that of wild-type Spt7. I engineered single copy (*cen*) and multicopy (*2μ*) plasmids carrying full length myc⁹-tagged wild-type or Spt7₁₂₁₋₁₃₃₂. After these constructs were transformed into the *spt7* knockout yeast strain FY1093, expression levels were checked by Western blot analysis using α -myc antibody following separation by SDS-PAGE. Spt7₁₂₁₋₁₃₃₂ expressed on a centromeric plasmid showed a notable decrease in levels compared to that of wild-type Spt7 also carried on a *cen* plasmid. Thus, a lack of the first 121 amino terminal residues decreased protein levels of Spt7. This decrease in protein expression was compensated for by expressing Spt7₁₂₁₋₁₃₃₂ on a multi-copy (*2μ*) plasmid, resulting in comparable protein levels to the wild-type expressed on a centromeric plasmid (**Figure 3.1**). Expression of the Spt7₇₃₋₁₃₃₂ mutant protein was also tested, and showed similar levels compared to Spt7₁₂₁₋₁₃₃₂ (data not shown). In addition, **Figure 3.1** showed no differences in expression between either of the lower-weight forms of Spt7 (Spt7^{SLIK} or Spt7^{form3}) when the wild-type (*cen*) and Spt7₁₂₁₋₁₃₃₂ (*2μ*) levels were compared. It should be noted however that Spt7₁₂₁₋₁₃₃₂ produced a greater quantity of protein fragments that ran below the molecular weight of Spt7^{form3} as compared

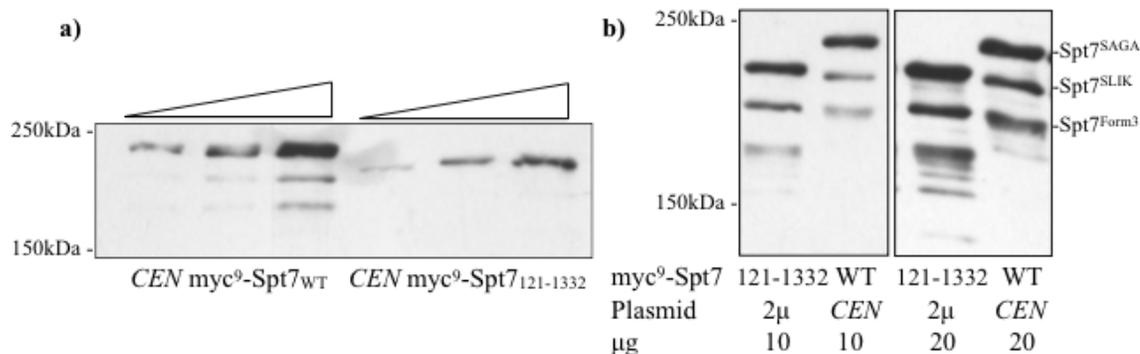


Figure 3.1 Comparing expression levels of Spt7_{WT} and Spt7₁₂₁₋₁₃₃₂. Yeast strains expressing myc⁹-tagged wild-type (CY4367) or N-terminally truncated Spt7₁₂₁₋₁₃₃₂ (CY4359) from centromeric plasmids were grown to mid-log phase in minimal medium. Likewise, yeast strains expressing myc⁹-tagged wild-type (CY4384) or N-terminally truncated Spt7₁₂₁₋₁₃₃₂ (CY4385) from multi-copy (2μ) plasmids were grown to mid-log phase in the same medium. Crude cell lysates were obtained, two-fold serial dilutions were separated by SDS-PAGE and Western blotted with α-myc Ab. **a)** Increasing dilutions of Spt7_{WT} and Spt7₁₂₁₋₁₃₃₂ expressed from a centromeric plasmid **b)** Comparing expression of myc⁹-tagged Spt7_{WT} from a centromeric plasmid versus Spt7₁₂₁₋₁₃₃₂ from a 2μ plasmid.

to the wild-type protein (**Figure 3.1**).

3.2 N-terminal truncations of Spt7 decrease growth under various conditions

Prior to determining specific functions of the N-terminal region of Spt7, it was important to test for general effects of Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂. I tested for the ability of wild-type (on a *cen* plasmid) and Spt7 deletions (on a 2μ plasmid) to grow on various media (**Figure 3.2**). Serial dilutions of strains CY4367 (*spt7* knockout carrying *myc*⁹-wild-type Spt7 on a centromeric plasmid), CY4385 (*spt7* knockout carrying *myc*⁹-Spt7₁₂₁₋₁₃₃₂ on 2μ plasmid), CY5616 (*spt7* knockout carrying *myc*⁹-Spt7₇₃₋₁₃₃₂ on 2μ plasmid), and FY1093 (*spt7* knockout, kindly provided by Fred Winston) were spotted onto the following media: YPD (at 30° and 37°), YPD containing low phosphate, YPD containing 4% and 6% ethanol, YP containing galactose as the carbon source, YPD containing 1.25M NaCl, and medium lacking inositol. Both truncations decreased growth under all tested conditions (**Figure 3.2a**). Some conditions however, such as medium lacking inositol, YPD containing 6% ethanol, and YPD containing 1.25M NaCl resulted in a more pronounced growth defect, which was comparable to that of the *spt7* knockout strain. Interestingly, growth at 37°C resulted in a more severe growth defect in context of both Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ truncation mutants than the knockout.

Given the decrease in growth due to the lack of the first 73 or 121 amino terminal residues, I asked whether expression of a fragment carrying the first 120 N-terminal amino acid residues (Spt7₁₋₁₂₀) would affect growth in the context of a wild-type strain. BY4742 (containing wild-type Spt7) was transformed with *myc*⁹-tagged wild-type Spt7₁₋₁₂₀

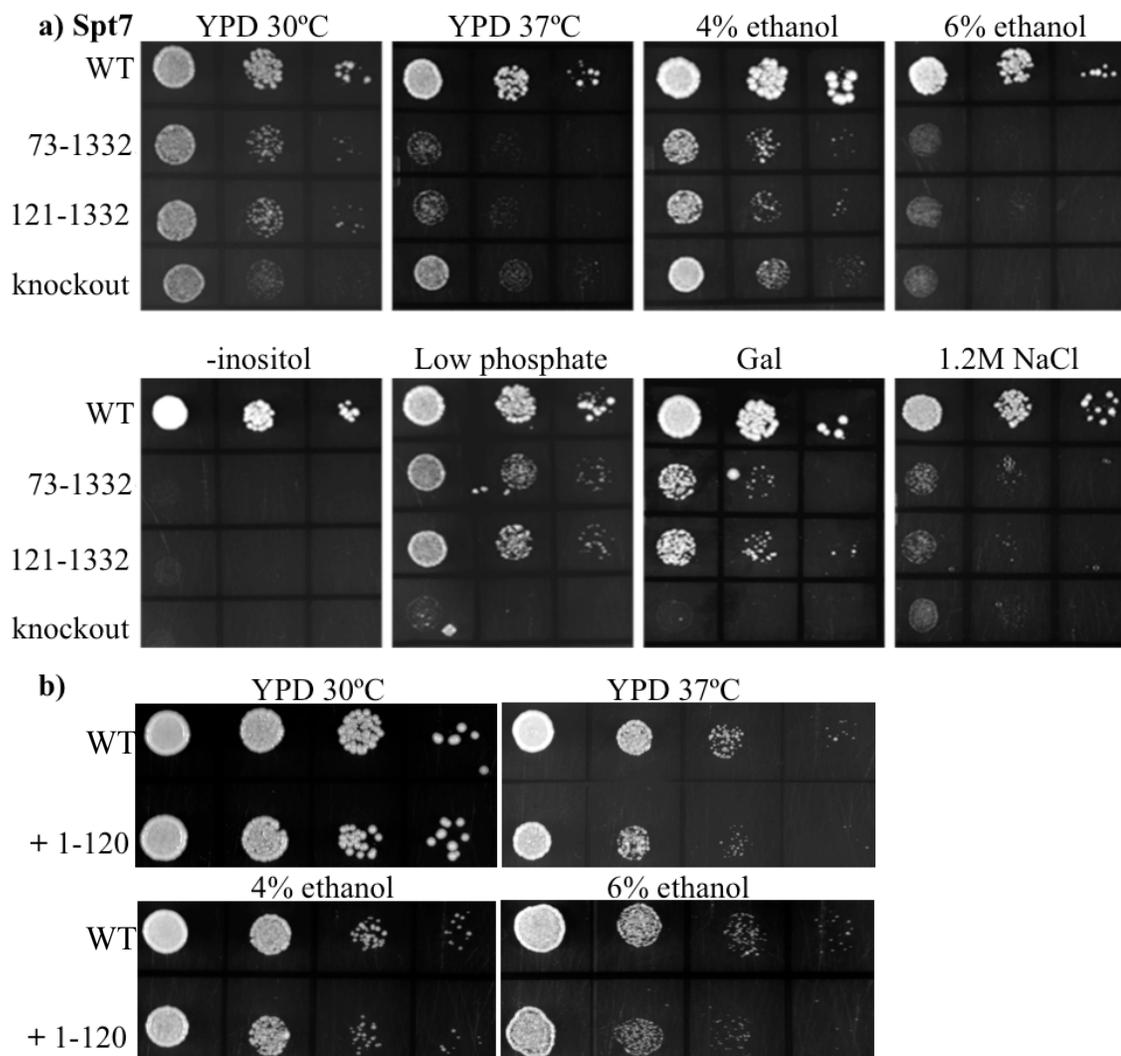


Figure 3.2 Phenotypes of N-terminal truncations of Spt7. **a)** Spt7 deletion strain FY1093 was transformed with full-length wild-type Spt7 expressed on *cen* plasmid. The Spt7₇₃₋₁₃₃₂, and Spt7₁₂₁₋₁₃₃₂ truncated-Spt7 mutants were expressed on 2μ plasmids. Saturated cultures were serially diluted and spotted onto YPD plates (30°C and 37°C), 4% and 6% ethanol, medium lacking inositol, low phosphate, galactose medium, and 1.2M sodium chloride. **b)** KY320 strain was transformed with an N-terminal wild-type 1-120 residue fragment of Spt7 expressed on a 2μ plasmid. Strains either carrying (+ 1-120) or lacking the 120 residue amino terminal fragment were diluted and spotted onto YPD plates (at 30°C and 37°C), as well as on 4% and 6% ethanol as described above.

fragment expressed from a 2 μ plasmid (strain CY6161). The expression of Spt7₁₋₁₂₀ (checked via Western Blot analysis; data not shown) in the context of the wild-type strain showed a minor decrease in growth on YPD and YPD containing 4% and 6% ethanol compared to the wild-type control alone, indicative of a dominant-negative phenotype (**Figure 3.2b**).

3.3 N-terminal truncations of Spt7 reduce transcriptional activation at SAGA-targeted promoters

The growth defects seen as a result of the missing 73 and 121 amino terminal residues of Spt7 suggest a broad effect on SAGA function. I performed β -galactosidase assays to determine whether a lack of the N-terminal 73 or 121 amino acid residues of Spt7 would affect transcriptional activity at *PHO5* and *INO1*. These genes were chosen based on the fact that their expression is SAGA-dependent. As illustrated in **Figure 3.2**, Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ caused notable growth defects on both low phosphate medium and medium lacking inositol. To test for activity at the *PHO5* promoter, cells were grown in YPD lacking phosphate; *INO1* promoter activity was tested in inositol depleted conditions (Barbaric *et al.* 2003; Lo *et al.* 2005). As shown in **Figure 3.3**, the truncation mutants decreased transcript levels comparable to that of the *spt7* knockout strain for both *PHO5* and *INO1*. Transcriptional activity was also tested at a promoter regulated by two SRE (stress responsive) elements. Similar to the results observed at *PHO5* and *INO1*, a loss of expression of approximately 8% of wild-type was seen after growth in 6% ethanol. The magnitude of this effect was also comparable to that of the *spt7* knockout.

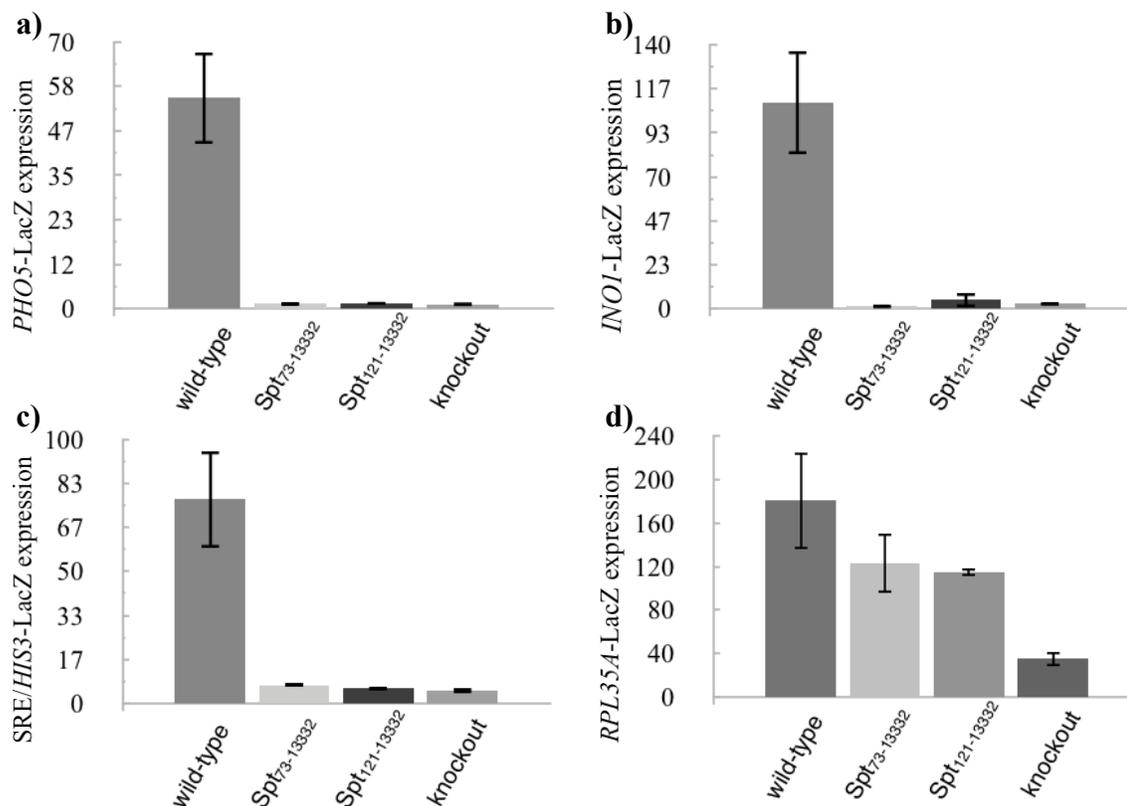
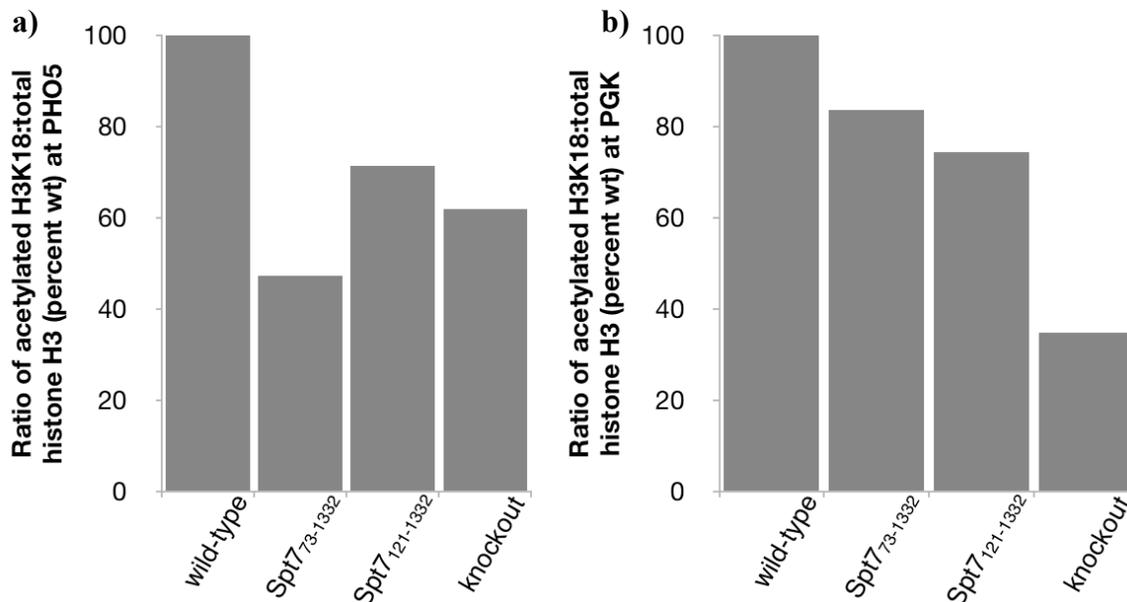


Figure 3.3 Effect of N-terminal Spt7 truncations on transcriptional activation at *PHO5*, *INO1*, an SRE-containing promoter, and *RPL35A*. Strains carrying wild-type Spt7 on *cen* plasmid, along with Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ mutants on 2μ plasmids and a *spt7* knockout strain were tested for transcriptional activation. All LacZ fusions were expressed from a *LEU2* centromeric plasmid. **a)** Strains carrying *PHO5*-LacZ were grown in YPD depleted of phosphate. **b)** Strains carrying *INO1*-LacZ were grown in medium lacking inositol. **c)** Strains carrying SRE/*HIS3*-LacZ were grown in 6% ethanol. **d)** Strains carrying *RPL35A*-LacZ were grown in YPD. β -galactosidase activity for each promoter was measured in triplicate and growth was for 16hrs.

To determine whether this decrease in transcriptional activity was SAGA-specific, transcriptional activation was also tested at *RPL35A*, a ribosomal gene not targeted by SAGA (Huisinga and Pugh, 2004). A partial decrease in activation (~65% of wild-type levels) was seen with both Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂, an effect lesser than that at *PHO5*, *INO1*, and the SRE-containing promoter. The *spt7* knockout strain showed the greatest loss in activity at *RPL35A* compared to either mutant, resulting in a decrease to 20% of wild-type levels (**Figure 3.3**).

3.4 N-terminal truncations of Spt7 partially decreases acetylation of H3K18 at *PHO5* *in vivo*

The severe decrease in transcriptional activity seen with LacZ-*PHO5*, as well as the other promoters tested prompted me to ask whether Gcn5 driven acetylation at the *PHO5* promoter was also affected. Thus, I proceeded to examine the effect of the N-terminal truncations of Spt7 on histone H3 (K18) acetylation expressed as a ratio of total histone H3 at the *PHO5* promoter; the *PGK* promoter was also tested as a control. **Figure 3.4a** shows the mean percentages of acetylated lysine 18 (AcH3K18) to total histone H3 for strains CY5616 (Spt7₇₃₋₁₃₃₂), CY4385 (Spt7₁₂₁₋₁₃₃₂), and FY1093 (*spt7* knockout), all normalized to the AcH3K18/H3 ratio of wild-type carrying strain CY4367. Acetylation at the *PHO5* gene by Gcn5 requires inducing conditions, thus all strains were grown in YPD depleted of phosphate prior to the assay (Barbaric *et al.* 2003). As seen in **Figure 3.4a**, the ratio of AcH3K18 to total H3 was reduced to approximately 50% with the Spt7₇₃₋₁₃₃₂ mutant and 70% in context of Spt7₁₂₁₋₁₃₃₂. A decrease to 60% of wild-type levels was seen in the



3.4 Effect of N-terminal truncations of *Spt7* on histone H3 acetylation at the *PHO5* and *PGK* promoters.

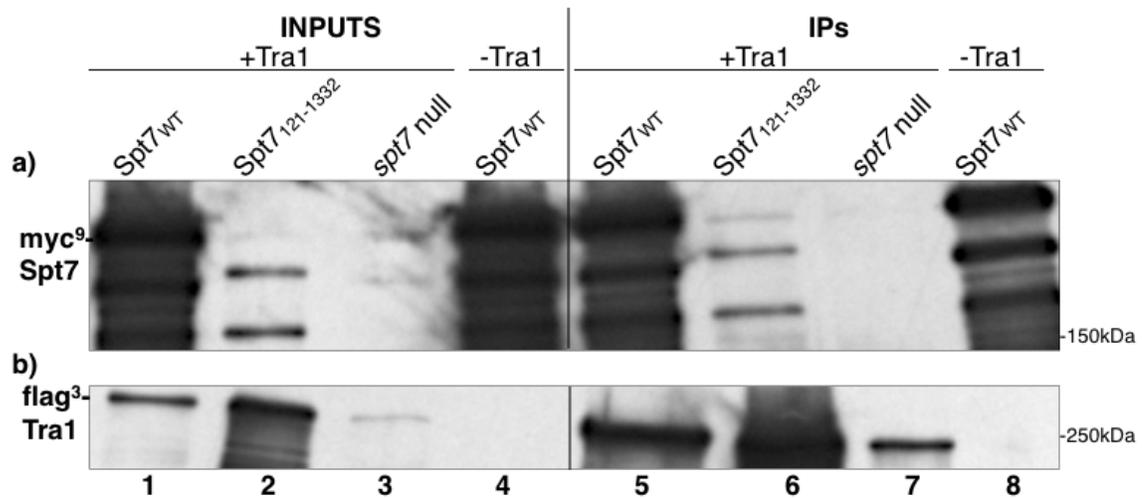
a) Histone H3K18 acetylation at the *PHO5* promoter. Yeast strains CY4367 (WT), CY5616 (*Spt7*⁷³⁻¹³³²), CY4385 (*Spt7*¹²¹⁻¹³³²), and FY1093 (*spt7* knockout) were grown to mid-log phase in YPD depleted of phosphate. ChIP was performed using anti-H3 and anti-H3K18 antibodies. Levels of acetylation at H3 and H3K18 were determined by PCR using primers flanking the *PHO5* gene, and analysis of the products was done following gel electrophoresis on agarose gels stained with ethidium bromide. Serial dilutions in triplicate were performed to ensure a linear range. The results are shown as a percentage of the total H3 levels of the CY4367 (wild-type *Spt7*) strain, and are the average of two independent experiments. **b)** Histone H3K18 acetylation at the *PGK* promoter. ChIP was performed the same way as with the *PHO5* promoter, except primers flanking the *PGK* gene were used; these results are based on a single experiment, with serial dilutions also done in triplicate.

FY1093 (*spt7* knockout) strain. Interestingly, the Spt7₇₃₋₁₃₃₂ mutant resulted in the lowest acetylation levels, even when compared with the *spt7* knockout.

At the *PGK* promoter, the ratio of AcH3K18 to total H3 was 85% in CY5616 (Spt7₇₃₋₁₃₃₂) and 75% in CY4385 (Spt7₁₂₁₋₁₃₃₂) relative to the wild-type strain, showing a slightly lesser decrease in acetylation as compared to the *PHO5* promoter. A decrease to 40% of wild-type levels was seen in the FY1093 (*spt7* knockout) strain, representing a lower ratio than was observed at *PHO5* (**Figure 3.4b**).

3.5 Effect of the N-terminal Spt7₁₂₁₋₁₃₃₂ truncation mutant on interaction with Tra1

The Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ mutants produced notable phenotypic effects (**Figure 3.2**), decreased transcriptional activation at SAGA-targeted promoters (**Figure 3.3**), and slightly reduced histone H3 acetylation at the *PHO5* gene (**Figure 3.4**). To determine whether these effects were due to compromised integrity of SAGA, I examined whether Spt7₁₂₁₋₁₃₃₂ would disrupt interaction with subunits of the complex. I tested for interaction with Tra1, which occupies a peripheral portion of the complex away from Spt7 (Wu *et al.* 2004). Flag³-wild-type Tra1 was transformed into the *spt7* knockout strain (CY5871) along with myc⁹-wild-type Spt7 on a centromeric plasmid (CY6132), and myc⁹-Spt7₁₂₁₋₁₃₃₂ on a 2 μ plasmid (CY6139). As controls, strains separately carrying flag³-wild-type Tra1 (CY6110) and myc⁹-wild-type Spt7 (CY6108) on plasmids were engineered. Flag³-Tra1 was pulled down (2.5mg of protein from all extracts) using anti-flag M2 magnetic beads followed by separation of eluted protein by SDS-PAGE and Western blot analysis. Membranes were probed using α -myc and anti-flag antibodies to detect for the presence of

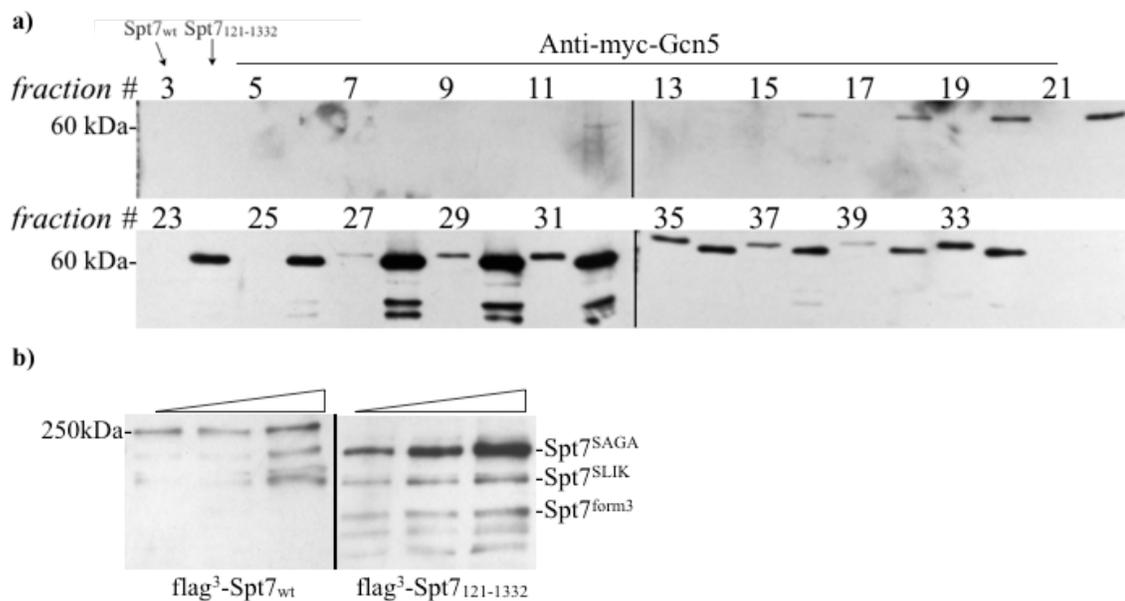


3.5 Tra1-Spt7 immunoprecipitation. Yeast strains CY6132 (flag³-Tra1 on *cen* plasmid and myc⁹-wild-type Spt7 on *cen* plasmid in the *spt7* knockout strain FY1093), CY6139 (flag³-Tra1 on *cen* plasmid and myc⁹-Spt7₁₂₁₋₁₃₃₂ on *2μ* plasmid) were grown up to mid-log phase in YPD along with control strains CY6110 (flag³-Tra1 on *cen* plasmid in FY1093) and CY6108 (myc⁹-wild-type Spt7 on *cen* plasmid in FY1093). **a)** The top gel shows the membrane probed against myc⁹-Spt7. **b)** The bottom gel shows the membrane probed against flag³-Tra1. On both top and bottom gels, lanes 1-4 show input protein, and lanes 5-8 show IP elutions as indicated by the dividing line. Each lane is marked to indicate the presence or absence of each tagged protein as labelled.

*myc*⁹-Spt7 and *flag*³-Tra1, respectively. Tra1 levels were slightly lower in the strain carrying wild-type Spt7 versus the strain carrying Spt7₁₂₁₋₁₃₃₂, as shown in lanes 1 versus 2 of **Figure 3.5b**, respectively. It should also be noted that the level of wild-type Spt7 was higher compared to Spt7₁₂₁₋₁₃₃₂ in the input protein extracts (lanes 1 versus 2 **Figure 3.5a**, respectively). Analysis of the membrane probed for *myc*⁹-Spt7 showed that both wild-type (lane 5) and mutant Spt7 (lane 6) co-immunoprecipitated with Tra1 (**Figure 3.5a**); interestingly, a greater amount of Tra1 was present in the strain carrying the Spt7₁₂₁₋₁₃₃₂ mutant (lane 6, **Figure 3.5b**). The results presented here indicate that the N-terminal truncation of Spt7 does not have an effect on its association with Tra1.

3.6 Investigating the effect of Spt7₁₂₁₋₁₃₃₂ on Spt7-Gcn5 interaction via gel filtration analysis

Given that a lack of the N-terminal 121 residues had no effect on Spt7's association with Tra1, I decided to test for interaction with a more proximal subunit within the SAGA complex, Gcn5. I had technical difficulties with immunoprecipitations, so I looked for co-fractionation after Superose 6 gel filtration chromatography. CY6099 carried *flag*³-wild-type Spt7 (*cen*) and *myc*⁹-Gcn5 (2 μ), and CY6308 carried *flag*³-Spt7₁₂₁₋₁₃₃₂ (2 μ) and *myc*⁷-Gcn5 (2 μ); the difference in tag length ensured that no confusion would arise between the strains. 2mg of protein extract from CY6099 and CY6308 were independently chromatographed on a Superose 6 HR10/30 size exclusion column. Every other fraction was separated by SDS-PAGE and the presence of *myc*-Gcn5 was analyzed by Western blot analysis. Note that the elution fractions from CY6099 (wild-type Spt7) and CY6308

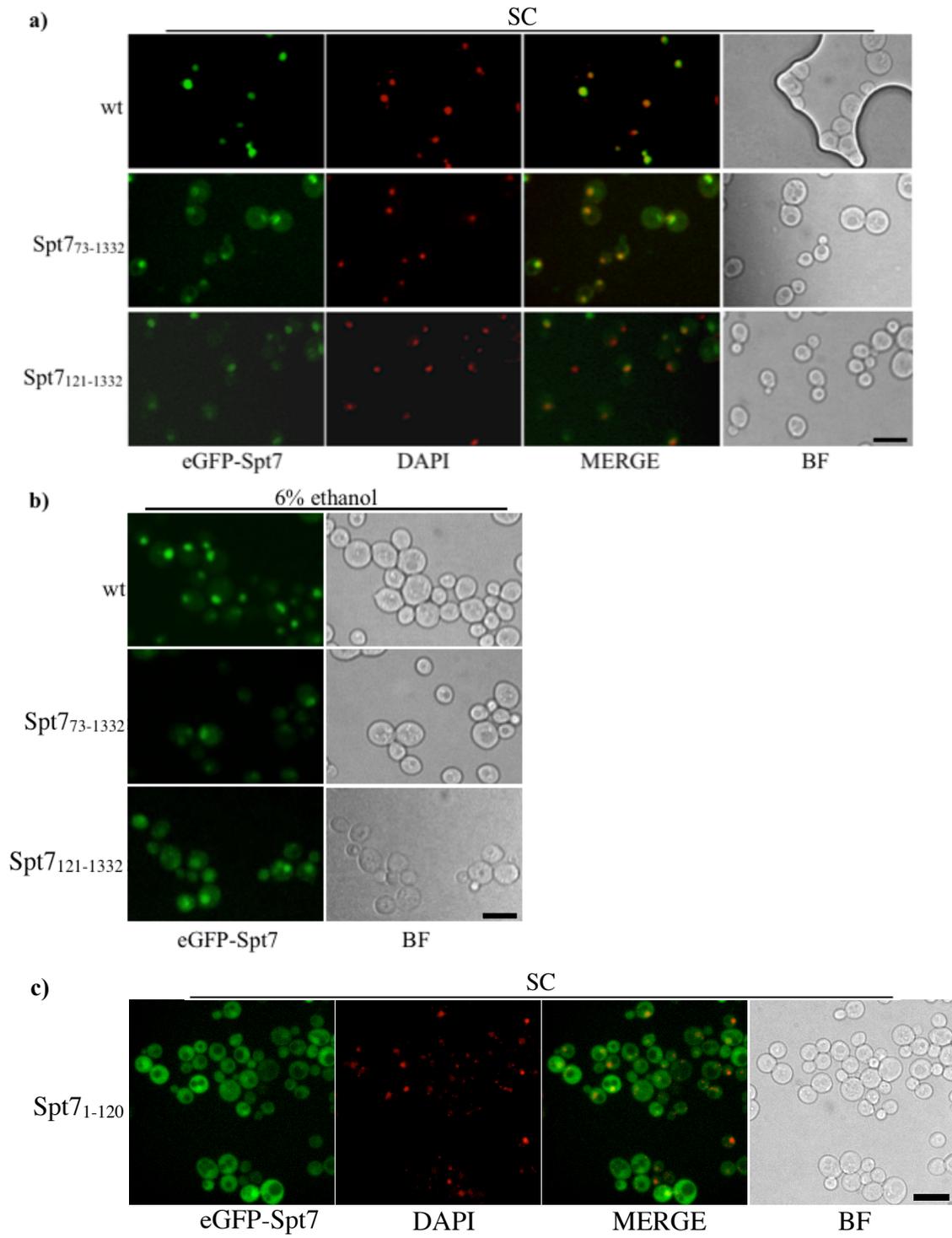


3.6 Fractionation of Gcn5 from a Superose 6 size exclusion column from strains carrying either Spt7_{WT} or Spt7₁₂₁₋₁₃₃₂. Yeast strains CY6099 (flag³-wild-type Spt7 on *cen* plasmid + myc⁹-Gcn5 on 2 μ) and CY6308 (flag³-Spt7₁₂₁₋₁₃₃₂ on 2 μ plasmid + myc⁷-Gcn5 on 2 μ) were grown to mid-log phase in YPD. 2mg of 6099 and 6308 lysed cell extract was separately applied over a Superose 6 HR10/30 size exclusion column and every other fraction was separated by SDS-PAGE followed by Western blot analysis. **a)** Spt7_{WT} represents extracts from CY6099 (every numbered lane) while Spt7₁₂₁₋₁₃₃₂ represents extracts from CY6038 (lanes lacking numbers), as indicated by the arrows. Membranes were probed with α -myc Ab to detect myc⁹-Gcn5 and **b)** anti-flag Ab to detect flag³-Spt7. Fractions #3-11, #13-21, #23-31, and #33-39 to be probed were loaded on separate gels due to well limit per gel, as illustrated by the black vertical lines separating each membrane. Note that fraction #33 is present at the end of the last gel due to an error in loading.

(Spt7₁₂₁₋₁₃₃₂) were loaded side-by-side for each fraction so that a more accurate comparison between Gcn5 levels could be made. Gcn5 signal was present in context of the Spt7₁₂₁₋₁₃₃₂ carrying mutant strain in the same fractions (#27-39) as the wild-type carrying strain (**Figure 3.6a**). The strain containing Spt7₁₂₁₋₁₃₃₂ showed a greater expression compared to that carrying the wild-type due to the fact that the Spt7₁₂₁₋₁₃₃₂ mutant was expressed on a 2 μ plasmid (**Figure 3.6b**). This result supports the view that Spt7₁₂₁₋₁₃₃₂ does not have an affect on the association of Gcn5 with Spt7.

3.7 Localization of Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ via fluorescence microscopy

Since Spt7₁₂₁₋₁₃₃₂ did not have an effect on its association with either Gcn5 or Tra1, perhaps the observed effects on expression, growth, transcriptional activation and acetylation could be due to a change in localization as a result of the missing N-terminal residues. To test this, yeast strains carrying N-terminally eGFP-tagged wild-type Spt7 (CY6079), Spt7₇₃₋₁₃₃₂ (CY5922) and Spt7₁₂₁₋₁₃₃₂ (CY5921) on 2 μ plasmids were engineered. All eGFP-tagged proteins were expressed in a yeast strain containing endogenous Spt7 (BY4741) to prevent potential complications as related to growth defects seen with a *spt7* knockout strain (**Figure 3.2**). When grown in YPD, wild-type Spt7 localized exclusively to the nucleus. No obvious difference in cellular localization was seen with either the Spt7₇₃₋₁₃₃₂ or Spt7₁₂₁₋₁₃₃₂ mutants. Both of the mutant strains showed a predominantly nuclear localization, although a decrease in relative signal intensity was seen compared to wild-type (**Figure 3.7a**). In addition, localization appeared to remain in the nucleus upon growth in YPD containing 6% ethanol (**Figure 3.7b**).

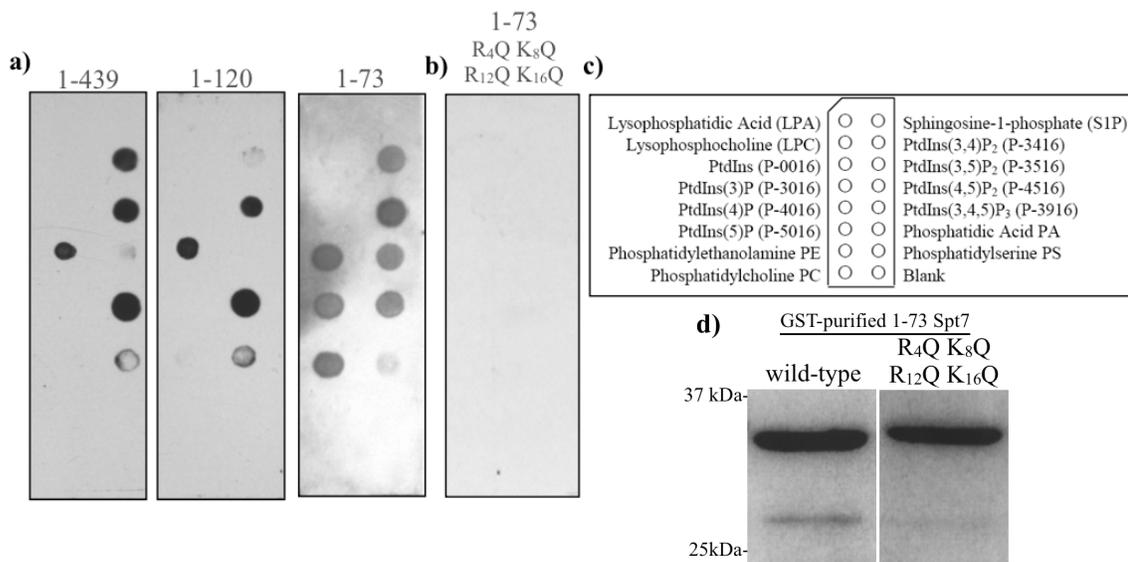


3.7 Localization of full-length Spt7_{WT}, Spt7₇₃₋₁₃₃₂, and Spt7₁₂₁₋₁₃₃₂. **a)** SC medium: Yeast strains CY6079 (eGFP-wild-type Spt7), CY5922 (eGFP-Spt7₇₃₋₁₃₃₂), and CY5921 (eGFP-Spt7₁₂₁₋₁₃₃₂) were grown to mid-log phase in medium lacking uracil to ensure plasmid maintenance, followed by DAPI staining. Excess DAPI was washed off with PBS, and cells were visualized by fluorescence microscopy. BF, bright field. Bar: 10 μ m (bottom right). Note that partial drying out of the slide occurred when analyzing the CY6079 strain. **b)** SC medium containing 6% ethanol. CY6079, CY5922 and CY5921 were grown to mid-log phase in medium lacking uracil, followed by dilution and growth in SC medium containing 6% ethanol for another 16hrs. Cells were subsequently visualized by fluorescent microscopy. DAPI staining is ineffective in the presence of ethanol. **c)** Diploid yeast strain containing Spt7₁₋₁₂₀ fragment/wild-type-Spt7 (CY6162) was grown in SC medium and stained with DAPI as described above, and visualized by fluorescence microscopy.

Although no change in localization was seen with respect to Spt7₇₃₋₁₃₃₂ or Spt7₁₂₁₋₁₃₃₂, I tested whether a GFP-tagged 1-120 amino residue fragment alone would also exhibit nuclear localization. Thus, yeast strain carrying eGFP-Spt7₁₋₁₂₀ fragment in context of a second wild-type copy of Spt7 (CY6162) was engineered and observed via fluorescence microscopy. The results indicate a diffuse distribution of eGFP-Spt7₁₋₁₂₀ throughout the cell that is not exclusive to the nucleus (**Figure 3.7c**).

3.8 Lipid binding by the extreme N-terminus of Spt7

We identified a potentially novel lipid-binding motif at the extreme N-terminus of Spt7 (**Figure 1.3b**). As mentioned previously, Hoke *et al.* (2008a) found that Spt7 bound phosphatidylserine (PS) independent of Ada2, which also binds PS. To follow this up, I sought to map the region of lipid-binding by Spt7, directing my focus on the putative N-terminal lipid binding domain. Plasmid constructs containing GST fusions of N-terminal 1-439, 1-121 and 1-73 fragments were generated and purified using an *E. coli* expression system. These fragments were purified using a GST column, followed by spotting onto PIP-strips: hydrophobic membranes spotted with 100pmol of phosphatidylinositols and other physiologically relevant lipids (**Figure 3.8c**). Two hundred micrograms of each protein was used and the PIP-strips were separately probed with anti-GST antibody. As shown in **Figure 3.8a**, all three N-terminal fragments showed lipid-binding to numerous PIPs, most notably PtdIns(3)P and PtdIns(3,5)P₂. Signal variability was observed due to the variable nature of the assay.

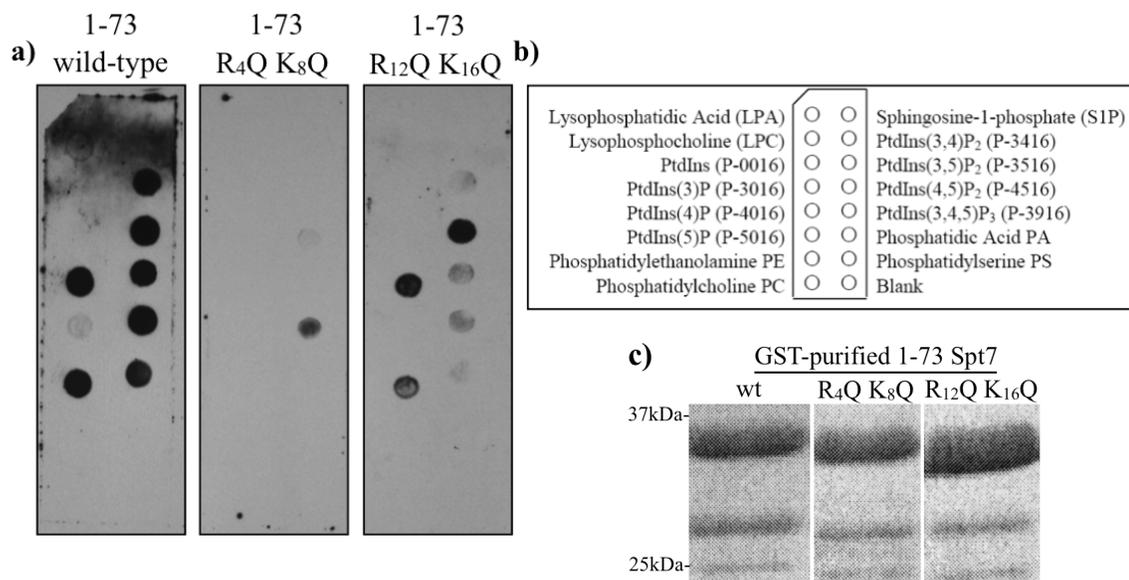


3.8 Lipid binding by N-terminal fragments of Spt7. **a)** Wild-type N-terminal Spt7 fragments. GST-tagged N-terminal fragments were purified using a GST-column and eluted with 25mM glutathione. 200 μ g of protein was added to PIP-strips, and probed with GST-antibody. **b)** 1-73 R₄QK₈QR₁₂QK₁₆Q mutant. Equal amounts (200 μ g) of wild-type 1-73 amino acid residue fragment and R₄QK₈QR₁₂QK₁₆Q mutant peptide were overlaid onto PIP-strips and probed with GST-antibody. **c)** PIP-strip schematic. Distribution of lipid species on PIP-strips used in lipid-overlay assay (Echelon Biosciences). **d)** Wild-type and R₄QK₈QR₁₂QK₁₆Q 1-73 Spt7 protein fragments were purified by GST-column using 0.5mM IPTG at 15°C. Purified protein was separated by SDS-PAGE and stained using Coomassie Brilliant Blue to ensure induction of protein expression prior to performing the lipid overlay assay.

The observation that the first 73 amino terminal residues were sufficient for lipid binding raised the possibility that the alternating pattern of positively charged residues at the extreme N-terminus of Spt7 coordinated this lipid binding. Thus, I asked whether specific mutations in these positively-charged residues would cause defects in binding. To this end, site-directed PCR mutagenesis was performed to mutate the first four positive residues to glutamines. Interestingly, the 1-73 R₄QK₈QR₁₂QK₁₆Q mutant reproducibly showed complete loss of binding to all lipid species on the PIP-strips (**Figure 3.8b**). The purified proteins used in the PIP-strip assay are illustrated in **Figure 3.8d**.

3.9 Changes in lipid binding specificity by Spt7 N-terminal mutant fragments

To further investigate the specificity of lipid binding of the first four positively-charged residues, two additional mutant fragments were engineered via site-directed mutagenic PCR. These mutants carried double mutations in the first two (Spt7-R₄QK₈Q), and third and fourth positively-charged residues in combination (Spt7-R₁₂QK₁₆Q). While the R₁₂QK₁₆Q mutant retained the ability to bind PtdIns(3)P and PtdIns(3,5)P₂, the R₄QK₈Q mutant lost binding to both lipid species (**Figure 3.9a**). The purified proteins used in the PIP-strip assay are shown in **Figure 3.9c**.



3.9 Lipid binding by N-terminal mutant fragments of Spt7. **a)** Double mutants. GST-tagged 1-73 N-terminal residue fragments carrying double mutations, R₄QK₈Q and R₁₂QK₁₆Q, were purified using a GST-column and eluted with 25mM glutathione, along with the 1-73 residue wild-type fragment. 200μg of protein was added to PIP-strips, and probed with GST-antibody to detect lipid-binding to various species. **b)** PIP-strip schematic. Distribution of lipid species on PIP-strips used in lipid-overlay assay (Echelon Biosciences). **c)** Wild-type, R₄QK₈Q, and R₁₂QK₁₆Q 1-73 Spt7 protein fragments were purified by GST-column using 0.5mM IPTG at 15°C. Purified protein was separated by SDS-PAGE and stained using Coomassie Brilliant Blue to ensure induction of protein expression prior to performing the lipid overlay assay.

3.10 Phenotypes of strains carrying N-terminal point mutations

The difference in lipid binding specificity observed by altering just two of the positively charged residues at the N-terminus of Spt7 allowed me to pinpoint the region necessary for binding PtdIns(3)P and PtdIns(3,5)P₂. To test whether loss of this binding affected the growth of yeast, strains containing full-length Spt7 carrying the R₄QK₈Q (CY5798), R₁₂QK₁₆Q (CY5795), and R₄QK₈QR₁₂QK₁₆Q (CY5797) mutations on plasmids were engineered, along with a strain containing wild-type Spt7 (CY5796). Cultures were grown to stationary phase, serially diluted, and spotted onto YPD, medium lacking inositol and YPD containing 6% ethanol. The *spt7* null strain (CY4465) showed the most severe growth defect. Although the mutants did not show nearly as severe an effect as that of the null strain, Spt7-R₄QK₈Q grew slower than either the R₁₂QK₁₆Q double-, or R₄QK₈QR₁₂QK₁₆Q quadruple mutants at 37°C and on medium lacking inositol (**Figure 3.10**). This was consistent with the loss of PtdIns(3)P and PtdIns(3,5)P₂ lipid binding seen with Spt7-R₄QK₈Q as compared to the R₁₂QK₁₆Q mutant (**Figure 3.9**). However, the fact that the R₄QK₈QR₁₂QK₁₆Q quadruple mutant showed a lesser phenotype compared to Spt7-R₄QK₈Q under these conditions presents an inconsistency with regards to the size of the mutation and effect on growth. On the other media tested (YPD at 30°C, YPD containing 6% ethanol), the three mutants showed a minor decrease in growth (**Figure 3.10**).

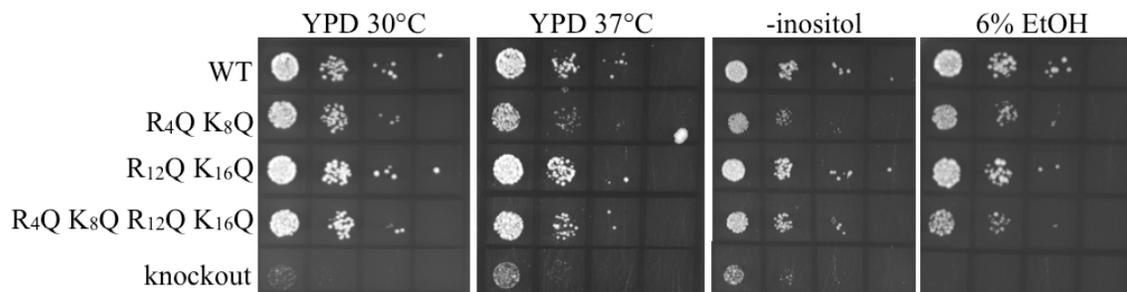


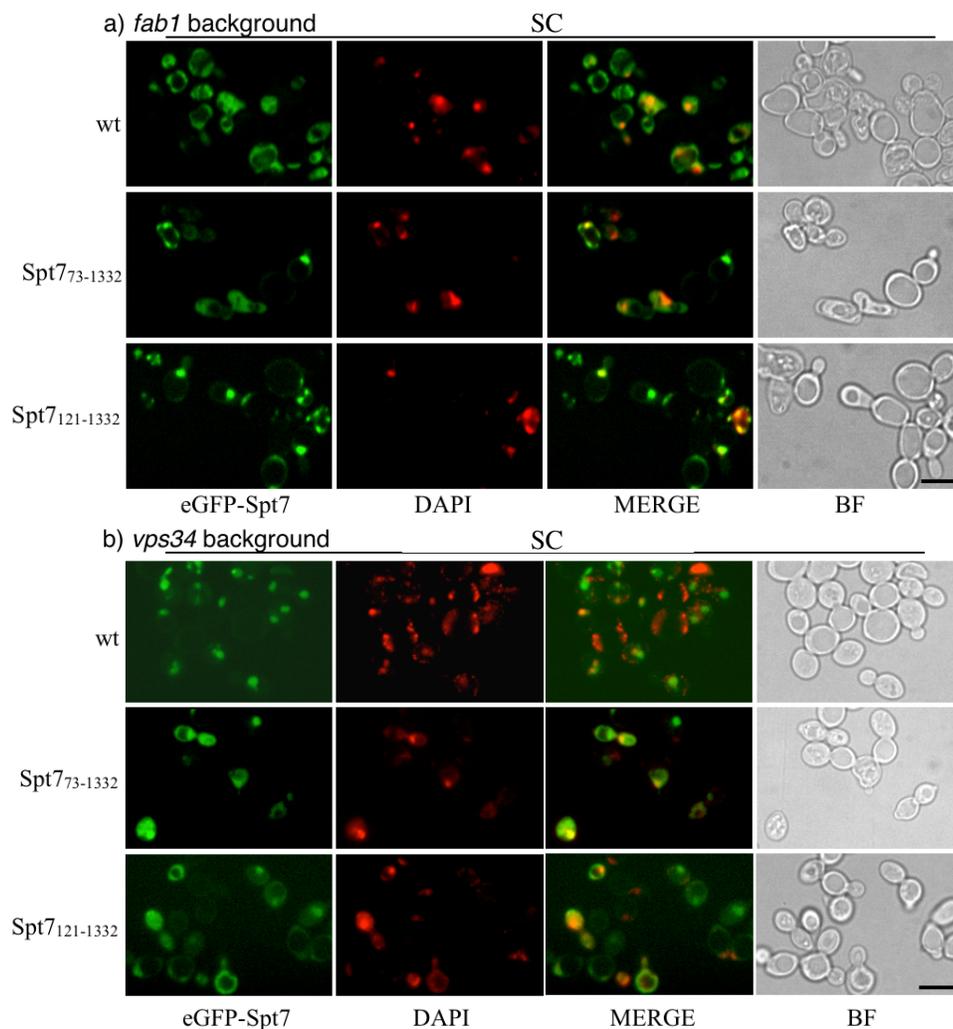
Figure 3.10 Phenotypes of N-terminal point mutations of full-length Spt7. Serial dilutions of yeast strains CY5796 (wild-type Spt7), CY5798 (full length Spt7 carrying R₄QK₈Q mutation), CY5795 (Spt7 carrying R₁₂QK₁₆Q mutation), CY5797 (Spt7 carrying R₄QK₈QR₁₂QK₁₆Q mutation), and CY4465 (*spt7* knockout) were spotted onto YPD plates at (30°C and 37°C), medium lacking inositol, and YPD containing 6% ethanol.

3.11 Spt7 localization in the context of *fab1* and *vps34* deletion backgrounds

I next asked whether the absence of PtdIns(3)P and PtdIns(3,5)P₂ lipid species in yeast strains would affect Spt7 localization in the cell. A *fab1* knockout strain containing eGFP-tagged wild-type (CY6085), Spt7₇₃₋₁₃₃₂ (CY6087) or Spt7₁₂₁₋₁₃₃₂ (CY6086) Spt7 was generated. Likewise, *vps34* knockout strains carrying eGFP-tagged wild-type Spt7 (CY6089), Spt7₇₃₋₁₃₃₂ (CY6091) or Spt7₁₂₁₋₁₃₃₂ (CY6090) were engineered. Vps34 is the kinase responsible for converting PtdIns to PtdIns(3)P, which is subsequently phosphorylated by Fab1 to form PtdIns(3,5)P₂ (Strahl and Thorner, 2007). As shown in **Figure 3.11**, both strains exhibited altered morphology, including greatly enlarged vacuoles. The eGFP-Spt7 signal showed nuclear as well as cytoplasmic localization in both knockout strains. Interestingly, the distribution of signal appeared more nuclear in the *vps34Δ* strain (**Figure 3.11b**) compared to the *fab1Δ* strain (**Figure 3.11a**).

3.12 Localization of Tti2 and Tti2-F328S

As part of our investigation into the function of the FATC domain of Tra1, we analyzed the suppression of the Tra1-F3744A mutant by Tti2-F328S. Apart from multiple biochemical assays performed by other members of our lab including ChIPs, β -galactosidase assays, and Western blots (Genereaux *et al.* 2012), I performed fluorescence microscopy to investigate whether the Tra1 mutation altered the protein's cellular localization, and in turn, whether a difference would be seen in context of the Tti2-F328S suppressor.



3.11 Localization of full-length Spt7_{WT}, Spt7₇₃₋₁₃₃₂, and Spt7₁₂₁₋₁₃₃₂ in the context of *fab1* and *vps34* knockout strains. **a)** Localization in *Fab1* Δ background. Yeast strains CY6085 (eGFP-wild-type Spt7), CY6087 (eGFP-Spt7₇₃₋₁₃₃₂), and CY6086 (eGFP-Spt7₁₂₁₋₁₃₃₂) were grown to mid-log phase in medium lacking uracil to promote plasmid maintenance, followed by DAPI staining. Following washing in PBS to get rid of excess DAPI, cells were visualized by fluorescence microscopy. BF, bright field. Bar: 10 μ m (bottom right). **b)** Localization in *Vps34* Δ background. Yeast strains CY6089 (eGFP-wild-type Spt7), CY6091 (eGFP-Spt7₇₃₋₁₃₃₂), and CY6090 (eGFP-Spt7₁₂₁₋₁₃₃₂) were grown to mid-log phase in medium lacking uracil, stained with DAPI and visualized by fluorescence microscopy as stated above.

Prior to tracking the localization of Tra1 however, I looked at the cellular distribution of eGFP-tagged wild-type Tti2 and Tti2-F328S, as this had not yet been performed; this would also provide us with more information to better analyze the nature of suppression of the Tra1-F3744A allele. **Figure 3.12** shows fluorescence microscopy for separate BY4741 strains carrying eGFP-wild-type-Tti2 (CY5854) and eGFP-Tti2-F328S (CY6062). After growth to mid-log phase in synthetic complete medium and staining by DAPI, cells were visualized under the microscope. Wild-type Tti2 was both nuclear and cytoplasmic. No apparent difference was observed with the Tti2-F328S mutant, except for a slight decrease in eGFP signal intensity (**Figure 3.12a**). Growth in synthetic complete medium containing 6% ethanol resulted in largely unchanged localization, except for the appearance of numerous foci near the vacuolar membrane within context of both wild-type and Tti2-F328S (**Figure 3.12b**).

3.13 Localization of Tra1, and Tra1-F3744A in presence and absence of Tti2-F328S

Yeast strains containing N-terminally eGFP-tagged Tra1 (CY6029) and Tra1-F3744A (CY6025) were engineered in the context of a heterozygous diploid strain carrying a wild-type copy of Tra1 to avoid potential complications of slow growth due to the Tra1-F3744A allele (refer to **Figure 1.5** for phenotypes of Tra1-F3744A). Note that these strains also carried two copies of wild-type Tti2. Wild-type Tra1 was almost exclusively nuclear when grown in synthetic complete medium. The Tra1-F3744A mutant localized to the nucleus, but also mislocalized to numerous foci in the cytoplasm (**Figure 3.13a**). When grown in synthetic complete medium containing 6% ethanol, eGFP-Tra1-F3744A showed a higher

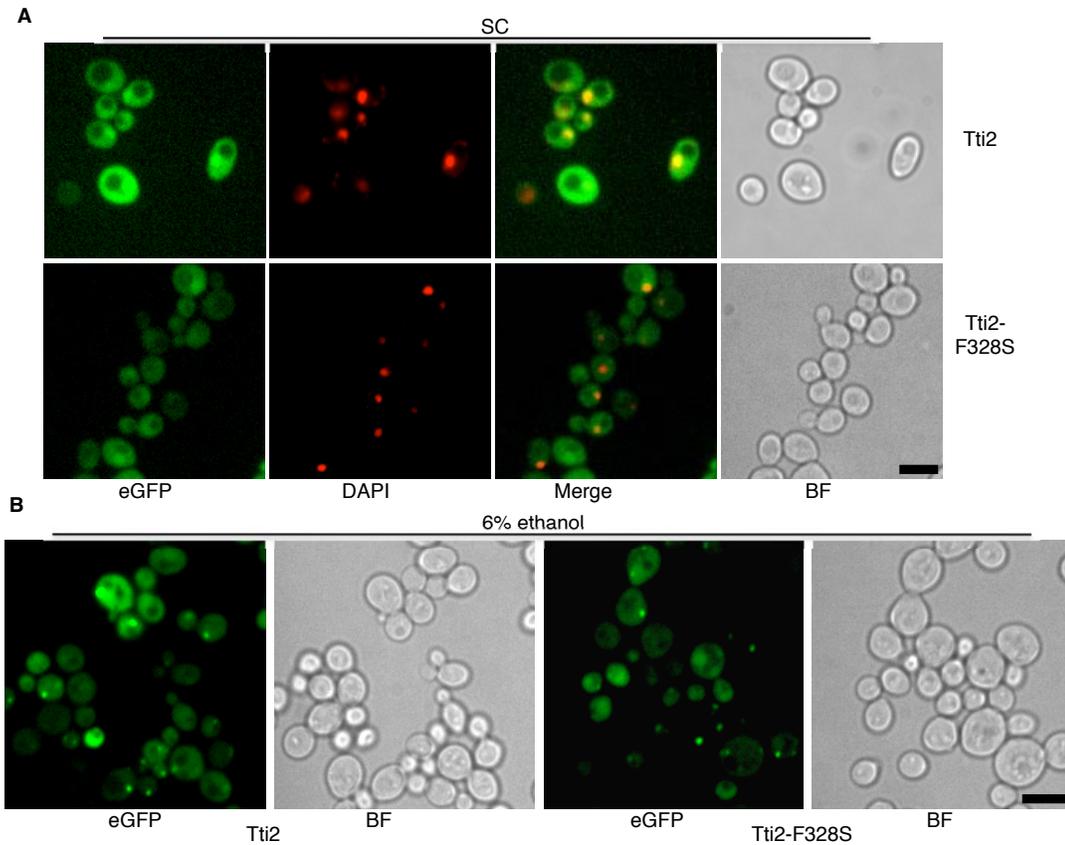


Figure 3.12 Localization of Tti2 and Tti2-F328S. a) BY4741 strains containing eGFP-wild-type-Tti2 (CY5854) or eGFP-Tti2-F328S (CY6062) were grown to mid-log phase in synthetic complete medium, followed by DAPI staining. Cells were washed in PBS and visualized by fluorescence microscopy. b) Cells were grown to stationary phase in SC medium, washed and grown until mid-log phase for another 18 hours in SC medium containing 6% ethanol. After washing in PBS, cells were visualized by fluorescence microscopy. DAPI staining was ineffective when grown in presence of ethanol. Bar, 10µm (bottom right). BF, bright field.

degree of diffusion to the cytoplasmic foci (**Figure 3.13b**). To test for the effect of the Tti2-F328S suppressor, another diploid strain carrying eGFP-Tra1-F3744A/wild-type-Tra1 and Tti2-F328S/wild-type-Tti2 (CY6063) was made. Interestingly, the levels of cytoplasmic eGFP-Tra1-F3744A signal decreased in synthetic complete medium upon introduction of the Tti2-F328S suppressor (**Figure 3.13a**). Likewise, the mislocalization was partially reversed in medium containing 6% ethanol (**Figure 3.13b**). I used imaging software to quantify the fluorescent intensity of eGFP-Tra1, eGFP-Tra1-F3744A and eGFP-Tra1-F3744A/Tti2-F328S per unit area in the nucleus as compared to the whole cell, taking an average of 20 cells per calculation. As **Table 3.1** shows, the ratio of nuclear to cytoplasmic GFP signal in synthetic complete medium, as well as medium containing 6% ethanol, increased upon introduction of the Tti2-F328S suppressor mutant, confirming the visual effect described above. Note that because DAPI staining was ineffective in ethanol-containing medium, I assumed that the area with the most concentrated GFP-signal represented the nucleus, alongside its shape and location within the cell.

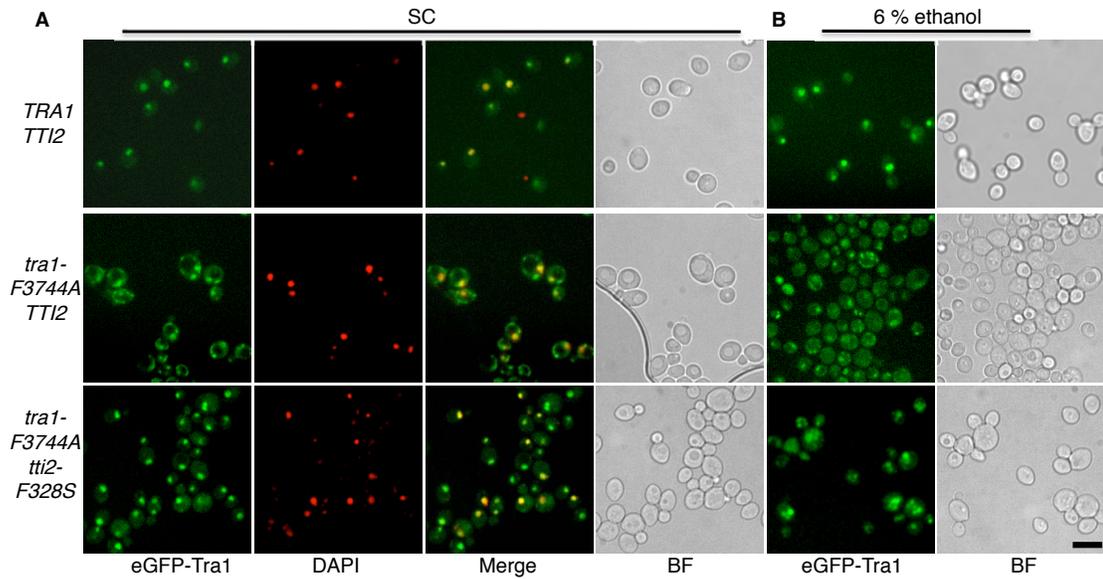


Figure 3.13 Localization of Tra1 and Tra1-F3744A in the presence of Tti2 or Tti2-F328S. **a)** Yeast strains containing eGFP-Tra1/Tra1 Tti2/Tti2 (CY6029), eGFP-Tra1-F3744A/Tra1 Tti2/Tti2 (CY6025), and eGFP-Tra1-F3744A/Tra1 Tti2-F328S/Tti2 (CY6063) were grown in synthetic complete medium to mid-log phase stained with DAPI and visualized by fluorescence microscopy. **b)** The two rightmost panels are strains grown in SC containing 6% ethanol. BF, bright field. Bar, 10 μ m (bottom right)

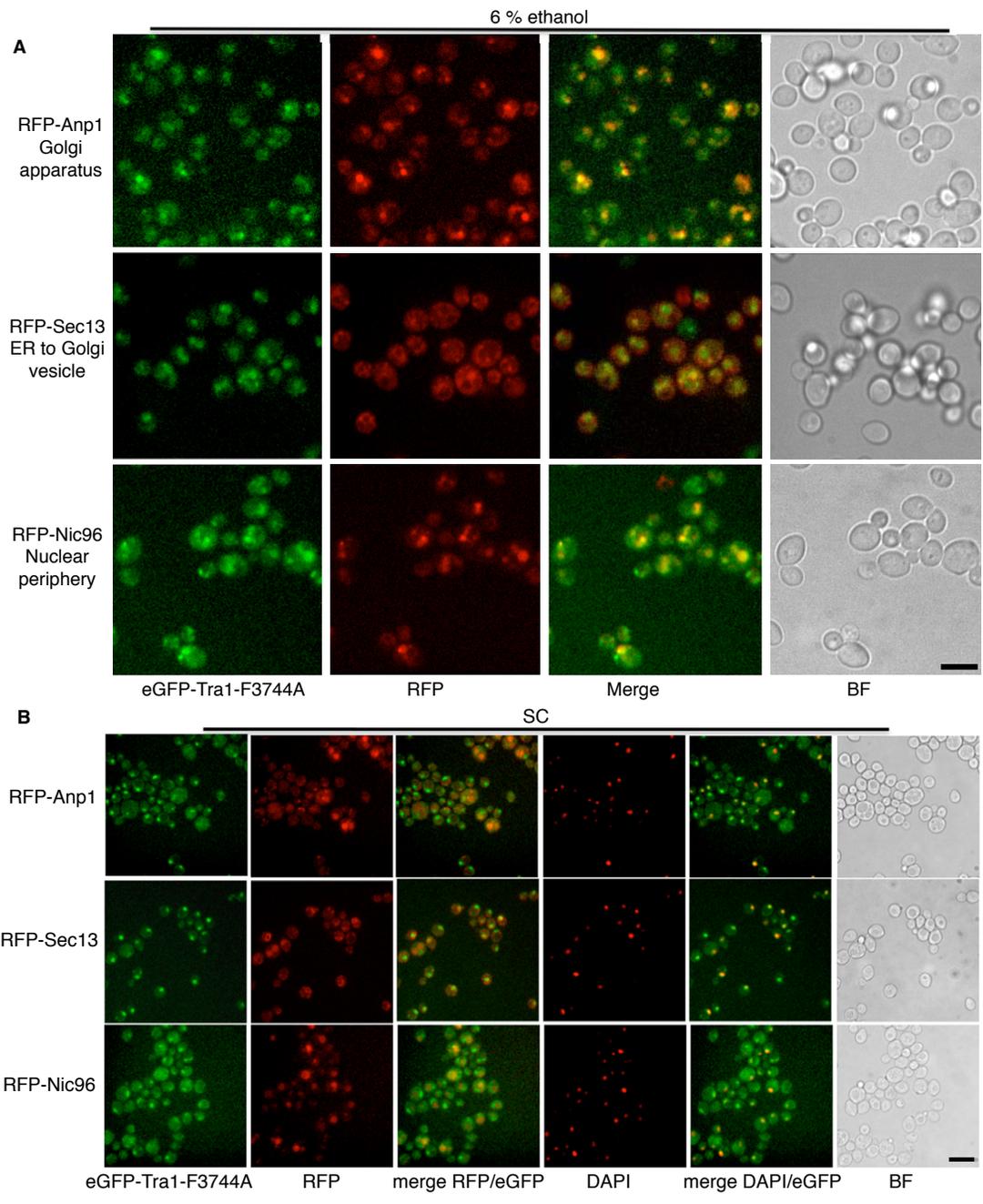
Table 3.1: Relative concentrations (fluorescence intensity per unit area) of eGFP-Tra1 (wild type or F3744A) in the nucleus vs. total cell

Tra1/Tti2 (strain)	[Nuclear eGFP-Tra1]/[total cell eGFP-Tra1]	
	SC medium	SC plus 6% ethanol
WT/WT (CY6029)	4.0 ± 0.6	2.4 ± 0.5
F3744A/WT (CY6025)	2.7 ± 0.5	2.0 ± 0.4
F3744A/F328S (CY6063)	3.5 ± 0.4	2.5 ± 0.5

Concentrations represent the intensity of eGFP fluorescence per unit area in the nucleus divided by the eGFP fluorescence per unit area for the cell (including the nucleus). Numbers represent the average for 20 cells. As DAPI staining was ineffective for the ethanol-grown cells, the most intense focus was assigned as the nucleus.

3.14 Comparing localization of Tra1-F3744A with membrane constituents.

In an effort to determine the nature of the foci in which Tra1-F3744A was found, diploid strains containing eGFP-Tra1-F3744A/wild-type-Tra1 and several RFP-tagged membrane constituents (kindly provided by Peter Arvidson) were generated. Localization of RFP-tagged Anp1 (Golgi apparatus), Sec13 (ER to Golgi vesicles), and Nic96 (nuclear periphery) was analyzed simultaneously with eGFP-Tra1-F3744A after growth in medium containing 6% ethanol (**Figure 3.14a**). All RFP-tagged constituents showed a general overlap with the eGFP-Tra1-F3744A signal, the most prominent of which was Anp1. I repeated this experiment in synthetic complete medium. As shown in **Figure 3.14b**, Tra1-F3744A retained mostly nuclear localization (as well as localization to cytoplasmic foci) confirming the results described above. In addition, I analyzed co-localization with three more RFP-tagged constituents, Cop1 (Early Golgi), Snf7 (endosome), and Pex3 (peroxisome). A general overlap was also seen (**Figure 3.14c**), rendering it difficult to pinpoint the exact type of membrane to which the Tra1-F3744A mutant localized.



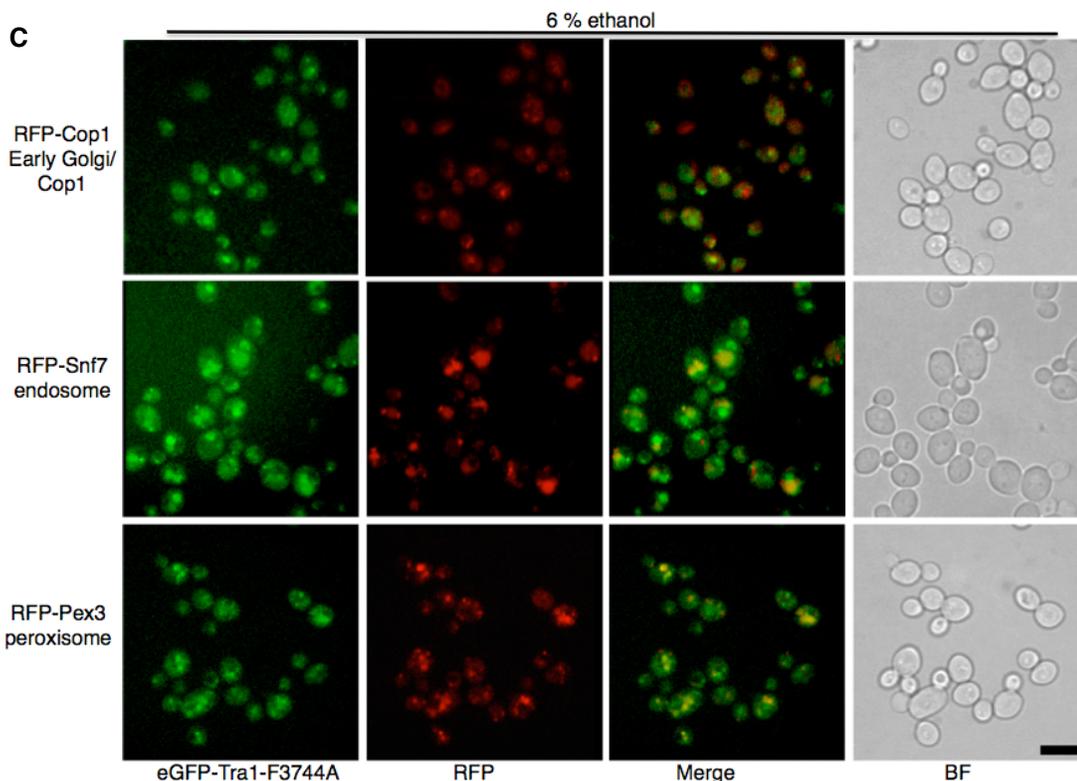


Figure 3.14 Localization of eGFP-Tra1-F3744A with RFP-tagged membrane constituents. **a)** Localization of eGFP-Tra1-F3744A and RFP-tagged Anp1, Sec13, and Nic9 in SC medium containing 6% ethanol. Diploid strains containing a single copy of each tagged allele were grown to stationary phase in SC medium. Cells were then washed with PBS and grown to mid-log phase in SC containing 6% ethanol, followed by visualization using fluorescence microscopy. **b)** Localization of eGFP-Tra1-F3744A and RFP-tagged Anp1, Sec13, and Nic9 in SC medium. Columns 3 and 5 from the left represent merged images between eGFP (green) and RFP or eGFP and DAPI, respectively. Bar, 10 μ m (bottom right). **c)** Localization of eGFP-Tra1-F3744A and RFP-tagged Cop1, Snf7, and Pex3 in SC medium containing 6% ethanol. Cells were grown to stationary phase in SC medium, washed with PBS and diluted in SC medium containing 8% ethanol (to make up a total concentration of 6% ethanol), grown a further 18 hours, and visualized by fluorescence microscopy. Bar, 10 μ m (bottom right)

Chapter 4: Discussion

Spt7

The first 121 amino terminal residues of Spt7 are crucial for its full expression, for transcriptional activation of target promoters and their histone acetylation in inducing conditions. Deletion of this region leads to numerous growth phenotypes. In addition, I found that the 1-121 amino terminal region does not play a role in maintaining interaction with the SAGA subunits, Tra1 (**Figure 3.5**) and Gcn5 (**Figure 3.6**). Likewise, the lack of this N-terminal region did not cause mislocalization of Spt7 away from the nucleus, as confirmed by fluorescence microscopy (**Figure 3.7**).

The extreme N-terminus binds PtdInsPs. Complete loss of binding was seen when the first four positively charged residues of Spt7 were mutated to glutamines (**Figure 3.8**). Specific binding to PtdIns(3)P and PtdIns(3,5)P₂ was lost upon mutating the first two of these residues (Spt-R₄QK₈Q), but remained unchanged in context of cells carrying the Spt7-R₁₂QK₁₆Q mutant (**Figure 3.9**). These results were consistent with a minor phenotypic effect seen with the Spt7-R₄QK₈Q mutant in medium lacking inositol and high temperature growth conditions (**Figure 3.10**). In strains lacking PtdIns(3)P and/or PtdIns(3,5)P₂, changes in eGFP-Spt7 localization were observed, further suggesting that PtdIns binding by Spt7 may be important for its function. By studying the N-terminal region of yeast Spt7, I have potentially uncovered new ways in which the SAGA complex is regulated in *S. cerevisiae*.

4.1 The N-terminal 121 residues of Spt7 are necessary for its full expression

Our initial finding that the Spt7₁₂₁₋₁₃₃₂ mutant decreased expression levels of Spt7 could be due to several factors. First, the lack of the N-terminal residues could result in decreased protein stability, as well as a potentially weaker association with the SAGA complex. This would likely render the truncation mutant more susceptible to proteolysis. The observation that Spt7₁₂₁₋₁₃₃₂ showed several bands below that of the indicated Spt7^{form3} band which were absent from the wild-type sample lane further validates the possibility of increased levels of degradation, and thus targeting for proteolysis. In addition, expression of the truncation mutant on a multi-copy plasmid could possibly result in over-saturation of Spt7 in the nucleus, thus leading to degradation and re-cycling of excess Spt7 not utilized by the SAGA complex.

Because no difference was observed in the relative levels of Spt7^{SLIK} and Spt7^{form3} between wild-type and mutant protein, the 1-121 amino terminal region of Spt7 has no effect on processing of full-length Spt7 into its Spt7^{SLIK} or Spt7^{form3} counterparts. This has previously been found by Hoke *et al*, who showed that the C-terminus is sufficient for processing of full-length Spt7 to its Spt7^{SLIK} and Spt7^{form3} counterparts, and does not require the N-terminus of the protein up to the region of the histone fold motif (residues 979-1045) (Hoke *et al*. 2007).

4.2 The lack of the first 73 and 121 amino terminal residues severely affects growth and transcriptional activation at SAGA targeted promoters

In a study by Wu and Winston (2002), a N-terminally truncated mutant of Spt7 was made which lacked the first 873 residues of the protein (spt7-100). A diploid strain carrying

two copies of this mutation (*spt7-100/spt7-100*) was tested for growth defects alongside a set of C-terminal truncations. This N-terminal truncation resulted in partially reduced growth on medium lacking inositol, and medium containing galactose. This defect in growth was nearly fully restored to that of wild-type levels through complementation by a second copy of Spt7 expressing the missing N-terminal residues (*spt7-873*). It is interesting to note that the *spt7-873/spt7-873* diploids (which lacked the N-terminus) showed a similar lack in growth as the *spt7-100/spt7-100* (lack of C-terminus). The biochemical effects of the N-terminal deletion were not pursued further however, as the paper was focused on specifics relating to the C-terminal portion of the protein (Wu and Winston, 2002).

Haploid strains carrying Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ mutant proteins showed a more drastic decrease in growth compared to wild-type Spt7 than Wu and Winston's strain carrying *spt7-873* (lacking the C-terminus) under growth conditions such as medium lacking inositol. Such a drastic effect due to lack of the first 73 and 121 N-terminal residues suggests an important role of this region in Spt7 function. It is possible that the N-terminus may fold back to interact with and regulate a downstream region of the protein. In such a case, deletion of the first 121 residues would leave the adjacent portion of the protein unregulated and cause potential destabilization of Spt7. This self regulation could be similar to the PIKKs, where folding takes place to facilitate the interaction of the C-terminal FATC domain with the PI3K region (Lempiäinen and Halazonetis 2009).

The greatest decrease in growth for the Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ mutants was observed in medium lacking inositol; this agrees with the fact that SAGA directly plays a role in the transcriptional activation of *INO1* (Marcus *et al.* 1996; Dietz *et al.* 2003;

Horiuchi *et al.* 2007). In the absence of cellular inositol, SAGA via its Gcn5 subunit acetylates lysine 14 of histone H3 after its recruitment to the promoter by Snf1 (Lo *et al.* 2005). Likewise, SAGA controls the response of the cell to varying phosphate levels through transcriptional activation at *PHO5* (Barbaric *et al.* 2003). The decreased growth under these conditions was consistent with the severe decrease in transcriptional activation at both *PHO5* and *INO1* (comparable in magnitude to that of an *spt7* knockout). This decreased transcription could have been a direct result of either i) a lack of physical recruitment of SAGA to the promoter, ii) altered function at the promoter preventing the necessary acetylation by Gcn5 to take place or iii) a loss of interaction with specific activators at the promoter. Combining the transcription defects with the observed growth effects strongly suggests that the N-terminus of Spt7 plays a role in regulating SAGA-driven activation at its target genes. This was also indirectly confirmed by testing for transcriptional activity at *RPL35A*, a component of the 60S large ribosomal subunit (Babiano and Cruz, 2010) whose transcription is regulated by the TFIID complex (Huisinga and Pugh, 2004). The milder effect on transcription seen as a result of the truncation mutants at this promoter shows that the effect of the N-terminal residues of Spt7 is SAGA-specific. Given the breadth of phenotypes as a result of the N-terminal truncated mutants of Spt7, combined with the severe decrease in transcriptional activation at SAGA-targeted genes, I conclude that the extreme N-terminus of Spt7 affects the general ability of SAGA to effectively regulate its target genes.

4.3 The extreme N-terminal region of Spt7 is necessary for acetylation at lysine 18 of histone H3 at the *PHO5* promoter

It is established that Gcn5 preferentially acetylates SAGA-targeted genes at histone H2B and H3 (Kuo *et al.* 1996; Grant *et al.* 1999). More specifically, acetylation at lysine 18 (K18) of histone H3 is essential for activation of genes such as *HXT2* and *HXT4* (van Oevelen *et al.* 2005) as well as *ARG1*, *CTT1*, *FUS1*, and *VTC1* (Hoke *et al.* 2008a). Testing for effects of N-terminal truncated mutants on K18 of histone H3 at the *PHO5* promoter, I found acetylation to be down in strains carrying Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ to ~50% and ~70%, respectively. This drop in levels was comparable to that of a *spt7* knockout strain (~60% of total H3 levels). In 1999, Eberharter *et al* showed that the ADA module, consisting of Ada2, Gcn5 and Ngg1/Ada3 is stable and active as its own complex in the cell. This partial drop in acetylation as a result of the missing 73 and 121 N-terminal residues of Spt7 suggests that acetylation was not completely inhibited due to retained Gcn5 activity in the context of the independent ADA module. In comparison with these results, Genereaux *et al* (2012) found that acetylation of H3K18 in an *ada2* knockout strain was drastically reduced to levels near zero. This finding confirms the necessity of Ada2 in regulating Gcn5, as previously hypothesized (Barbaric *et al.* 2003), as well as confirming that unlike Ada2, the N-terminus of Spt7 does not regulate Gcn5 acetylation outside of its association with the SAGA complex.

The observed decrease in acetylation to approximately 80% of wild-type levels seen with both truncation mutants at the *PGK1* promoter (40% of wild-type levels with the *spt7* knockout) may indicate indirect/off target effects as a result of not having fully functional

SAGA in the nucleus or a general role for SAGA in non-targeted histone acetylation. A recent study done in our lab showed a ~40% decrease with the Tra1-F3744A mutation at the *PHO5* promoter (AcH4K8) compared to the wild-type ratio of acetylated/total histone H4, which is consistent with the ChiP results presented in this study (Genereaux *et al.* 2012). In contrast, they found the level of acetylated K8 of H4 to be ~90% of wild-type levels at *PGK1* as a result of the Tra1-F3744A mutation. This is comparable to the effect seen with my Spt7 mutants (80%). Interestingly, a publication by Downey *et al* (2013) showed that *IFH1*, a gene which controls ribosomal protein (RP) transcription is only acetylated by Gcn5 in the context of intact SAGA complex and requires Spt7 to be present. The N-terminus of Spt7 may regulate the Gcn5 subunit in such a way that it is required for full regulation at the *PHO5* promoter and therefore suggests that most of the acetylation is mediated through SAGA rather than the ADA complex.

4.4 The N-terminal truncation of Spt7 has no effect on its interaction with Tra1 and Gcn5

A direct way for us to determine whether the effects described thus far were due to a loss of subunits from SAGA as a result of the missing 121 N-terminal residues was to perform immunoprecipitation assays. Previous studies by Wu *et al* (2004) and Lee *et al* (2011) mapped the numerous subunits of SAGA to specific modules. Tra1 was located away from Spt7 as part of its own module within the complex. Hoke *et al* (2010) found that an addition of a glycine at the C-terminus of Tra1 decreased association with Spt7, hinting at a structural connection between the two subunits in the context of SAGA. However, the

Tra1-L3733A mutant had no effect on interaction. My results suggest that a lack of the first 121 amino-terminal residues of Spt7 does not affect the presence of Tra1 within SAGA.

The ADA module, which contains Gcn5, is found directly adjacent to where Spt7 has been mapped (Wu *et al.* 2004). Thus, I predicted that a loss of interaction, or dissociation of Gcn5 from SAGA, would be more probable due to its close proximity to Spt7. In addition, a decrease in transcription (**Figure 3.3**) and acetylation (**Figure 3.4**) at SAGA-targeted promoters might coincide with dissociation of the ADA module from SAGA. The presence of Gcn5 in the context of the strain carrying Spt7₁₂₁₋₁₃₃₂ indicates that the mutation has no effect on Gcn5's association with SAGA. In addition, combining this result with the fact that Tra1 association is not affected suggests that the extreme N-terminus (up to residue 121) does not play a role in the maintenance of structural integrity of SAGA, although the effect on other SAGA subunits cannot be dismissed.

Overall, the severe decrease in growth and transcriptional levels, as well as lowered acetylation at lysine 18 of histone H3 at *PHO5* are not due to a dissociation of Gcn5 from SAGA. Rather, it is likely that the N-terminus of Spt7 regulates Gcn5 in a more subtle fashion, perhaps by partially coordinating the recruitment of SAGA to target promoters. Another possibility could be that since the bromodomain of Gcn5 confers specificity of binding to targeted genes via H3 and H4 amino terminal peptides (Synthichaki *et al.* 2000), the N-terminus of Spt7 may help coordinate Gcn5's binding to histones and facilitate transcriptional activation.

4.5 Spt7 localization is unaltered in the context of N-terminal truncations

The fact that eGFP-Spt7₇₃₋₁₃₃₂ and eGFP-Spt7₁₂₁₋₁₃₃₂ mutants were prevalent in the nucleus under regular growth conditions, as well as in the presence of 6% ethanol leads to the conclusion that the defects caused by the lack of the extreme N-terminus of Spt7 were not due to a mislocalization of the protein. This does not dismiss the possibility that the mutants may have caused a mislocalization within the nucleus itself. Such an effect could not be detected due to limitations in resolution with fluorescence microscopy. It was interesting to find that the eGFP-Spt7₁₋₁₂₀ N-terminal fragment only partially localized to the nucleus, with the rest of the signal being found in the cytoplasm. This result is somewhat inconsistent with the dominant negative growth phenotype observed in **Figure 3.2b** as a result of the expression of the Spt7₁₋₁₂₀ fragment, since I would predict it to be more prominently found in the nucleus if it is to affect SAGA function. However, perhaps it is possible that the small amount of the Spt7₁₋₁₂₀ fragment which localized to the nucleus could interfere with regular SAGA function. Another explanation for this effect on growth could be that the Spt7₁₋₁₂₀ fragment interfered with an aspect of formation of the SAGA complex within the cytoplasm. Expressing this fragment could potentially result in its binding and thus sequestering of SAGA subunits away from the complex, although this is unlikely given the previous observation that Spt7₁₂₁₋₁₃₃₂ did not affect Tra1 or Gcn5 interaction. The possibility of Spt7₁₋₁₂₀ binding to another subunit of SAGA cannot be dismissed however. Also, binding of the fragment to PtdInsPs could prevent binding by SAGA, and thus affect proper functioning of the complex.

4.6 Lipid binding by Spt7 is important for its function

Our discovery of a potential novel lipid binding domain of Spt7 sparked interest in studying the role of lipid binding in the functioning of SAGA. In fact, Ada2 was previously found to bind phosphatidylserine, although the biochemical consequences of this binding are unknown (Hoke *et al.* 2008a). Every fourth residue of the first 28 residues of Spt7 is either a lysine or arginine (**Figure 1.3b**). A minimum number of two basic amino acids, including at least one aromatic residue is a general feature of lipid binding domains, as mentioned in a review by Rosenhouse-Dantsker and Logothetis (2007). However, from multiple *in silico* analyses, the potential lipid binding domain of Spt7 did not match any known lipid binding domains (Chris Brandl, personal communication). In addition, analysis of the first 120 N-terminal residues of Spt7 via *raptor x* structure prediction software showed that this region was unstructured. It should also be noted that previous unpublished data in our lab showed that the downstream portion of Spt7 (fragment encompassing residues 558 to 869) also bound PtdInsPs. This indicates that lipid binding by Spt7 is not exclusive to its extreme N-terminal portion.

The 1-73 residue wild-type Spt7 peptide bound to several species of PtdInsPs, including PtdIns(3)P and PtdIns(3,5)P₂ which are relevant lipid species in yeast. PtdIns(3)P is mostly found at the endosomal membranes and in multivesicular bodies, while PtdIns(3,5)P₂ is found on vacuolar membranes (Strahl and Thorner, 2007). However, since Spt7/SAGA acts in the nucleus, it is important to focus our analysis of this binding on its connection to nuclear processes affected by PtdInsPs. PtdInsPs have been found to affect regulation of proteins within the nucleus such that binding by transcription factors

facilitates transcriptional activation of specific genes. One example can be found in a study by Han and Emr (2011), where they showed PtdIns(3,5)P₂ binding by a triad of interacting proteins Cyc8, Cti6 and the GTF Tup1 on late endosomal membranes. This binding results in a switch from a Tup1 corepressor state (Cyc8-Tup1) to a coactivator state (Cti6-Cyc8-Tup1 complex), facilitating its import back into the nucleus to recruit SAGA to the *GALI* promoter, leading to transcriptional activation (Han and Emr, 2011). As suggested by Han and Emr (2011), tight spatial and temporal control of specific signalling events within a highly complex intracellular environment would benefit from the coordinated binding of proteins with rare PIP species.

The observation that all lipid binding activity was lost upon mutation of the first four positively charged residues to glutamines highlights the importance of these residues to lipid binding. More specifically, it was interesting to see that mutation of the first two basic residues (R₄QK₈Q) led to a loss of binding to both PtdIns(3)P and PtdIns(3,5)P₂, while mutation of the third and fourth residues in combination (R₁₂QK₁₆Q) showed unaltered binding. Although results based on the lipid-overlay assay are variable in nature (Yu *et al.* 2004), each overlay assay was repeated to ensure a general consistency in experimental outcome. The fact that the R₄QK₈Q mutant showed a greater phenotype on medium lacking inositol and YPD at 37°C compared with both R₁₂QK₁₆Q, and R₄QK₈QR₁₂QK₁₆Q mutants was unexpected, since it would be predicted that the allele carrying four mutated residues would confer a more notable growth defect compared with one carrying only two mutations. This is likely explained by the fact that no binding to lipid is more acceptable to the cell than partial binding, since strict binding specificity is likely

required by Spt7 to properly aid SAGA in carrying out its regulatory functions. Brickner and Walter (2004) found that transcription of *INO1* occurs at the nuclear periphery. Since PtdInsPs are usually found at membranes (Strahl and Thorner, 2007), PtdIns(3)P and/or PtdIns(3,5)P₂ may aid in recruitment of SAGA via the N-terminus of Spt7 to coordinate *INO1* activation under inducing conditions. If this binding is affected by partial specificity as a result of N-terminal mutations of Spt7, the necessary cues would likely be missing to support full regulation of SAGA by Spt7. The results presented in this study not only narrow down the region necessary for PtdInsP binding within the N-terminus of Spt7, but also show that a loss of binding to PtdIns(3)P and PtdIns(3,5)P₂ may cause a minor decrease in cellular growth through affecting a specific aspect(s) of Spt7 function. Another way to test whether this lipid binding by Spt7 directly affects cell growth would be to utilize a lipid binding domain of another protein known to bind PtdIns(3)P and PtdIns(3,5)P₂, such as the FRRGT domain of Atg18 (Krick *et al.* 2006). The FRRGT domain of Atg18 could be cloned in to replace the missing 73 N-terminal residues of Spt7, and PIP-strip assays as well as growth assays could be performed to test for a gain of binding to PtdIns(3)P and PtdIns(3,5)P₂, as well as recovery from the minor growth defects observed, respectively. It would also be interesting to generate Spt7 mutants harbouring single mutations, such as Spt7-R₄Q alone in an attempt to map the specific residues responsible for binding PtdIns(3)P and PtdIns(3,5)P₂.

4.7 eGFP-tagged Spt7 mislocalizes in *fab1* and *vps34* deletion strains

Since Spt7 binds PtdInsPs, a change in the lipid balance of the cell might affect its function. Thus, I asked whether cells lacking both PtdIns(3)P and PtdIns(3,5)P₂, or PtdIns(3,5)P₂ alone would alter Spt7 localization. Interestingly, while both *Fab1*Δ [lacking PtdIns(3,5)P₂] and *Vps34*Δ [lacking both PtdIns(3)P and PtdIns(3,5)P₂] strains caused a mislocalization with both mutants and wild-type, the eGFP signal was slightly more dispersed in the context of the *Fab1*Δ strain compared to the *Vps34*Δ strain. The *Vps34*Δ strain showed the majority of eGFP to be in the nucleus. This result was intriguing, as it suggests that localization of Spt7 is more affected in a strain lacking only the PtdIns(3,5)P₂ lipid species. This brings about the possibility that a lack of binding due to absence of both lipid species results in Spt7 being shuttled back to the nucleus by chance, since it is not being held back in the cytoplasm by either lipid alone. In contrast, the *Fab1*Δ strain still contains PtdIns(3)P, which may result in sequestering of Spt7 on the endosomal membrane. Without PtdIns(3,5)P₂, the necessary cue may be lacking to facilitate import of the protein into the nucleus. It should be noted that both *fab1* and *vps34* deletions cause transcriptional defects at SAGA-targeted promoters. Previous research in our lab showed a decrease in transcriptional activation at both *PHO5* and *INO1* to approximately 50% and 10% of wild-type levels in *Fab1*Δ and *Vps34*Δ strains, respectively (Chris Brandl, unpublished). Such a finding provides a further link (direct or indirect) between PtdInsP regulation and SAGA function.

Tra1

Apart from investigating the functions of the N-terminus of Spt7, I contributed to furthering our understanding of how the FATC domain of Tra1 regulates this 3744 residue subunit of SAGA. Both wild-type Tti2 and the Tti2-F328S suppressor mutant showed nuclear as well as dispersed cytoplasmic eGFP signal. While wild-type Tra1 was exclusively nuclear, the Tra1-F3744A mutant partially mislocalized to cytoplasmic foci, especially when cells were grown in SC medium containing 6% ethanol. This mislocalization was partially reversed in a strain also carrying the Tti2-F328S suppressor. Lastly, the exact nature of the foci to which the FATC mutant of Tra1 mislocalized could not be specified, as its signal overlapped in a general fashion with numerous membrane components, including Anp1, Sec13, and Nic96. These results are briefly discussed below, drawing from the rest of our publication (Genereaux *et al.* 2012) and other research to discuss possible models in which the FATC domain regulates Tra1 via analysis of suppression by the Tti2-F328S allele.

4.8 Suppression of Tra1-F3744A by Tti2-F328S is partially achieved via an effect on its localization

Mislocalization of eGFP-Tra1-F3744A to cytoplasmic foci was observed compared to wild-type eGFP-Tra1. In our publication, we proposed that these foci are spots on membranes where Tra1 gets tethered as a result of the F3744A mutation (Genereaux *et al.* 2012). This observation leads to a presumption that wild-type Tra1 also likely binds to membrane components, potentially to stabilize and/or facilitate its folding, or assembly of

complexes it has been found to be a part of. Since wild-type Tra1 shows nuclear localization, this binding is likely too temporary to be detected. In contrast, the F3744A mutant may render the binding to membranes more permanent, hence our ability to detect the cytoplasmic presence of the protein. The pattern of Tra1-F3744A localization to cytoplasmic foci seems to lack specificity to any particular membrane, since eGFP-Tra1-F3744A signal did not exclusively overlap with any of the RFP-tagged membrane constituents alone. Rather, a general overlap was observed with RFP-tagged Anp1 (Golgi apparatus), Sec13 (ER-to-Golgi vesicles), Nic96 (nuclear periphery), Cop1 (retrograde vesicles), Pex3 (peroxisome), and Snf7 (late endosome to vacuole transport).

It was clear from my results that the Tti2-F328S suppressor reversed the mislocalization of Tra1-F3744A. Based on this, and other results in our paper (Genereaux *et al.* 2012), we concluded that Tti2 likely aids in the folding of Tra1. It is possible that decreased stability/folding efficiency experienced as a result of the Tra1 F3744A mutation may force the protein to stay on membranes for a longer period of time in order to fix this defect. The presence of Tti2-F328S likely results in accelerated folding of Tra1, facilitating its import into the nucleus to carry out its functions.

4.9 Model and Conclusions

This study demonstrates that the extreme N-terminus of Spt7 plays an important role(s) in the function of SAGA through regulating transcriptional activation and acetylation at SAGA targeted promoters, as well as binding to phosphatidylinositol phosphates. The exact mechanisms by which the N-terminus regulates these processes

cannot be pinpointed based on this study alone. However, it is likely, as suggested by Gansheroff *et al* (1995), that Spt7 plays a role in coordinating transcription at the level of initiation, besides also being required for structural integrity of SAGA (Wu and Winston, 2002). I ruled out the possibility of these effects being caused by a loss of interaction with Tra1 and Gen5, since the missing N-terminal residues did not alter Spt7/SAGA's association with these two subunits. To find out if the same applies for other subunits of SAGA, additional immunoprecipitation or gel filtration assays would need to be performed. Localization of Spt7₇₃₋₁₃₃₂ and Spt7₁₂₁₋₁₃₃₂ was unchanged in comparison to wild-type protein, leading to the conclusion that the lack of the extreme N-terminal residues did not cause a mislocalization of Spt7 away from the nucleus. Mislocalization within the nucleus itself should not be ruled out however, especially the possibility of the truncation mutants affecting Spt7 function whereby the N-terminus is required for proper recruitment of SAGA to its target promoters, or sites of transcriptional initiation at the nuclear membrane.

I mapped the region required for PtdIns(3)P and PtdIns(3,5)P₂ binding by the N-terminus of Spt7 to the first two positively charged N-terminal residues R4 and K8. The fact that mutation at these two residues to glutamines caused a minor phenotype when grown in medium lacking inositol suggests that lipid binding by Spt7 plays a role in SAGA-driven transcriptional regulation by potentially targeting/anchoring Spt7, and thus SAGA, to specific sites within the nuclear membrane. The presence of PtdIns(3)P in the nuclear membrane (as well as nuclear speckles) presents a plausible scenario for the direct regulation of SAGA by PtdInsPs, as it has been established that the nuclear envelope is important for structural organization and spatial arrangement of proteins and protein

complexes which coordinate transcription (Schink *et al.* 2013; Steglich *et al.* 2013).

Although PtdIns(3,5)P₂ has not yet been found in the nucleus, it is indirectly important in facilitating cellular processes in which SAGA is involved (Han and Emr, 2011). Lastly, Ada2 has already been found to bind phosphatidylserine (Hoke *et al.* 2008a), making the notion of SAGA regulation by lipid binding that much more attractive.

The exact function of the FATC domain of Tra1 has yet to be established, although it likely folds back to interact with the PI3K domain, as has been demonstrated by Yang *et al.* (2013) in the context of mTOR. It would be interesting to test for lipid binding by the FATC domain, since the Tra1-F3744A mutant was found to mislocalize to numerous foci within the cytoplasm, and since the nature of this binding to membrane components is not known. The association of Tra1 with membrane components makes lipid binding an attractive possibility, given the sensitivity that lipids and membranes exhibit in response to the environmental state. However, because the PI3K region does not bind lipid and since Tra1 does not contain any other regions likely involved in such binding, the direct binding of lipid by Tra1 is unlikely (Chris Brandl, unpublished). Alternatively, localization of Tra1 to membrane components may be a direct consequence of Spt7's affinity for lipid. Temporary docking to membranes via binding of Spt7 to lipids could serve as a structural docking site for the assembly of complexes within which Tra1 is found. Also, there lies the possibility that wild-type Tra1, which showed an exclusively nuclear signal could be specifically targeted within the nucleus by Spt7 to aid in the coordination of Tra1-containing complexes to sites of transcriptional regulation. It would be interesting to

explore this connection by tracking the localization of Tra1-F3744A in the context of strains carrying N-terminal truncation mutants of Spt7.

I showed that the Tti2-F328S suppressor partially reversed mislocalization of Tra1-F3744A from cytoplasmic foci. Separate assays performed in our lab suggest that this suppression effect is not due to increased interaction between the two proteins, or increased expression of the suppressor (Genereaux *et al.* 2012). Thus, we propose that Tti2 likely acts to aid in the folding of Tra1 via stabilization of the FATC domain. The effect of the Tra1-F3744A mutant on transcriptional activation at the *PHO5* promoter has also been tested, showing decreased levels to approximately one third of wild-type (Kvas, 2012). Acetylation at histone H3 of *PHO5* yielded very little effect with the same mutant (Genereaux *et al.* 2012). As seen with Spt7₁₂₁₋₁₃₃₂, the lack of a severe effect on acetylation is likely attributed to Gcn5 acting in the context of the ADA complex. The general resemblance in effects seen with both Tra1-F3744A and N-terminal Spt7 truncation mutants suggests a partial overlap in their requirement for facilitating SAGA-specific transcriptional activation.

Overall, the work presented in this thesis offers new perspectives on yeast Spt7, shedding light on previously unfound roles of its largely unstudied N-terminus. In addition, the discovery of lipid binding by the N-terminal region could prove to facilitate future research efforts into uncovering the subtleties of phosphatidylinositol phosphate binding on transcriptional regulation.

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Appendix

Table S1: *Saccharomyces cerevisiae* strains used in this study

Strain	Description	Plasmids	References
KY320	MATa ura3-52 ade2-101 trp1-Δ1 lys2-801 his3-Δ200 leu2::PET56	-	Chen, 1988
FY630	MATa ura3-52 ade8 trp-Δ63 his917 Δlys2-801173R2 leu2Δ1	-	Gansheroff <i>et al.</i> 1995
FY1093	MATa spt7-402::LEU2 his4-917- lys2-173R2 leu2-1 ura3-52 trp1-63 ade8	-	Gansheroff <i>et al.</i> 1995
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	-	Winzeler and Davis, 1997
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	-	Winzeler and Davis, 1997
CY4359	isogenic to FY1093	CB2154	this study (JG)
CY4367	isogenic to FY1093	CB2157	this study (JG)
CY4384	isogenic to FY1093	CB2162	this study (JG)
CY4385	isogenic to FY1093	CB2161	this study (JG)
CY4465	MATa spt7-402::URA3 his4-917- lys2-173R2 leu2-1 ura3-52 trp1-63 ade8	-	this study (JG)
CY5616	isogenic to FY1093	CB2216	this study (JG)
CY5795	isogenic to CY4465	DD20	this study (JG)
CY5796	isogenic to CY4465	CB2157	this study (JG)
CY5797	isogenic to CY4465	CB2273	this study (JG)
CY5798	isogenic to CY4465	DD19	this study (JG)
CY5921	isogenic to BY4741	DD28	this study (DD)
CY5922	isogenic to BY4741	DD29	this study (DD)
CY5854	isogenic to BY4741	CB2282	this study (DD)
CY6025	MATα ura3D0 his3D0 leu2D0 URA3-eGFP-tra1-F3744A-HIS3 x BY4741	-	Genereaux <i>et al.</i> 2012
CY6029	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 URA3-eGFP-TRA1 x BY4741	-	Genereaux <i>et al.</i> 2012
CY6062	isogenic to BY4741	CB2322	Genereaux <i>et al.</i> 2012
CY6063	MATα ura3D0 his3D0 leu2D0 tti2-F328S URA3-eGFP-tra1-F3744A-HIS3 x BY4741	CY1932	Genereaux <i>et al.</i> 2012

CY6079	isogenic to BY4741	CB1966	this study (DD)
CY6085	isogenic to BY4742 except spt7-402::LEU2 and fab1Δ::KanMX4	CB1966	this study (DD)
CY6086	isogenic to BY4742 except spt7-402::LEU2 and fab1Δ::KanMX4	DD28	this study (DD)
CY6087	isogenic to BY4742 except spt7-402::LEU2 and fab1Δ::KanMX4	DD29	this study (DD)
CY6089	isogenic to CY6085 except vps34Δ::KanMX4	CB1966	this study (DD)
CY6090	isogenic to CY6086 except vps34Δ::KanMX4	DD28	this study (DD)
CY6091	isogenic to CY6087 except vps34Δ::KanMX4	DD29	this study (DD)
CY6099	isogenic to FY1093	DD31 & DD33	this study (DD)
CY6108	MATa spt7-402::TRP1 his4-917- lys2-173R2 leu2-1 ura3-52 trp1-63 ade8	CB2288	this study (JG)
CY6110	MATa spt7-402::TRP1 his4-917- lys2-173R2 leu2-1 ura3-52 trp1-63 ade8	CB2293	this study (JG)
CY6139	isogenic to CY5871	CB1779 & CB2293	this study (DD)
CY6161	isogenic to BY4742	DD37	this study (DD)
CY6162	isogenic to BY4742	DD38	this study (DD)
CY6308	isogenic to FY630	DD33 & DD39	this study (DD)

*All strains referenced as from “this study” were generated by the author of this thesis, Dominik Dobransky (DD), or Julie Genereaux (JG) as indicated. All strains indicated as from Genereaux *et al* (2012) were engineered by Dr. Brandl.

Table S2: DNA constructs used in this study

Name	Description
DD6	1-73 residue fragment of Spt7 in pGEX-KGT expression vector (Miller <i>et al.</i> 2001)
DD7	1-73 R ₄ QK ₈ QR ₁₂ QK ₁₆ Q fragment of Spt7 in GEX-KGT expression vector
DD13	1-73-R ₄ QK ₈ Q mutant fragment of Spt7 in pGEX-KGT expression vector
DD14	1-73-R ₁₂ QK ₁₆ Q mutant fragment of Spt7 in GEX-KGT expression vector
DD18	intermediate molecule containing 1441 bp piece from CB2157 cloned into DD14 <i>HindIII/HindIII</i>
DD19	Full length Spt7 carrying R ₄ QK ₈ Q mutation in YCplac22 (N-terminal myc ⁹ trp ⁺ <i>cen DED1</i> promoter)
DD20	Full length Spt7 carrying R ₁₂ QK ₁₆ Q mutation in YCplac22 (N-terminal myc ⁹ trp ⁺ <i>cen DED1</i> promoter)
DD21	eGFP-Spt7 carrying R ₁₂ QK ₁₆ Q mutation in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD22	eGFP-Spt7 carrying R ₄ QK ₈ Q mutation in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD27	eGFP-Spt7 carrying R ₄ QK ₈ QR ₁₂ QK ₁₆ Q mutation in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD28	eGFP-Spt7 carrying 121-1332 truncation in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD29	eGFP-Spt7 carrying 73-1332 truncation in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD31	Spt7 _{WT} in YCplac22 (N-terminal flag ³ TRP <i>CEN DED1</i> promoter)
DD32	Spt7 _{1-121Δ} in YCplac22 (N-terminal flag ³ TRP <i>CEN DED1</i> promoter)
DD33	Gcn5 _{WT} in YEplac195 (N-terminal myc ⁹ URA 2μ <i>DED1</i> promoter)
DD36	N-terminal 1-120 residue fragment of Spt7 in YCplac22 N-terminal flag ³ TRP <i>cen DED1</i> promoter
DD37	N-terminal 1-120 residue fragment of Spt7 in YEplac195 (N-terminal myc ⁹ URA 2μ <i>DED1</i> promoter)
DD38	eGFP-tagged 1-120 residue fragment of Spt7 in pEGFP-C3 (Clontech Laboratories, Inc; Hoke <i>et al.</i> 2008b)
DD39	Spt7 ₁₂₁₋₁₃₃₂ YEplac112 (TRP 2μ flag ³)
CB1779	wild-type Tra1 in YCplac22 (N-terminal flag ³ TRP <i>cen DED1</i> promoter)
CB2157	Spt7 _{WT} in YCplac22 (N-terminal myc ⁹ TRP <i>cen DED1</i> promoter)
CB2161	Spt7 ₁₂₁₋₁₃₃₂ in YCplac112 (N-terminal myc ⁹ TRP 2μ <i>DED1</i> promoter)
CB2216	Spt7 ₇₃₋₁₃₃₂ in YCplac112 (N-terminal myc ⁹ TRP 2μ <i>DED1</i> promoter)
CB2288	Spt7 _{WT} in YEplac195 (N-terminal myc ⁹ URA 2μ <i>DED1</i> promoter)

CB2293	Spt7 ₁₂₁₋₁₃₃₂ in YEplac195 (N-terminal myc ⁹ URA 2 μ <i>DED1</i> promoter)
CB1503	LacZ-PHO5 fusion in <i>LEU2</i> centromeric plasmid YCp87 (Brandl <i>et al.</i> 1993)
CB1467	LacZ-INO1 fusion in <i>LEU2</i> centromeric plasmid YCp87 (Brandl <i>et al.</i> 1993)
CB2112	LacZ-SRE/His3 fusion in <i>LEU2</i> centromeric plasmid YCp87 (Hoke <i>et al.</i> 2010)
CB1533	LacZ-Rpl35A fusion in <i>LEU2</i> centromeric plasmid YCp87

Table S3: Oligonucleotides used in this study

Name	Sequence (5' to 3')	Description
3203-1	cgggatcctaagaggccaacgtcgatg	primer for 5' end of promoter region of RPL35A into His4. Has a <i>Bam</i> HI site in frame for LacZ fusion
3203-2	cggaagctccatttgcaatatactgtctcac	primer for 3' end of promoter region of RPL35A into His3. Has a <i>Hind</i> III site in frame for LacZ fusion
5077-1	atcgggatccacaatggtgagcaaggcgagg	primer for amplifying eGFP from the eGFP-C3 plasmid. Contains <i>Bam</i> HI site
5077-2	tttcttttgcgccgctgtacagctcgtccatg	primer for amplifying eGFP from the eGFP-C3 plasmid. Contains <i>Not</i> I site
5914-1	gatccatgacggaacagattcctattcagaactcaacagacgaatgcgca	primer to kinase, anneal to 5914-2 and ligate into DD5-Spt7 construct - first four positive residues changed to Gln
5914-2	agcttgcgcatctctgtgtgatagttctgaataggaatctgttccgcatg	primer to kinase, anneal to 5914-1 and ligate into DD5-Spt7 construct - first four positive residues changed to Gln
5939-1	agggatccatgactgaacagataccaatacagaattatcaaagaac	N-term Spt7 mutant (R ₄ QK ₈ Q) to amplify off of the wild type strand (1-73 wild-type Spt7 in Y240 vector with filled in <i>Hind</i> III; DNA DD6)
5939-2	agggatccatgacggaagaattcctattaagaactcaacagac	N-term Spt7 mutant (R ₁₂ QK ₁₆ Q) to amplify off of the DD7 mutant strand (1-73 R ₄ QK ₈ QR ₁₂ QK ₁₆ Q Spt7 in Y240 vector with filled in <i>Hind</i> III
5799-1	ggaattctattttcgacatttaatgctatgtt	primer to clone piece of Spt7 into y61 as GST fusion. Non-coding strand, stops at residue 73 (has engineered stop codon).
6001-1	ataagaatcggcgcccatgactgaacagataccaa	Forward primer with <i>Not</i> I site to PCR up DD17 (intermediate molecule carrying R ₄ K ₈ mutation of Spt7 N-term), to clone mutation into full length Spt7 for yeast studies

6001-2	ataagaatcggccgcatgacggaagaattcc	Forward primer with <i>NotI</i> site to PCR up DD18 (intermediate molecule carrying R12K16 mutation of Spt7 N-term), to clone mutation into full length Spt7 for yeast studies
5741-1	ataagaatcggccgctgtgaaaatgaaattgaagataaag	primer to clone piece of Spt7 into N-terminal 9-myc plasmid with <i>NotI</i> at 5' end
5741-2	cagtattcaggtccatggact	3' primer in Spt7 at base pair 1471. Non-coding strand, includes native <i>NcoI</i> site.
4051-1	gcggccgcaatgactgaagaataccaataaag	5' primer of Spt7 gene
5705-1	ggaattctaattgatttcattgctgatactgg	primer to clone piece of Spt7 into y61 as GST fusion. non-coding strand, stops at residue 120 (has engineered stop codon).

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