INVESTIGATING THE ROLE OF HISTONE ACETYLATION IN YEAST FACT COMPLEX FUNCTION

by

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Abstract

The FACT (facilitates chromatin transactions) complex, a heterodimer comprised of Spt16p and Pob3p, is involved in a number of processes involving DNA, including transcription initiation, elongation, and repression, DNA replication, and DNA repair. FACT is thought to facilitate these processes through its ability to bind histones and reversibly alter nucleosomal structure modulating DNA accessibility. Recent data has shown that the Spt16p subunit of FACT interacts with Sas3p of the NuA3 HAT (histone acetyl transferase) complex. Interestingly, mutants of NuA3 share none of the many phenotypes observed in FACT mutants, suggesting that this putative interaction may be redundant to other unknown FACT interactions. In the first part of this study, we confirmed the interaction between FACT and NuA3, and proceeded to describe, for the first time, an interaction between FACT and the ADA HAT complex both *in vivo* and *in vitro*. The interaction of FACT with NuA3 and ADA was then mapped to the N-terminal 482 residues of the Spt16p FACT subunit. However, attempts to implicate this interaction with known FACT functions were unsuccessful. Biochemical approaches similarly failed to demonstrate a role for FACT in NuA3 and ADA complex function.

The second part of this study focused on the novel *spt16Δ922* mutant, which expresses 182 residues more of the Spt16p polypeptide than the *spt16ΔNTD* allele, yet possesses a number of mutant phenotypes. Our results show that the *spt16Δ922* mutation causes a loss in histone H3 acetylation, supporting the hypothesis that the protein encoded by *spt16Δ922* is interfering with normal histone interactions. This loss in histone acetylation was shown to parallel the generation of an *spt* phenotype, suggesting a possible role for histone acetylation in preventing aberrant transcription. However, further work examining the role of histone acetylation with respect to FACT yielded conflicting results. We conclude that *spt16Δ922* is interfering with histone modifications, although the molecular basis of this is as of yet unknown.

Table of Contents

List of Tables

List of Figures

Nomenclature

The text follows the conventional method for designating genetic symbols and protein products. Dominant alleles of wild type genetic loci are designated by italicized upper case letters (e.g. *SAS3*) while mutant genes are designated with italicized lower case letters (e.g. *sas3*). Gene deletions are written as a mutation with a Greek 'delta' following the designation (e.g. *sas3Δ* indicates deletion of the *SAS3* locus). Insertion mutations are indicated with the symbol ::. For example *sas3::HISMX6* indicates that the *HISMX6* gene is inserted within the *SAS3* locus. Gene products are not italicized and only the first letter is capitalized followed by a 'p' postscript (e.g. Sas3p is the gene product of the *SAS3* locus).

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As always, difficult times are overcome during any long-term project. Thank you to all the friends (many of whom have already been mentioned) and family that have always listened, despite often not completely understanding what it was I was talking about.

Dedication

This thesis is dedicated to my parents and grandparents.

Chapter 1 – Introduction

1.1 Chromatin – Structure and Function

1.1.1 The Nucleosome and Higher Order Structure

The basic repeating subunit of chromatin is the nucleosome core particle. It is made up of 147 base pairs (bp) of DNA wound 1.7 times around a histone octamer (1). Separating each core particle is a 10-60 bp stretch of linker DNA, the length of which varies depending on a number of factors including the presence of DNA binding proteins (2). This repeating structure of the nucleosome core and linker DNA forms the beads-on-a-string model and is the foundation for all higher order chromatin structure.

The histone octamer is composed of two copies each of four highly conserved histone proteins. The central tetramer is made up of two copies each of histones H3 and H4. Two H2A-H2B dimers flank the central $(H3)_2(H4)_2$ tetramer and complete the histone octamer (3-5). The interactions of histone proteins with other histones and with DNA are the result of a common core histone motif termed the histone fold (6). The association with DNA is through the phophodiester backbone, forming a coiled structure around the histone core of 1.7 left-handed superhelical turns (2). Sticking out of the histone core are the largely unstructured, highly basic, N-terminal tails of each histone protein (8). These tails are thought to modulate nucleosomal folding and higher order chromatin structure through a variety of mechanisms, including protein-protein interactions, and charge neutralization of DNA (9).

The next order of chromatin structure believed to form *in vivo* is the 30 nm fibre (1). This structure and further fibre-fibre interactions are modulated by both histone tails and the

actions of linker histones. These linker histones are non-core histone proteins, and associate at the periphery of the nucleosome. They are believed to impart stability to the core complex and play a role in organizing greater oligomeric interactions between nucleosomes. In vertebrates, linker histones H1 and H5 are essential, and associate at a 1:1 ratio with the nucleosome core (9). In yeast, the linker histone Hho1p has not been shown to have any clear ratio (10). In fact, linker histones are not essential in yeast or lower organisms (11, 12), suggesting that histone tails may play a more fundamental role in chromatin condensation (1).

Additional structural organization beyond the 30 nm fibre also involves non-histone proteins. These include Sir3p and Tup1p, two yeast silencing proteins that function through interactions with histone tails (13, 14). These proteins function by cross-linking chromatin fibres through the interaction of other silencing proteins. This causes an overall coming together of nucleosomes over long distances, resulting in the formation of a highly ordered structure (9). These highly condensed and tightly packaged regions of chromatin are referred to as heterochromatin, and generally have a lower gene density. Conversely, less ordered chromatin, or euchromatin, has a higher gene density (15).

1.1.2 The Regulation of Chromatin Structure – Links to Gene Expression

The structure of chromatin inherently restricts the access of DNA by cellular machinery for processes like transcription or replication (16, 17). The regulation of chromatin structure, then, has an important role in the control of gene expression (18-20). The repressive effects of chromatin structure are thought to be mediated by two sets of interactions, those between histones and DNA, and those involving the histone tails. Mechanisms that modulate these

interactions include the incorporation of histone variants, the action of ATP-dependent chromatin remodelers, and post-translational modification of histones (2).

In yeast, the histone variant Htz1p has been shown to positively regulate gene expression by destabilizing nucleosomes at the promoters of genes (21). Loss of the nucleosome unpackages the promoter and allows it to interact with transcription binding factors, leading to gene expression. Whereas histone variants are substituting actual components of the nucleosome, ATP-dependent chromatin remodelers affect the position of the nucleosome with respect to DNA. There are a variety of remodeling complexes that are thought to function through slightly different mechanisms. The first of the ATP-dependent chromatin-remodelers to be discovered was from the SWI/SNF family, revealed through studies selecting for genes whose products counteract histone-mediated transcriptional repression (22). SWI/SNF complexes disrupt DNA-histone contacts, allowing uniform accessibility of DNA (23, 24). Another complex of the SWI/SNF family, RSC, is thought to function by transferring the entire histone octamer to another molecule of DNA (25). Yet another mode of chromatin-remodeling is observed in the ISWI family of complexes, which enable movement or sliding of the histone octamer to adjacent positions on DNA (26). The final mechanism of modulating chromatin structure is the use of post-translational covalent histone modifications, these include lysine and arginine methylation, lysine acetylation, serine phosphorylation, and ubiquitination (27).

1.1.3 The Histone Code Hypothesis

Originally, post-translational histone modifications were generally thought to alter histone-DNA and histone-histone interactions, through the actions of charge neutralization and addition, thus altering nucleosomal structure (28). However, subsequent work has demonstrated a degree of complexity that cannot be explained solely by the direct effects of modifications

themselves. For example, histone acetylation, generally perceived as disrupting DNA-histone interactions through histone tail charge neutralization, is observed in transcriptionally silent regions of chromatin (28). This has led to the development of the histone code hypothesis, which states that post-translational histone modifications may modulate gene expression by encoding docking sites or recruitment signals recognized by other proteins.

Evidence supporting the histone code hypothesis includes the identification of the bromodomain, a DNA binding motif that specifically recognizes acetylated lysines on the histone tail (27). Both SWI/SNF and SAGA (Spt, Ada, and Gcn5 Acetyltransferase) complexes contain the bromodomain, and preferentially associate with acetylated nucleosomes over unacetylated nucleosomes (28). A more recent example of the histone code is the discovery that Set2p methylation of histone H3 lysine 36 causes the recruitment of a histone deacetylase Rpd3S complex. This recruitment is dependent on a chromodomain of the Eaf3p subunit of the Rpd3S complex (29, 30). Although the implications of the histone code hypothesis are still only beginning to be understood, it is becoming increasingly clear that the modification of histones can cause widespread downstream effects including transcriptional activation and repression.

1.2 Histone Acetylation

1.2.1 Histone Acetylation

The most well studied of all the post-translational histone modifications is acetylation. This modification is catalyzed by a group of enzymes called histone acetyltransferases (HATs) which transfer an acetyl group from acetyl co-enzyme A to lysine residues of histone proteins (31). The sites of modification reside primarily on the histone tails, though recent work has

shown the globular domains of histone proteins are targets of acetylation as well (32-34). The association between histone acetylation and transcription has existed for quite some time (35, 36), and the discovery that some transcription activators and co-activators, like Gcn5p (general control non-deprepressible 5), are actually HATs only served to strengthen this view (37-40). However, more recent work has shown acetylation to be involved with both transcriptional activation and repression, indicating that the effect of histone acetylation cannot be entirely generalized (28, 41).

Table 1.1 Sites of Histone Acetylation in Yeast (34, 42, 43)

	Histones			
	H2A	**H2B**	**H3**	**H4**
Acetylation Sites	K5	K5		K5
				K8
	K9		K9	
		K12		K12
			K14	
		K15		
				K16
			K18	
		K20		
			K23	
			K27	
			K56	

Histone acetylation is generally believed to alter chromatin structure in two ways. The first is that acetylation of histone tails neutralizes the positively charged lysine residues, disrupting interactions with DNA causing altered nucleosomal folding and chromatin structure (44, 45). Although this model does fit with known histone-DNA interactions, the actual effect of acetylation on nucleosome structure directly appears rather modest (46). Additionally, the simplicity of this model fails to explain seemingly contradictory results concerning acetylation and gene expression. The second, mechanism of histone acetylation relates to its function within the histone code hypothesis, specifically that modified residues modulate the activity of

activators and repressors of gene activity (47). As has already been described, both SWI/SNF and SAGA complexes specifically recognize and bind acetylated histones.

Histone acetyltransferase complexes are also subject to recruitment themselves. Yeast HATs have been shown to associate with the transactivation domains of activators including VP16 and Gal4 (48), and SAGA and NuA4 are recruited to the nucleosome through a shared Tra1p subunit (49). It is thought that this sequence-specific targeting via DNA-binding proteins adds another level of control over transcription (50).

1.2.2 HAT and HDAC Complexes

The HATs responsible for post-translational acetylation of histones are categorized as type-A HATs and are located in the nucleus. Type-B HATs are found in the cytoplasm and acetylate histones prior to transport into the nucleus. In the yeast *Saccharomyces cerevisiae* there are at least eight different proteins shown to have HAT activity in *in vitro* or *in vivo* assays (34, 37, 51). Two of these HATs relevant to this study are the H3 specific Gcn5p and Sas3p (something about silencing 3).

Gcn5p is part of the GNAT (Gcn5p-related N-acetyltransferases) super family, which also includes Hat1p, Elp3p, and Hpa2p (37). Studies have shown that Gcn5p contains a bromodomain, Ada2p (adaptor deficiency or alteration) interaction domain, and a HAT domain essential for acetyltransferase activity (52, 53). In yeast, Gcn5p is closely associated with the adaptor proteins Ada2p and Ada3p (54). These three proteins form a trimeric module that forms the basis of all known Gcn5p HAT complexes, including SAGA, ADA, SLIK/SALSA, and HAT-A2 (55-59). As shown *in vitro,* Gcn5p alone has the ability to acetylate free histone H3 at residue K14 (60). Once incorporated into HAT complexes, though, Gcn5p not only gains the ability to acetylate nucleosomal histones (59), but also expands the specificity of those residues

acetylated (61). Both SAGA and ADA have been shown to acetylate K9, K14, K18 and K23 on histone H3.

Table 1.2 Components of the Gcn5p Containing HAT Complexes SAGA, SLIK/SALSA, and ADA (42) and the NuA3 Complex (62, 63)

	Complex				
	SAGA	**SLIK/SALSA**	**ADA**	**HAT-A2**	**NuA3**
Subunits	Gcn5	Gcn5	Gcn5	Gcn5	Sas3
	Ada2	Ada2	Ada2	Ada3	Yng1
	Ada3	Ada3	Ada3	Ada3	Nto1
	Ada1	Ada1			
	Tra1	Tra1			Taf14
	Spt20	Spt20			Eaf6
	Spt3	Spt3			
	Spt7	Spt7			
	Spt8				
	Taf5	Taf5			
	Taf6	Taf6			
	Taf9	Taf9			
	Taf10	Taf10			
	Taf12	Taf12			
	Sgf11	Sgf11			
	Sgf29	Sgf29	Sgf29		
	Sgf73	Sgf73			
	Ubp8	Ubp8			
		Rtg2			
			Ahc1		

Originally identified in a screen for silencing defects in a *sir1Δ* background, Sas3p has subsequently been shown to function as both a positive and negative regulator of transcription depending on the gene locus (63). Sas3p belongs to the MYST (MOZ, Sas3p/Ybf2p, Sas2p, and Tip60p) family of histone acetyltransferases (37). As with Gcn5p, Sas3p possesses HAT activity, but can only acetylate nucleosomal histones when incorporated into NuA3, a 400 KDa complex made up of Sas3p, Yng1p, Nto1p, Taf14p, and Eaf6p (Howe, L. unpublished data, (62, 63). NuA3 has been shown *in vitro* to preferentially acetylate histone H3 K14 and, slightly less effectively, K23 (64).

It is interesting to note that although histone acetylation is well conserved and seems to be involved in important cellular processes, only one HAT is essential, Esa1p of the HAT complexes NuA4 and Piccolo NuA4 (65, 66). It has also been shown that the combination of deleting *GCN5* and *SAS3* is synthetically lethal, so called because the lethal phenotype is being "synthesized" (64). This lethality is thought to be the result of a loss in some essential acetylation activity that requires at least one of Gcn5p or Sas3p. This lack of serious phenotypes in HAT mutants does suggest a certain degree of redundancy in their actions. Indeed, analysis of acetylated residues targeted by each HAT complex has revealed many similarities, for instance those between SAGA and ADA (61). Again, this serves as a reminder of the complexity and still developing understanding we have of histone acetylation.

To counteract the effects of HATs, there exist histone deacetylase complexes (HDACs). Just as histone acetylation has largely been associated with transcription, deacetylation has long been linked with silencing and repression (67). More recently, however, the Hos2p HDAC has been shown to deacetylate nucleosomes of coding regions during gene expression (68). In yeast, there are at least ten HDACs in three phylogenetic classes, including Rpd3p, Hda1p, Sir2p, as well as the Hos and Hts proteins (69). One of the better characterized HDACs is Rpd3p, which has recently been shown to be a part of two distinct complexes, Rpd3S and Rpd3L (29, 30). These two complexes have both been shown to deacetylate nucleosomes in coding regions, preventing spurious transcription. Rpd3S has been previously mentioned for its ability to be recruited by Set2p methylation in a transcriptionally repressive mechanism.

1.3 Spt16p and the Yeast FACT Complex

1.3.1 The *SPT* Genes

In yeast, the homologous recombination of Ty-retrotransposons with the genome can result in the insertion of δ-elements adjacent to or within genes (70). Two such genes include *HIS4* and *LYS2*, resulting in the *his4-912δ* and *lys2-128δ* alleles (71). In normal cells, transcription of these genes is disrupted by the presence of promoter and termination sequences within the δ-element, for example, in the *lys2-128δ* allele, the δ-element inserts downstream of the start of the *LYS2* gene. Transcription initiates and proceeds as normal but is prematurely terminated upon reaching the δ-element, causing the production of a non-functional *LYS2* transcript, resulting in lysine auxotrophy (72). The mutation of *SPT* (suppressor of Ty) genes somehow alleviates the effects of δ-element insertion, rescuing production of a functional gene transcript (71). In terms of the *lys2-128δ* allele, *SPT* gene mutants are thought to lose the ability to repress transcription from promoter elements in the δ-element. This cryptic initiation produces a truncated, though still functional *LYS2* transcript, rescuing lysine auxotrophy. This loss of transcriptional repression is thought to be representative of a genome wide effect, as aberrant transcription has been observed at other loci besides *lys2-128δ* in mutants of *SPT* genes (73-75).

SPT genes are categorized into two groups defined by the Ty elements they suppress (71). The first includes those genes that are related to TFIID function. The second, and more relevant to this study, are the histone related *SPT* genes. These include *SPT11* and *SPT12*, later identified as the histone H2A and H2B encoding *HTA1-HTB1* locus (76), *SPT4, SPT5, SPT6,* and *SPT16* (77-79). These *SPT* gene mutants share similar characteristics, including counteracting the transcriptional defects of SWI/SNF mutants, and suppressing the loss of

upstream activator sequence (UAS) regions, needed for binding of transcriptional activators (71). Additionally, the histone related *SPT* gene mutants share many of the same effects as an imbalance in histone protein ratio and nucleosome disruption, suggesting that these *SPT* genes function to properly maintain nucleosome structure, and in so doing repress spurious gene expression (76).

1.3.2 The Yeast FACT Complex – Spt16p and Pob3p

The yeast FACT complex (facilitates chromatin transactions - hereafter referred to as FACT) is comprised of two components, Spt16p and Pob3p (polymerase one-binding 3) (80). Both subunits are essential in yeast, and show a high level of conservation throughout eukaryotes as seen in the human FACT complex and the frog DUF1 complex (81, 82). The identification of *SPT16* actually occurred simultaneously through two different screens, one for suppressors of Ty, and the other for cell division cycle (CDC) mutants (78, 79). Surprisingly, both screens picked out the same G132D mutant of *SPT16/CDC68*. These initial screens demonstrated that *SPT16* could affect transcription of different genes both positively and negatively, providing the first clues to Spt16p and FACT function. Recent domain analysis of Spt16p has shown it has three domains, a C-terminal (CTD), a mid, and an N-terminal domain (NTD) (83). The CTD was found to be essential for cell viability, suggesting it is required for FACT's essential function, and the mid-domain is responsible for mediating interactions with the other FACT subunit, Pob3p. As for the NTD, it is not required for interactions within FACT, and is suggested to interact with other proteins (83, 84).

Pob3p is the smaller of the two FACT subunits, and was identified through its ability to bind DNA polymerase α (85). One difference of the yeast Pob3p is that it lacks a high mobility group (HMG) box domain, a DNA-binding motif found in other Pob3p homologs SSRP and

DUF87 (in human FACT and DUF1 respectively) (86). Instead, yeast FACT utilizes the HMG1 motifs of Nhp6p (non-histone chromosomal protein 6), a ubiquitous nucleosomal binding protein involved in altering nucleosome properties (86). However, strains with deletions of Nhp6p are viable (87), suggesting that the two essential subunits of FACT proper do not require Nhp6p activity for function.

1.3.3 FACT Functions in Processes Involving DNA

FACT has been implicated in a variety of processes involving DNA. Initially, the complex was identified through its ability to facilitate RNA polymerase II transcription of nucleosomal DNA *in vitro* (88). Further *in vitro* research has shown that FACT binds H2A-H2B dimers, destabilizing their interaction with $(H3\text{-}H4)_2$ tetramers (82, 84, 89). *In vivo*, FACT is distributed in parallel with RNA polymerase II, further supporting its role in facilitating elongation (73, 90). In addition, genetic studies have shown FACT to be important in mediating nucleosome structure and transcription (86, 91). Currently, FACT is believed to associate with the nucleosome, facilitating the displacement of H2A-H2B, disrupting nucleosomal structure and allowing RNA polymerase II progress (89, 92).

FACT also has a repressive effect on transcription, as demonstrated by the initial studies of *SPT16* as a repressor of aberrant transcription (78, 79). Further work has supported this role as *spt16* mutants allow the production of aberrant transcripts from other genes in addition to *lys2-128δ* (74). It is suggested that FACT mutants fail to properly maintain chromatin structure, thus allowing accessibility to normally non-permissive promoter regions. This may be a result of improper re-packaging of the nucleosome following FACT's role in transcriptional elongation (74, 92). This failure in preventing aberrant transcription may also be related to FACT's putative role in moderating the fidelity of transcriptional initiation. It has been shown that, for certain

genes, FACT is required for proper promoter association with the preinitiation complex, likely through nucleosomal alterations at promoter sequences (73). Mutants of *SPT16,* then, fail to prevent similar initiation from hidden promoters which are normally repressed.

Other processes FACT is associated with are DNA replication and DNA repair. In addition to the association of FACT with subunits of the DNA polymerase α complex (80, 93), mutations of FACT have been shown to interact genetically with genes important in DNA replication (94). Depletion of the FACT complex in *Xenopus* oocytes has also been shown to compromise DNA synthesis *in vitro* (81). FACT also interacts with the protein kinase CK2, which phosphorylates and activates p53 during times of replication stress (95).

Whether these functions of FACT are independent of each other or representative of a more general function is currently not clear. Considering the above processes all involve DNA, and therefore must overcome the non-permissive state of chromatin, it is conceivable that FACT is simply involved in maintaining nucleosome structure. Recent work has suggested that FACT may be important in nucleosome reassembly, as mutations in *SPT16* and *POB3* caused dependence on the Hir/Hpc nucleosome reassembly pathway (91). In any case, FACT remains an integral facilitator of important cellular processes, although the mechanisms by which it acts are not completely elucidated.

The research described in this study aimed to understand the possible relationship between histone acetylation and FACT complex function, first suggested in recent studies showing a putative interaction between Sas3p (of NuA3) and Spt16p (63, 96). In the first part of this thesis, we describe the confirmation of this interaction, and investigate FACT's association with other HAT complexes and their possible role in FACT function. The second half focuses on the novel *spt16Δ922* mutant, specifically examining the hypothesis that this partially truncated Spt16p interferes with normal histone modifications.

Chapter 2 – Materials and Methods

2.1 Preparation of Yeast Strains and Plasmids

The manipulation of yeast strains in this study was carried out using standard protocols (97). These include all transformations, sporulation, screening, and plating. All yeast strains used in this study are listed in table 2.1, and all plasmids in table 2.2.

The insertion of both TAP tags and HA tags downstream of *HTB1, POB3,* and *AHC1* required additional PCR product. Consequently, 3x 100 μL reactions were concentrated to 40 μL for transformation into the cell. PCR purifications and gel purifications were done using the Wizard SV Gel and PCR Clean-up system (Promega Corporation, Madison, WI).

The *spt16* null strain SNL314 and all *SPT16* plasmids were gifts from our collaborators in the R.A. Singer Lab at Dalhousie University. The haploid *spt16::KANMX4* strain is maintained by a wild type *SPT16* locus on the p*SNLCDC68(3)* plasmid. This plasmid consists of the 4.6 kb BglI/BamHI wild type *SPT16* fragment from p68BSX1 cloned into pSLncrΔBS. The p*SNLCDC68(3)* plasmid includes the *URA3* gene and the *ADE3* gene, allowing for selection against it using 5`-FOA plates (0.1% of 5`-fluoroorotic acid) media and red/white colour-screening respectively. All pRS315 wild type and mutant *SPT16* plasmids were created by inserting a BamHI/SstI *SPT16* fragment from a pRS316 plasmid into pRS315. The *spt16Δ922* truncation mutant was originally created using an SstI/EagI *SPT16* fragment which was subsequently mutagenized and subject to in-frame deletion with ClaI before KpnI/EagI digestion yielded the *spt16Δ922* gene fragment (98). The *spt16ΔNTD* truncation mutant was originally created from a NcoI/BtgI digestion of full-length *SPT16* on pJW212 (83).

The p*SPT8HA* plasmid contains the *SPT8* ORF plus ~1,000 bp of upstream sequences including the endogenous promoter, PCR amplified from genomic DNA. The fragment was then digested by ApaI/SalI and cloned into a *CEN/ARS TRP1* vector pRS314 fused to a triple hemagglutinin (HA)-CYC cassette.

The wild type *HHT2-HHF2* plasmid p*HHT2* was created by SpeI digestion of pDM18 (99), followed by ligation of the largest fragment into the SpeI site of the vector pRS414 (100). All plasmids with *HHT2* point mutations were created by site directed mutagenesis using Stragatene's QuickChange Site-Directed Mutagenesis Kit (101).

Table 2.1 Yeast Strains Used in This Study

Strain	Mating Type	Genotype
SNL314	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pSNLCDC68(3).URA*
YJS101	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68NM2(4).LEU*
YJS102	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68NM3(4).LEU*
YJS103	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68M3(12).LEU*
YJS104	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68M3(19).LEU*
YJS105	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68ΔNTD.LEU*
YJS106	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68A4.LEU*
YJS107	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68NM2(4).LEU*
YJS108	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68NM3(4).LEU*
YJS109	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68M3(12).LEU*
YJS110	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68M3(19).LEU*
YJS111	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68ΔNTD.LEU*
YJS112	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68Δ922.LEU*
YJS113	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU*
YJS114	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68ΔNTD.LEU*
YJS115	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68Δ922.LEU*
YJS120	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU pAHC1HA.URA*
YJS121	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68ΔNTD.LEU pAHC1HA.URA*
YJS122	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68Δ922.LEU pAHC1HA.URA*
YJS127	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68A4.LEU*
YJS128	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68ΔNTD.LEU*
YJS129	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68Δ922.LEU*
YJS130	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68NM2(4).LEU*
YJS131	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68NM3(4).LEU*
YJS132	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68M3(12).LEU*
YJS133	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pCDC68M3(19).LEU*
YJS134	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 cdc68::KANMX4 HTB1-3HA::HIS3MX6 POB3TAP::TRP1 pSNLCDC68(3).URA*

YJS137	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 cdc68::KANMX4 HTB1-3HA::HIS3MX6POB3TAP::TRP1 pCDC68A4.LEU*
YJS138	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 cdc68::KANMX4 HTB1-3HA::HIS3MX6 POB3TAP::TRP1 pCDC68ΔNTD.LEU*
YJS139	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 cdc68::KANMX4 HTB1-3HA::HIS3MX6 POB3TAP::TRP1 pCDC68Δ922.LEU*
YJS141	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 POB3-3HA::HIS3MX6*
YJS145	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 HTB1-3HA::HIS3MX6 POB3TAP::TRP1 ada2::KANMX4*
YJS146	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 HTB1-3HA::HIS3MX6 POB3TAP::TRP1 sas3::KANMX4*
YJS148	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 rpd3::URA3 pHHT2(K14R).TRP*
YJS149	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 rpd3::URA3 pHHT2(K23R).TRP*
YJS153	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 AHC1-3HA::HIS3MX6*
YJS155	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU pADA2HA.URA*
YJS156	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU pSPT8HA.URA*
YJS157	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 NTO1-3HA::HIS3MX6 pFLAG-SPT16.TRP*
YJS158	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 YNG1HA::KANMX6 pFLAG-SPT16.TRP*
YJS160	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68A4.LEU pAHC1HA.URA*
YJS161	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68ΔNTD.LEU pAHC1HA.URA*
YJS162	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68Δ922.LEU pAHC1HA.URA*
YJS163	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pAHC1HA.URA*
YJS164	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pAHA2HA.URA*
YJS165	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pSPT8HA.URA*
YJS166	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 SAS3-3HA::KAN pFLAG-SPT16.TRP*
YJS167	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pFLAG-SPT16.TRP*
YJS168	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pFLAG-SPT16.TRP pAHC1HA.URA*
YJS169	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pFLAG-SPT16.TRP pADA2HA.URA*
YJS170	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pFLAG-SPT16.TRP pSPT8HA.URA*
YJS171	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 AHC1-3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS172	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 AHC1-3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS173	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 SAS3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS174	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 SAS3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS176	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 AHC1TAP::TRP*
YJS177	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 NTO1-3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS178	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 NTO1-3HA::HIS3MX6 pSNLCDC68(3).URA*
YJS181	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU pYNG1HA.URA*
YJS182	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pSNLCDC68(3).URA pSAS3HA.LEU*
YJS183	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pYNG1HA.URA*
YJS184	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pSAS3HA.URA*
YJS185	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 AHC1-3HA::HIS3MX6 pCDC68A4.LEU*

YJS186	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 AHC1-3HA::HIS3MX6 pCDC68ΔNTD.LEU*
YJS187	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 AHC1-3HA::HIS3MX6 pCDC68Δ922.LEU*
YJS188	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 SAS3HA::HIS3MX6 pCDC68A4.LEU*
YJS189	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 SAS3HA::HIS3MX6 pCDC68ΔNTD.LEU*
YJS190	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 SAS3HA::HIS3MX6 pCDC68Δ922.LEU*
YJS194	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 POB3-3HA::HIS3MX6 pFLAG-SPT16.URA*
YJS195	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 pYNG1HA.URA pFLAG-SPT16.URA*
YJS196	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pSPT8HA.URA pCDC68A4.LEU*
YJS197	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pSPT8HA.URA pCDC68ΔNTD.LEU*
YJS198	Mat a	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pSPT8HA.URA pCDC68Δ922.LEU*
YJS199	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68A4.LEU pSAS3.URA*
YJS200	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68ΔNTD.LEU pSAS3HA.URA*
YJS201	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 pCDC68Δ922.LEU pSAS3HA.URA*
YJS202	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68A4.LEU pSAS3HA.URA*
YJS203	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68ΔNTD.LEU pSAS3HA.URA*
YJS204	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pCDC68Δ922.LEU pSAS3HA.URA*
YLH101	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63*
YLH102	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63*
YLH104	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 gcn5::HIS3*
YLH106	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 sas3::HIS3MX6*
YLH139	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 NTO1-3HA::HIS3MX6*
YLH144	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 ada2::HIS3*
YLH145	Mat α	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 ada2::HIS3 sas3::HIS3MX6*
YLH158	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 ahc1::HIS3MX6*
YLH160	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 ahc1::HIS3MX6 sas3::HIS3MX6*
YLH176	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 YNG1HA::KANMX6*
YLH224	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4*
YLH262	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 EAF6HA::HIS3MX6*
YLH267	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pSNLCDC68(3).URA*
YLH268	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68A4.LEU*
YLH269	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 HTB1TAP::TRP1 pCDC68Δ922.LEU*
YLH286	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 POB3TAP::TRP1 pSNLCDC68(3).URA*
YLH307	Mat α	*his4-912δ leu2-3 leu2-112 lys2-128δ ura3-52 trp1Δ1 ade2::HISG ade3::HIS cdc68::KANMX4 rpd3::TRP1 pSNLCDC68(3).URA*
YLH319	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 HTB1-3HA::HIS3MX6*
YLH322	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 HTB1-3HA::HIS3MX6 POB3TAP::TRP1*
YDM126	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 SAS3-3HA::HIS3MX6*
YDM147	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 SAS3-3HA::KAN*
YDM175	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2(K4R)HHF2.TRP*

YDM176	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2(K14R)HHF2.TRP*
YDM177	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2(K23R)HHF2.TRP*
YDM203	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2(K36R)HHF2.TRP*
YDM204	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2(K4R, K36R)HHF2.TRP*
YDG010	Mat a	*his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ35::his4 pHHT2HHF2.TRP*

Table 2.2 Plasmids Used in This Study

Plasmid	Description	Source or Reference
p*GSTSPT16*	GST-Spt16 fusion protein for amino acids 1-464 on pGEX2T vector	(63)
p*SNLCDC68(3)*	*CDC68* insert cloned into pSLncrDBS vector	(83)
p*CDC68A4*	*CDC68* WT fragment inserted into pRS315 vector	R. Singer Lab
p*CDC68ΔNTD*	mutant *cdc68 Δ2-484* inserted into pRS315 vector	R. Singer Lab
p*CDC68Δ922*	mutant *cdc68 Δ6-306* inserted into pRS315 vector	R. Singer Lab
p*CDC68NM2(4)*	mutant *cdc68 E736G* inserted into pRS315 vector	R. Singer Lab
p*CDC68NM3(4)*	mutant *cdc68 E857K* inserted into pRS315 vector	R. Singer Lab
p*CDC68M3(12)*	mutant *cdc68 E763G, R784G, S819P* inserted into pRS315 vector	R. Singer Lab
p*CDC68M3(19)*	mutant *cdc68 L804P, L946S, E1004G* inserted into pRS315 vector	R. Singer Lab
p*HHT2*	*HHT2-HHF2* locus on pRS414 vector	(101)
p*hht2K4R*	substitution mutation K4R of H3 on pHHT2	(101)
p*hht2K14R*	substitution mutation K14R of H3 on pHHT2	(101)
p*hht2K23R*	substitution mutation K23 of H3 on pHHT2	(101)
p*hht2K36R*	substitution mutation K36R of H3 on pHHT2	(101)
p*hht2K4/36R*	substitution mutations K4R and K36R of H3 on pHHT2	(101)
p*AHC1HA*	*AHC1*ORF on pRS314 fused to C-terminal triple HA cassette	(58)
p*ADA2HA*	*ADA2* ORF on YCp88 fused to N-terminal triple HA cassette	(102)
p*SAS3HA*	*SAS3* ORF on pRS314 fused to C-terminal triple HA cassette	(62)
p*SPT8HA*	*SPT8* ORF on pRS314 fused to C-terminal triple HA cassette	this study
p*SPT16FLAG*	full length *SPT16* fused to C-terminal FLAG epitope	(83)

2.2 PCR Techniques

The tagging and deletion of genes was achieved using high efficiency transformation of PCR product. All PCR reactions were done on an Eppendorf Mastercycler Gradient PCR machine (Eppendorf North America, NY) using Taq polymerase enzyme. A 100 µL reaction was the normal volume for PCR product to be used for insertions or deletions, and typically contained 77 µl of sdH_2O, 10 µl of 10X Taq buffer, 8 µl of 2.5 mM dNTPs, 0.5 µM primers, 1 µl of template DNA, 1 µl of Taq enzyme. Screening for insertions or deletions was also done with PCR, using genomic DNA of transformed strains as a template. Standard elongation protocol was used for all reactions using Taq polymerase enzyme (92°C for 30 s. followed by 30 cycles of 92°C for 45 s., 55°C for 45 s., and 72°C for 1 min).

2.3 Plasmid Shuffle

In this study, generation of *spt16* mutant strains was accomplished by transforming a pRS315 plasmid containing an *spt16* mutation into strains with the genomic copy of *SPT16* deleted and a wild type *SPT16* gene carried on the p*SNLCDC68(3)* plasmid. The p*SNLCDC68(3)* plasmid contains both the *URA3* and *ADE3* genes, allowing for selection against it in two steps. First, transformants of the *spt16* mutant plasmid with *LEU2* marker underwent red/white colour-screening. Selection of white colonies in an *ade2::HISG, ade3::HIS* strain indicated absence of *ADE3* gene, and p*SNLCDC68(3)* plasmid. Growth on 5`-FOA plates (0.1% of 5`-flouroorotic acid) used to verify the absence of the *URA3* gene on the p*SNLCDC68(3)* plasmid.

2.4 Preparation of Whole Cell Extracts for FLAG and TAP Pull-downs

Strains were inoculated from freezer stocks into 5 mL of yeast extract-peptone dextrose (YPD) or, in the case of maintaining a plasmid, appropriate drop out media and allowed to grow overnight at 30°C with shaking. Fifty milliliter cultures were then inoculated using the 5 mL overnight culture to an OD_{600} of 0.5, and grown at 30°C with shaking to an OD_{600} of 2.0. Cells were harvested by centrifugation (4,000 rpm for 3 min) at 4°C, followed by 1x wash with 25 mL distilled water. Harvested cells were resuspended in 500 µL of IPP 150 buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% NP40 with 1 mM PMSF and 2 µg/mL pepstatin A) and an equivalent volume of glass beads was added. Samples were vortexed for 3 min using a Vortex Genie (Scientific Industries, Bohemia, NY). Lysates were then clarified by centrifugation (14,000 rpm for 5 min) at 4°C.

2.5 Pull-downs of FLAG- and TAP-Tagged Proteins

Whole cell extracts were normalized for bulk protein content by Bradford assay. IgG sepharose 6 Fast Flow resin (Amersham Biosciences, Piscataway, NJ) or αFLAG M2 resin (Sigma Chemical Co., St. Louis, MO) was pre-washed 4x with cold IPP 150 buffer and aliquoted in 20 µL volumes for each pull-down. Four hundred microlitres of normalized whole cell extracts were added and allowed to rotate at 4°C for 2 h. Following binding, resin was washed 5x with 20 volumes of cold IPP 150 buffer. Thirty microlitres of 2.5x SDS loading buffer was then added to the resin and boiled for 5 min at 100 °C. Precipitated proteins were then analyzed using western blot analysis with anti-HA antibodies (Roche Diagnostics Canada, Laval, Qc). Normalization of TAP pull-downs was done by western blot analysis with peroxidase anti-peroxidase (PAP) antibodies (Sigma Chemical Co., St. Louis, MO).

Normalization of FLAG pull-downs was done by coomassie staining of SDS polyacrylamide gel (PAGE).

2.6 Preparation of Whole Cell Extracts for Calmodulin Pull-downs

Strains used for calmodulin pull-downs were inoculated in 5 mL of YPD and grown overnight at 30°C with shaking. One litres cultures of YPD were then prepared by inoculating the overnight culture and growing overnight at 30°C with shaking to an OD_{600} of 2.0. One litre cultures were harvested by centrifugation (10 min at 4,000 rpm) at 4°C and washed in 100 mL of distilled water. Cells from each 1 L culture was resuspended in 25 mL of calmodulin binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$, and 0.1% NP40 with 1 mM PMSF and 2 μg/mL pepstatin A). Cells were broken by adding cell suspension to a 30 mL bead-beater chamber (BSP bead-beater, Biospec Products, Bartlesville, OK) with ~10 mL of glass beads. Ten cycles of bead-beating (15s. bead-beating followed by 1 min rest) produced cellular lysates, which were then clarified by centrifugation (10,000 rpm for 15 min). Resultant supernatant made up whole cell extracts.

2.7 Calmodulin Pull-down Purifications of Protein Complexes

For each calmodulin pull-down purification, 70 μL calmodulin affinity resin slurry (Stratagene, Cedar Creek, TX) was pre-washed 4x with cold calmodulin binding buffer. Twenty-five mililitres of whole cell extract was then added to the resin and allowed to rotate at 4°C for 2 h. Resin was washed 4x with 20 volumes of cold calmodulin binding buffer. Bound proteins were then eluted off the calmodulin resin by washing with 100 μL of calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-Cl pH8.0, 150 mM NaCl, 1 mM Mg-

acetate, 1 mM imidazole, 2 mM EGTA, and 0.1% NP40) except for Ahc1TAP (purified ADA complex), which required 4mM EGTA calmodulin elution buffer. Three elution washes were performed by rotating for 30 min at 4°C. Relative amount of purified protein in each elution was determined by western blot analysis with peroxidase anti-peroxidase (PAP) antibodies (Sigma Chemical Co., St. Louis, MO).

2.8 Preparation of GST-Spt16p Fusion Protein

The p*GSTSPT16* plasmid expressing GST fusion protein and was transformed into BL21 (DE3) cells and inoculated into 10 mL LB ampicillin media (50 μg/mL ampicillin) for growth overnight at 37°C with shaking. Five hundred milliliters of LB ampicillin were inoculated with 5 mL saturated overnight culture and grown at 37°C with shaking to an OD_{600} = 0.6. IPTG (isopropyl-beta-D-thiogalactopyranoside) was then added to the 500 mL culture to a final concentration of 0.1mM causing induction of fusion protein. Cells were grown an additional 3 h. at 37°C with shaking. Cells were harvested by centrifugation (4000 rpm for 10 min) and washed with 25 mL 1x phosphate buffered saline (PBS) solution. Cells were resuspended in 25 mL 1x PBS with 1 mM PMSF and 2 μg/mL pepstatin A and lysed by sonication using an Ultrasonic Processor XL (Mandel, Guelph, Ont) on setting 4 for 3x 30 s. with 1 min rests. Triton-X-100 was added to the lysates to a final concentration of 1% and allowed to rotate for 20 min at 4°C. Lysates were then clarified by centrifugation (10,000 rpm for 15 min at 4°C).

GST-Spt16p was purified using glutathione sepharose resin (Amersham Biosciences, Piscataway, NJ). Two hundred and fifty microlitres of glutathione resin was pre-washed 3x with 1x PBS. Prepared lysates were then added and rotated for 2 h. at 4°C to allow binding.

Glutathione resin was washed 3x with cold 1x PBS and transferred to a new 1.5 mL microfuge tube.

2.9 The Spt16p Pull-down (*in vitro* analysis)

In vitro pull-downs were performed using the prepared GST-Spt16p fusion glutathione sepharose resin from above. Twenty microlitres each of purified NuA3, ADA, and SAGA (from calmodulin purification pull-down) were diluted into 180 µL of IPP 150 buffer (described above) and added to 10 µL of glutathione resin (containing immobilized GST-Spt16p). Tubes were rotated for 2 h. at 4°C to allow binding. Resin was washed 3x with 20 volumes of IPP 150 buffer. Thirty microlitres of 2.5x SDS loading buffer was then added to the resin and boiled for 5 min at 100 °C. Precipitation of ADA, NuA3, and SAGA with GST-Spt16p was then detected by western blot analysis using peroxidase anti-peroxidase (PAP) antibodies (Sigma Chemical Co., St. Louis, MO).

2.10 Histone H3 Acetylation Analysis

Western blots for H3 acetylation were performed on isogenic wild type and *spt16* mutant strains. Strains were grown in 50 mL cultures and harvested as described previously. Whole cell extracts were prepared in 400 µL of IPP 150 buffer with PMSF and pepstatin. Bulk protein content of whole cell extracts was normalized using Bradford assay. Thirty microlitres of 5x SDS loading buffer was then added to 20 µL of normalized sample and boiled for 5 min at 100 °C. Western blots were performed using anti-acetyl-H3 antibodies (Upstate Biotech, Lake Placid, NY).

Chapter 3 – Results

3.1 FACT Interacts with the HAT Complexes NuA3 and ADA

The first clue to an interaction between the FACT complex and a histone acetyltransferase came in a yeast two-hybrid screen (96). In it, gene fragments of *SAS3,* the catalytic subunit of NuA3, were pulled out using a LexA-Spt16$_{(1-464)}$ fusion protein as bait. This was followed by evidence suggesting the entire NuA3 complex interacts with Spt16p *in vitro,* using a GST-Spt16$_{(1-464)}$ fusion protein and purified NuA3, and *in vivo,* using anti-Spt16p polysera and HA-tagged Sas3p (63).

Although the above mentioned studies strongly suggest a physical interaction between NuA3 and FACT, no corresponding results have been published supporting the requirement of Sas3p for FACT function. Mutants of the essential gene *SPT16* show a variety of phenotypes, including sensitivities to hydroxyurea (HU) and 6-azauracil (6-AU), and an *spt* phenotype (63, 78, 79, 103). Yet, mutants of *SAS3,* or genes encoding other components of NuA3, show no such phenotypes. If these two complexes are in fact associating with each other for some process or mechanism, one would expect that disruption of either complex would result in similar phenotypes. The fact that this is not the case suggested to us two possibilities. First, the Sas3p-Spt16p interaction is an artifact and not actually indicative of a real NuA3-FACT association. The second possibility is that FACT is interacting with more than one HAT complex, and this other HAT acts in a role redundant to NuA3. However, before exploring this interesting hypothesis, we first aimed to eliminate any doubts regarding the interaction between NuA3 and FACT.

3.1.1 NuA3 Interacts with FACT

To confirm an interaction between NuA3 and FACT, we first wanted to determine whether Sas3p was associating with the entire FACT complex and not just Spt16p. To this end, the C-terminus of Pob3p, a second subunit of FACT, was TAP-tagged and tested for co-precipitation with Sas3p. The TAP tag consists of IgG-binding units of protein A (ProtA) from *Staphylococcus aureus* and a Calmodulin Binding Peptide (CBP). Due to the interaction between ProtA and immunoglobins, proteins with a fused TAP tag will bind IgG sepharose. Although not typical of a classical immunoprecipitation (IP), we will be referring to the precipitation of TAP-tagged proteins with IgG resin as an IP. To detect Sas3p, an HA epitope tag was attached to the C-terminus of Sas3p expressed from its endogenous promoter on a centromeric plasmid (p*SAS3HA*), and was transformed into the Pob3TAP strain. Pull-downs were performed using strains expressing either Sas3HA$_3$ or Pob3TAP by themselves (as negative controls), and a strain expressing both Sas3HA$_3$ and Pob3TAP. This was followed by anti-HA and peroxidase-anti-peroxidase (PAP) western blot analysis (Figure 3.1A). The anti-HA western blot clearly showed that Sas3HA$_3$ was being co-precipitated with Pob3TAP. No Sas3HA$_3$ signal was observed in the pull-down of the non-TAP tagged strain, revealing no visible non-specific interaction of Sas3HA with the resin. Although the anti-HA antibody did recognize the TAP epitope attached to Pob3p in the IP lanes (presumably due to an interaction between the HA antibody and the protein A motif of the TAP tag), we chose to also use a PAP western to ensure equal precipitation of Pob3p between samples in this experiment.

This *in vivo* interaction between Sas3p and Pob3p served to verify past data suggesting a Sas3p-FACT physical association. It also determined that the entire FACT complex, and not just Spt16p, was involved in this interaction. When taken together with the fact that GST-Pob3 fails

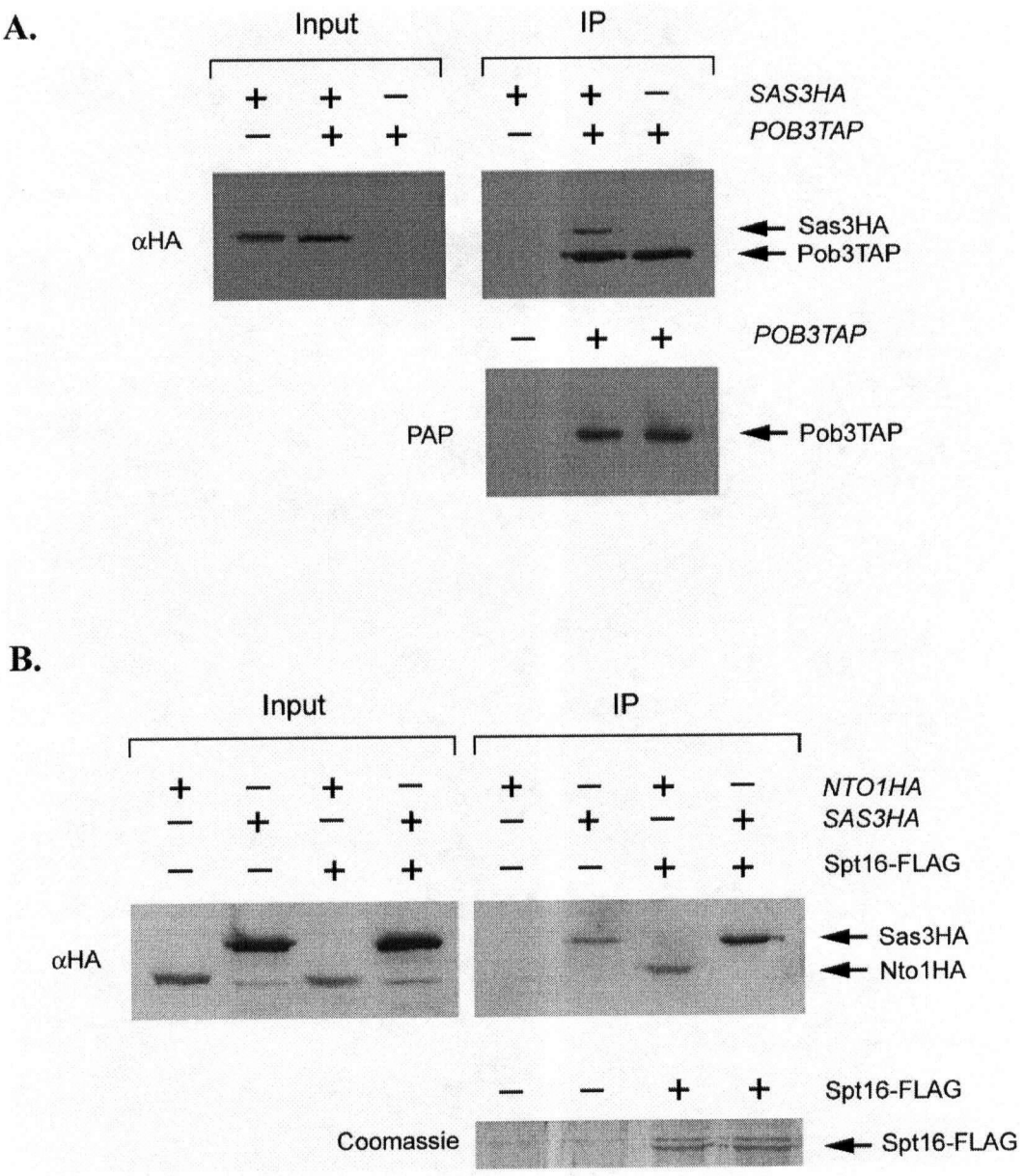

Figure 3.1 NuA3 interacts with the FACT Complex.

(A) The interaction of Pob3p with Sas3HA$_3$ was detected by anti-HA western blot analysis following pull-down of TAP-tagged Pob3p (1% of input sample and 50% of IP). Precipitated samples were also probed by PAP western blot analysis to verify levels of Pob3TAP in samples (5% of IP). (B) Spt16-FLAG was expressed on a plasmid and used in αFLAG immuno-precipitation experiments in strains expressing Nto1HA$_3$ and Sas3HA$_3$. Interaction was detected by anti-HA western blot analysis (1% of input and 50% IP). The precipitation of Spt16-FLAG was verified by coomassie blue staining of IP samples (50% of IP).

to associate with purified NuA3 *in vitro* (63) this data suggests that although Pob3TAP is pulling-down Sas3p, it is doing so through the interaction with Spt16p.

Although satisfied with the results of the Pob3TAP co-precipitation, we wished to determine whether it is the entire NuA3 complex, not just Sas3p that is interacting with FACT. To do this, we chose to demonstrate a NuA3-FACT physical interaction using different subunits of each complex. In addition to Sas3p, the NuA3 HAT complex is known to contain at least four other subunits, Yng1p, Nto1p, TAF14p and Eaf6p (62, 63). Thus far, no other subunit of NuA3 has been shown to interact with either component of FACT. Initially, we attempted to use a strain expressing HA-tagged Yng1p in this set of immunoprecipitation experiments, however results lacked consistency. Furthermore, recent work has suggested that the HA epitope interferes with Yng1p function (Martin *et al.*, submitted). Consequently, we focused our attention on the Nto1p subunit of NuA3 in our attempts to further confirm a NuA3-FACT association.

In this experiment, a centromeric plasmid containing a C-terminal FLAG-tagged Spt16p expressed from its endogenous promoter was transformed into strains expressing $Sas3HA_3$ (YDM147) and $Nto1HA_3$ (YLH139). Whole cell extracts were prepared from the resulting strains and Spt16-FLAG was immunoprecipitated using αFLAG resin. As seen in Figure 3.1B, we successfully showed another NuA3 subunit, $Nto1HA_3$, interacts with Spt16p *in vivo*. As a positive control, $Sas3HA_3$ was also included in this immunoprecipitation experiment. Although significant non-specific binding of $Sas3HA_3$ was observed in the negative control, the signal was at least two-fold higher in the presence of Spt16-FLAG. Coomassie staining for each IP sample was used to demonstrate equal levels of the Spt16-FLAG fusion protein in the precipitates.

After confirming the NuA3-FACT interaction using tagged fusion proteins of both subunits of FACT, and Sas3p and Nto1p of the NuA3 complex, we were confident in discarding

the first possibility, that the Spt16p-Sas3p interaction observed by two-hybrid is an artifact. Next, we moved onto exploring the second possibility that FACT interacts with other HAT complexes.

3.1.2 Spt16p Interacts Directly with both NuA3 and ADA

If FACT does have other interacting partners redundant with NuA3, it seemed logical that these partners share a similar function with NuA3. Deletion of *SAS3* results in only minor phenotypes, whereas deletion of *SAS3* in combination with loss of *GCN5* results in a synthetic lethality. Therefore, the possibility exists that NuA3 shares an overlapping function with a *GCN5*-dependent HAT complex. Gcn5p is the catalytic subunit of at least four HAT complexes including SAGA, ADA, SLIK/SALSA, and HAT-A2 (55-59), although there is some debate as to whether the later two are bona fide complexes or subcomplexes of SAGA (55, 57, 104). Thus SAGA and ADA alone were selected as candidates for FACT association.

To investigate a possible interaction with FACT, we chose to revisit the *in vitro* GST pull-down system used in the original Sas3p interaction study (63). This would allow us to reproduce the initial data, while also testing the direct interaction between HAT complexes and Spt16p in a highly purified system. Accordingly, GST (negative control) and GST-Spt16$_{(1-464)}$ fusion proteins were expressed and immobilized on glutathione sepharose beads. For purification of HATs, strains were created containing TAP-tagged subunits of all complexes of interest: Nto1TAP (YLH134) for NuA3, Ahc1TAP (YJS153) for ADA, and Spt8TAP (YLH339) for SAGA. HAT complexes were then purified using calmodulin affinity chromatography.

The presence of purified HAT complexes bound the GST beads was verified through PAP western blot analysis (Figure 3.2). Both Nto1TAP and Ahc1TAP displayed some level of

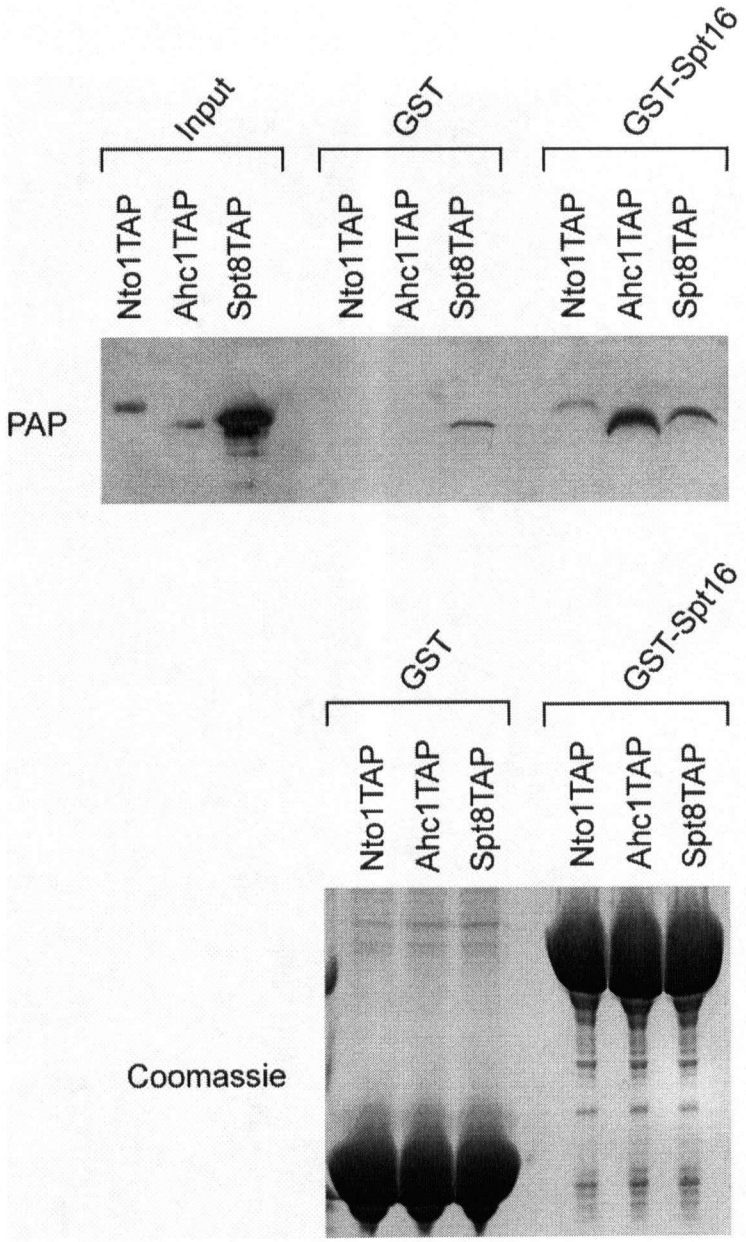

Figure 3.2 Spt16p interacts with purified NuA3 and ADA *in vitro*.

GST and GST-Spt16 fusion proteins were bound to glutathione resin and used in co-precipitation experiments with purified NuA3 (Nto1TAP), ADA (Ahc1TAP), and SAGA (Spt8TAP). Association of HAT complexes with GST and GST-Spt16 was visualized through PAP western blot analysis (5% of input sample and 50% of pull-down) and the presence of GST and GST-Spt16 fusion proteins was verified through coomassie blue staining (25% of pull-down).

association with GST-Spt16, but not GST, suggesting that ADA, in addition to NuA3, interacts with Spt16p. Interestingly, the interaction of GST-Spt16 appears to be stronger with ADA compared to NuA3, especially when levels of input signal are considered. This however could be due to differential effects of the TAP tags on each complex and on the binding of each HAT to Spt16p. The results of the test for SAGA-Spt16 interaction were less clear. Spt8TAP showed a significant interaction with the GST beads alone and input samples also showed a considerably higher concentration of SAGA compared to both NuA3 and ADA. This latter observation could either be a result of SAGA being more abundant in the cell, or better suited for the purification process. The significant level of non-specific binding observed for Spt8TAP with the GST alone may be a consequence of the higher concentration of SAGA in the input sample. Although the amount of Spt8TAP bound to GST-Spt16 appeared to be slightly higher than the control, it was not definitive enough to argue for or against an interaction of SAGA with Spt16p.

The *in vitro* GST-Spt16 co-precipitation experiment used a highly purified system to detect protein associations, thus allowing for analysis of the direct interaction these HAT complexes have with FACT. In addition to again verifying the association of NuA3, results obtained were highly supportive of an interaction between ADA and Spt16p, supporting the initial hypothesis that FACT associates with more than one histone H3 specific HAT. Unfortunately, data regarding SAGA was not conclusive. To further investigate these results, we again turned to *in vivo* pull-down experiments.

3.1.3 The ADA HAT Complex Interacts with FACT *in vivo*

Having already successfully used both TAP-tagged Pob3p and FLAG-tagged Spt16p in verifying the interaction between NuA3 and FACT, we decided to employ the same strategy in confirming the putative interaction with ADA, and the inconclusive standing of SAGA.

For the Pob3TAP co-precipitation experiment, we transformed the same Pob3TAP expressing strain with centromeric plasmids carrying the ORFs of *AHC1, ADA2,* and *SPT8* (expressed from their endogenous promoters) attached to a triple-HA cassette (p*AHC1HA*, p*ADA2HA*, and p*SPT8HA* respectively). Overnight cultures were grown in uracil drop-out media to ensure stability of the plasmid, and subjected to pull-downs using the TAP tag. As Figure 3.3A shows, both Ahc1HA_3 and Ada2HA_3 exhibited a specific interaction with Pob3TAP, further suggesting an interaction between ADA and FACT. Considering that ADA was observed to interact with the N-terminal fragment of *SPT16* in the GST-Spt16 *in vitro* experiment, this result confirms that, like NuA3, ADA interacts with the entire FACT complex via the Spt16p subunit.

Unfortunately, no such conclusions could be made regarding SAGA due to the fact that Spt8HA_3 migrates similarly to Pob3TAP (~100 kD), which the anti-HA antibody cross-reacts with (Figure 3.3A upper right panel). Careful scrutiny, however, does suggest Spt8HA_3 was not present at any significant level in the Pob3TAP pull-down lane. The PAP western blot shows relatively equal levels of Pob3TAP in all pull-down samples, as does the anti-HA signal associated with Pob3TAP in the upper panel. If observable amounts of Spt8HA_3 were precipitating out with Pob3TAP, one would expect a slightly stronger anti-HA signal in the Spt8HA_3 lane, which does not seem to be the case.

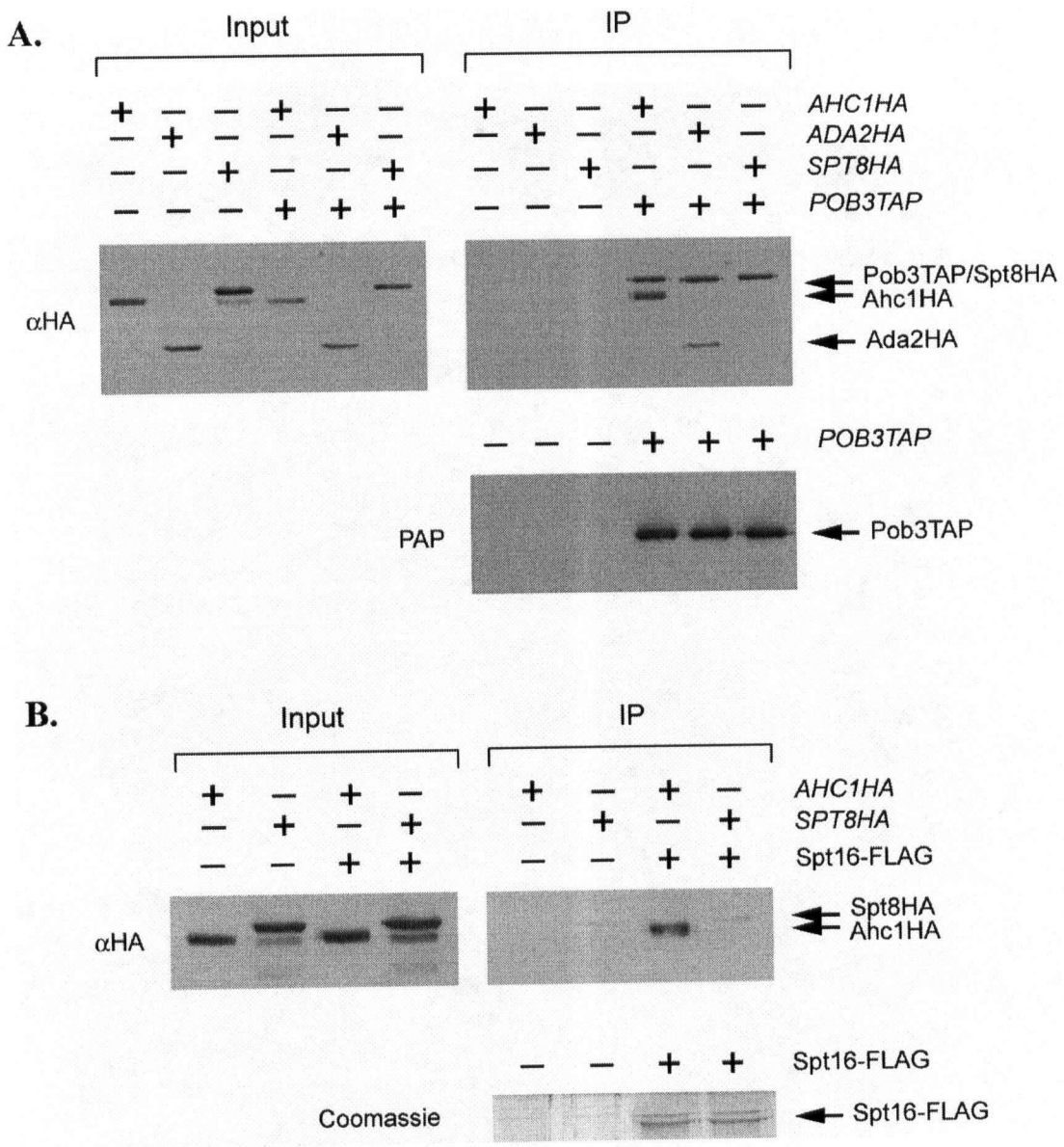

Figure 3.3 ADA interacts with the FACT complex.

(A) Pob3TAP pull-downs from cells expressing Ahc1HA$_3$, Ada2HA$_3$, and Spt8HA$_3$ probed by anti-HA western blot analysis (1% of input sample and 50% of IP). IP samples were also probed by PAP western blot analysis to verify precipitation of Pob3TAP in all samples (5% of IP). (B) Spt16-FLAG was expressed from a plasmid and used in co-precipitation experiments using strains expressing Ahc1HA$_3$ and Spt8HA$_3$ from plasmids. Interaction was detected using anti-HA western blot analysis (1% of input and 50% of IP). The precipitation of Spt16-FLAG was verified by coomassie blue staining of IP samples (50% of IP).

As a final attempt to decipher whether SAGA interacts with FACT, we utilized the Spt16-FLAG pull-down once more. This time, p*SPT16-FLAG* was transformed into strains containing plasmids expressing Ahc1HA_3 and Spt8HA_3. As expected, Ahc1HA_3 showed a clear interaction with Spt16-FLAG. Spt8HA_3, on the other hand, failed to show any significant association with the FLAG-tagged FACT subunit. Though there was a very faint signal in the IP sample, a similar level of association is seen in the negative control IP lacking Spt16-FLAG (Figure 3.3B upper right panel), suggesting a certain level of non-specific association of the HA-tagged Spt8p. Taken together with the previous data involving Spt8p, we concluded that, unlike NuA3 and ADA, SAGA does not interact with the FACT complex.

3.1.4 Deleting the NTD of Spt16p Causes a Loss of HAT Interaction

Initial experiments started with a suggested interaction between NuA3 and FACT. After confirming this interaction, we demonstrated, through both *in vitro* and *in vivo* techniques that FACT also interacts with the ADA complex, another histone H3 specific HAT. The next aim was to examine the possible function of the association between FACT and the HATs NuA3 and ADA. The simplest way to ascertain the significance of FACT's interaction with these HATs would be to study an *spt16* mutant lacking this interaction. In order to do this, we wished to first map the HAT interaction domain of Spt16p, thereby identifying an *spt16* mutant that might give us insight into which aspect of FACT function NuA3 and ADA are involved with.

The N-terminal domain was a reasonable starting point in assessing which part of Spt16p was required for HAT interaction for several reasons. First, the original yeast two-hybrid screen used the N-terminal 464 residues encoded by *SPT16* as bait (96). Second, the GST-Spt16 fusion protein used to identify an interaction with both NuA3 (63) and ADA (this study) *in vitro* was made up of the same Spt16p N-terminal residues. Third, domain analysis of Spt16p has

suggested that the first 484 amino acids make up an independently folded N-terminal polypeptide that is non-essential for FACT complex stability, and likely involved in other protein interactions (83).

A strain expressing spt16ΔNTD was received as a gift from our collaborators in the R.A. Singer Lab at Dalhousie University. Their recent work with the Spt16p N-terminal domain revealed convenient restriction sites allowing for the generation of the *spt16ΔNTD* mutant (83). This N-terminal truncation, lacking amino acids 2-484, was revealed to supply all essential functions of Spt16p (83). All work using *SPT16* and its mutants in this study used a "plasmid shuffle" system, where *spt16::KANMX4* strains were maintained by a wild type *SPT16* locus carried on a *URA3* plasmid. This plasmid was then selected against during growth on 5-FOA following transformation of *spt16* alleles carried on a *LEU2* plasmid.

In order to assess NuA3 and ADA interactions with the FACT complex in an *spt16ΔNTD* mutant, a protein besides Spt16p needed to be epitope tagged. Previous work has shown that the *spt16ΔNTD* mutant does not affect FACT stability, therefore we decided to employ the Pob3TAP co-precipitation experimental system used previously. To this end, Pob3 was TAP-tagged in a strain with the *ΔNTD* mutant *spt16* allele, and either carrying the plasmid expressing Sas3HA$_3$ (YJS203), or Ahc1HA$_3$ (YJS121). A pull-down of TAP-tagged proteins was done to evaluate NuA3 and ADA binding. As seen in Figure 3.4A, all association between Pob3TAP and Sas3HA$_3$ was lost in the *spt16ΔNTD* mutant strain, suggesting that the NTD of Spt16p is required for interaction between FACT and NuA3. Similarly, the *spt16ΔNTD* mutation resulted in loss of association between Pob3TAP and Ahc1HA$_3$ of ADA (Figure 3.4B).

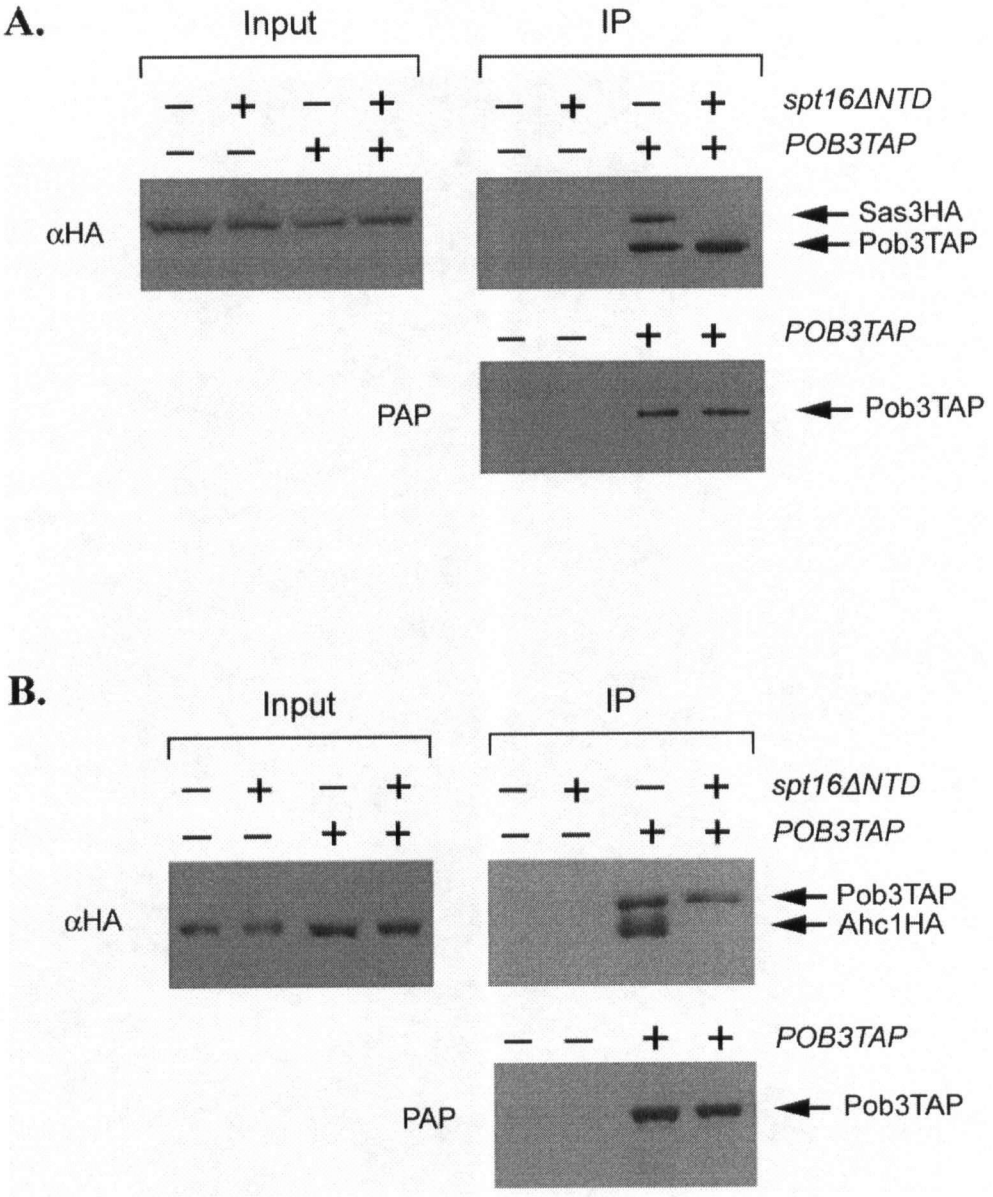

Figure 3.4 The N-terminal domain of Spt16p is required for NuA3 and ADA interaction.

(A) Pob3TAP pull-downs of plasmid expressed $Sas3HA_3$ in wild type and *spt16ΔNTD* strains. Western blot analysis using anti-HA antibodies was used to visualize $Sas3HA_3$ presence (1% of input sample and 50% of IP). Probing an identical blot with PAP was done to ensure precipitation of Pob3TAP in IP samples (5% of IP). (B) Pob3TAP pull-down of plasmid expressed $Ahc1HA_3$ in wild type and *spt16ΔNTD* strains. Western blot analysis using anti-HA antibodies was used to visualize $Ahc1HA_3$ presence (1% of input and 50% of IP). A PAP western blot was used to verify the presence of Pob3TAP in IP samples (5% of IP).

These results suggested that the N-terminal domain described previously (83) is essential for the association of both ADA and NuA3 with Spt16p and the entire FACT complex. Having now identified a mutant *SPT16* deficient in HAT binding, we wished to investigate possible phenotypes in order to gain insight into the function of FACT's interaction with the NuA3 and ADA HAT complexes.

3.1.5 Phenotype Analysis Shows that Interaction with HATs is not Required for Known FACT Functions

As mentioned previously, mutants of *SPT16* have been observed to display sensitivities to HU, 6-AU, and have an *spt* phenotype (63, 78, 79, 103). HU is a free-radical scavenger that inhibits ribonucleotide reductase, causing replication stress and sensitivity in strains deficient in processes related to replication. Sensitivity to 6-AU, a uracil analog that causes an imbalance in ribonucleotide triphosphates, indicates defects in transcription elongation. The *spt* phenotype is observed in strains able to grow on lysine drop-out media through aberrant transcription initiation from a δ-element in the normally transcriptionally repressed *lys2-128δ* allele. Possessing an *spt* phenotype is often indicative of a failure to properly repress gene transcription in a chromatin regulated manner (76, 77). Therefore, these phenotypes have implicated Spt16p and FACT in a variety of DNA processes, including transcription initiation, elongation, and repression, and DNA replication. Our initial strategy was to test the *spt16ΔNTD* mutant for these

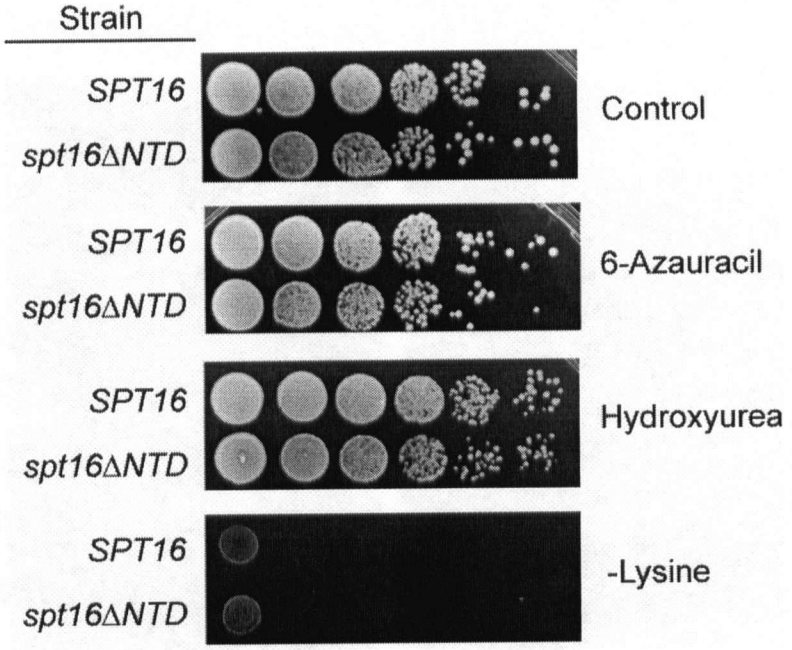

Figure 3.5 *spt16ΔNTD* shows no FACT related mutant phenotypes.

Wild type and *spt16ΔNTD* strains were serially diluted ten-fold and spotted on YPD (control), uracil drop-out media with 50 μg/mL 6-azauracil, YPD with 100 mM hydroxyurea, and lysine drop-out media and incubated at 30°C for 3 days.

aforementioned phenotypes in the hopes they might suggest a function for the HAT interaction domain of Spt16p. Accordingly, wild type and *spt16ΔNTD* strains were subjected to tenfold serial dilution and spotted on appropriate media and YPD as a control. As Figure 3.5 shows, there was no observable difference in growth for any of the tested phenotypes. Previously, the *spt16ΔNTD* mutant has been shown to be HU sensitive at 37°C (83), unfortunately we were unable to replicate this effect.

The presumption that an Spt16p mutant lacking its ability to interact with HATs would show a mutant phenotype was based on the assumption that FACT's interaction with ADA and NuA3 is essential for some process with measurable deficiencies. The fact that no phenotypes were observed may indicate that FACT-HAT interaction is required for a function yet to be identified or the ability of FACT to interact with HATs is redundant with the function of other factors in the cell. Just as ADA and NuA3 may act superfluously in their association with FACT, other aspects of FACT function may compensate for the loss of function in the *spt16ΔNTD* mutant. This would explain a lack of any genetic phenotypes upon loss of the N-terminal domain of Spt16p. However, the *spt16ΔNTD* mutant could still be affecting interactions at the nucleosome at a level measurable biochemically. With this in mind, we chose to characterize FACT's interaction with NuA3 and ADA using a biochemical approach.

3.1.6 NuA3 and ADA Mutants do not affect FACT Association with the Nucleosome

The interactions between FACT and HATs raise another intriguing possibility that perhaps histone acetylation is required to mediate the interaction of FACT with chromatin. This would be an example of the "histone code" in which histone post-translational modification serve as docking sites for factors that mediate chromatin remodeling. Although a direct interaction between FACT and ADA or NuA3 may facilitate this process, it may not be

absolutely critical, which may explain why loss of the Spt16p NTD does not result in any known *spt16* related phenotypes.

To determine whether NuA3 and ADA HAT activity are required to modulate the association of FACT with the nucleosome, the null mutants *sas3::HIS3MX6* and *ada2::HIS3* were mated with the strain expressing $H2BHA_3$ and Pob3TAP. The deletion of *SAS3* causes a loss of both NuA3 complex stability and H3 HAT activity (63), and deletion of *ADA2* affects the HAT activity of the ADA and SAGA HAT complexes as well as SLIK and HAT-A2 (61). An attempt was made to construct an *ada2Δsas3Δ* double mutant expressing both Pob3TAP and $H2BHA_3$, however mating and random spore analysis failed to produce any strains carrying the necessary markers possibly indicating this strain was too sick. Pob3TAP pull-downs of $H2BHA_3$ were done followed by anti-HA western blot analysis. As Figure 3.6 shows, no visible difference was seen in the levels of association between Pob3TAP and $H2BHA_3$ in either of the *sas3*Δ or *ada2*Δ mutants. This suggested that the HAT activity of NuA3, ADA, SAGA, SLIK, or HAT-A2 is not involved in the modulation of FACT's association with the nucleosome. That being said, failure to perform the co-precipitation experiment in an *ada2Δsas3Δ* double mutant meant we could not disregard the possibility that activity of these HATs is compensatory to each other. Thus, although we were able to conclusively show that FACT interacts with the NuA3 and ADA HAT complexes *in vitro* and *in vivo*, at this time we have no evidence that this interaction is required for FACT function.

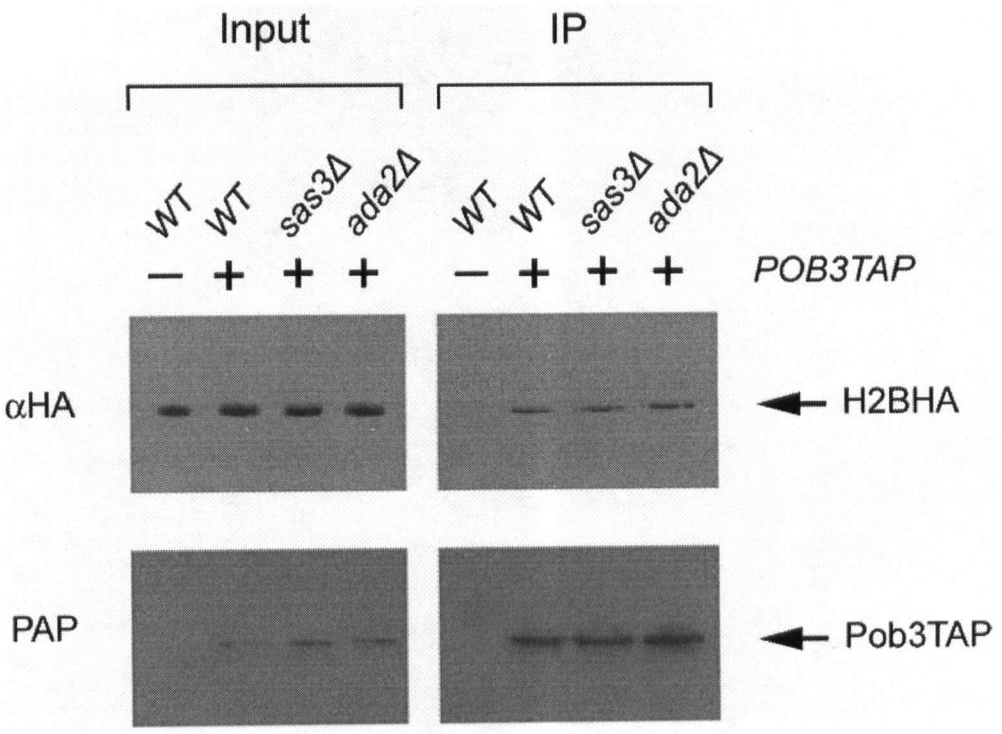

Figure 3.6 HAT Mutants do not affect the association between FACT and the nucleosome.

ADA2 and *SAS3* deletion strains expressing Pob3TAP and $H2BHA_3$ were subjected to TAP-tag pull-down. Following co-precipitation, anti-HA western blot analysis was used to visualize levels of $H2BHA_3$ associating with Pob3TAP (1% of input sample and 50% of IP). PAP western blot analysis was used to verify precipitation of Pob3p of IP samples (5% of IP).

3.1.7 HAT Interactions with the Nucleosome are Unaffected in *spt16ΔNTD* Mutants

Our results thus far do not support the hypothesis that the function of FACT is dependent on the ability of this complex to interact with HATs. An alternative hypothesis would be that FACT is required to facilitate the function of the ADA and NuA3 HAT complexes. Unfortunately the function of the ADA and NuA3 complexes in the cell is currently unknown and thus there are no suitable phenotypes available to analyze *spt16ΔNTD* mutants for. We therefore decided to address this question using two independent biochemical approaches.

One of the processes FACT is implicated in is the binding and chaperoning of histone H2A-H2B dimers at the nucleosome during transcript elongation (89). Our results demonstrate that FACT also interacts with NuA3 and ADA, and thus the obvious question was whether FACT is recruiting NuA3 and ADA to the nucleosome for acetylation of histone targets. Research has already shown that the HATs SAGA and NuA4 interact with a number of acidic transcriptional activators and general transcription factors in a recruiting fashion to modulate their HAT activity (105, 106), it is possible that the FACT complex has a similar function with respect to NuA3 and ADA. In order to examine the association of NuA3 and ADA with the nucleosome, we employed a chromatin pull-down assay that had been developed and used in our lab to show that Sas3p interaction with chromatin requires *YNG1* (101). The histone protein H2B was C-terminally TAP-tagged in strains containing *spt16ΔNTD* and *SPT16*. These were crossed with strains with integrated *SAS3-3HA* and *AHC1-3HA* tags to produce haploid strains expressing either Sas3HA_3 or Ahc1HA_3 and H2BTAP with either wild type or mutant *SPT16* alleles. Htb1p-Sas3p co-precipitation experiments were done and anti-HA and PAP western blot analysis was used to visualize results (Figure 3.7A and B).

As Figure 3.7A shows, loss of the N-terminal HAT interaction domain of Spt16p did not affect the level of Sas3HA_3 co-precipitated with H2BTAP. The same result was observed for

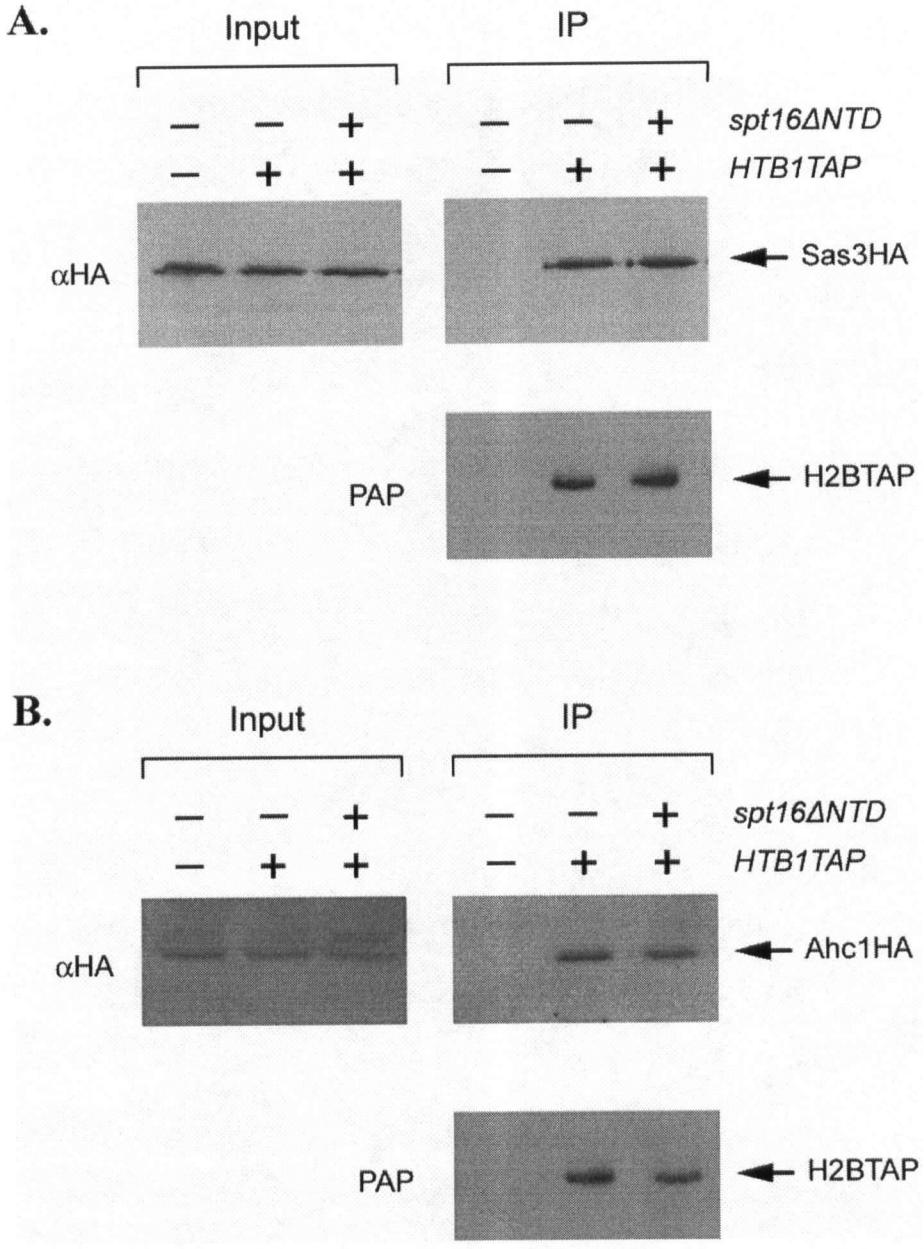

Figure 3.7 Loss of the Spt16p NTD does not affect NuA3 or ADA association with the nucleosome.

(A) Chromatin pull-downs using H2BTAP of Sas3HA$_3$ in *SPT16* and *spt16ΔNTD* strains. Anti-HA western blot analysis was used to visualize levels of Sas3HA$_3$ pulled-down (1% of input sample and 50% of IP). PAP western blot analysis of IP samples was used to verify precipitation of H2BTAP (5% of IP). (B) Chromatin pull-down using H2BTAP of Ahc1HA$_3$ in *SPT16* and *spt16ΔNTD* strains. Anti-HA western blot analysis was used to visualize levels of Ahc1HA$_3$ pulled down (1% of input and 50% of IP). PAP western blot analysis of IP samples used to verify the precipitation of H2BTAP (5% of IP).

Ahc1HA$_3$ (Figure 3.7B). In both pull-down experiments, input samples showed equivalent levels of the HA epitope tagged protein, and no non-specific binding was observed in the non-TAP-tagged control strain. Levels of Ahc1HA$_3$ are slightly lower in the *spt16ΔNTD* precipitate, although this minor difference was paralleled in the PAP western blot for H2BTAP levels (Figure 3.7B). These results suggest that the deletion of the NTD of Spt16p has no effect on the association of NuA3 and ADA with the nucleosome.

3.1.8 Global Histone Acetylation is Unchanged in the *spt16ΔNTD* Mutant

Although FACT is not required for the binding of ADA or NuA3 to chromatin, it may still be required for the HAT activity of these complexes. FACT has been shown to remodel nucleosomes, and this remodeling may be required for NuA3 or ADA to access the histone tails for acetylation. In order to directly determine whether FACT is required for the HAT activity of NuA3 and ADA, an anti-acetylated H3 antibody was used to test levels of acetylated histone H3 in both *SPT16* and *spt16ΔNTD* strains. Unfortunately, since the specific genes bound by ADA or NuA3 have not been determined, we were limited to examining histone acetylation of bulk histones.

The previous work of others have shown that both ADA and NuA3 preferentially acetylate lysine 14 of histone H3 (61, 62), as well as other sites to a lesser extent, however an antibody that can detect this modification alone by western blot is not available. Therefore we chose to use an antibody raised against peptides corresponding to the histone H3 tail acetylated at lysines 9 and 14. Whole cell extracts from *SPT16* and *spt16ΔNTD* strains were normalized for total protein levels and subjected to western blot analysis using these anti-acetylated H3 antibodies. Unfortunately, the results showed no significant change in the levels of acetylated-H3 in a strain lacking the Spt16p NTD HAT interaction domain (Figure 3.8). However, as there

are other HATs in the cell that target lysines 9 and 14, but do not interact with FACT, it is still formally possibly that deletion of the NTD does result in minor losses of histone acetylation that are not detectable using this assay. Alternatively, FACT may be required for acetylation of sites other than lysine 14 which have not been previously been detected due to absence of FACT in the HAT assays used. In addition, there is evidence that certain HATs also have non-histone targets (107-109), and this may be the case with NuA3 and ADA. Thus at this time we have no evidence that FACT is required for the function of ADA or NuA3 in the cell, and although we have been able to conclusively show that FACT interacts with the ADA and NuA3 HAT complexes *in vitro* and *in vivo*, we have been unable to reveal the functional relevance of this interaction.

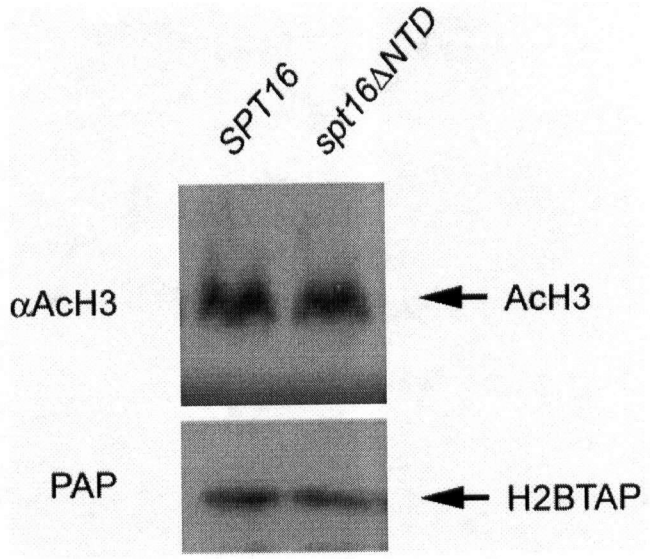

Figure 3.8 Loss of the Spt16p NTD does not affect global H3 histone acetylation.

Whole cell extracts from strains with wild type *SPT16* and mutant *spt16ΔNTD* alleles were normalized for bulk protein content using Bradford assay (10 mg/mL), and levels of H3 acetylation were determined by anti-acetylated H3 western blot analysis. Sample normalization was verified by PAP western blot analysis for H2BTAP.

3.2 The *spt16Δ922* Mutant Causes Interference of Histone Acetylation

It has been proposed that FACT not only has the ability to destabilize nucleosomes and allow passage of RNA pol II, but also to reassemble chromatin following transcription, which is required for proper repression of certain genes (89, 91, 103). This later hypothesis is based on the observations that in some *SPT16* mutants transcription can initiate from "cryptic" intragenic sites within multiple genes, such as *lys2-128δ*. The fact that the *spt16ΔNTD* mutant does not exhibit an *spt* phenotype is interesting when one considers that another well characterized mutant, *spt16Δ922,* which has a smaller truncation to *spt16ΔNTD*, does show this phenotype (83, 98). These results may suggest that while the *spt16ΔNTD* mutant lacks the entire N-terminal domain of Spt16p, the *spt16Δ922* mutant still expresses a remnant of this domain which becomes misfolded, and interferes with some aspect of normal chromatin structure regulation (83). In other words, the *spt* phenotype seen in an *spt16Δ922* mutant may not be due to a defect in FACT's ability to repackage chromatin, but rather to interference in the function of an unrelated chromatin modifying complex by aberrant Spt16p structure (83). Due to the role of histone acetylation in transcription regulation, we postulated that it may be possible that the product of the *spt16Δ922* may be interfering with normal histone acetylation. The goal of our research with the *spt16Δ922* mutant was to investigate this interference model.

3.2.1 FACT-Nucleosome Association is not Affected in the *spt16Δ922* Mutant

Our hypothesis was that the *spt16Δ922* mutant interferes with proper histone acetylation thus causing mutant phenotypes. Before testing this, however, we needed to ensure that the partial NTD mutant was not interfering with FACT-nucleosome interactions directly. To this end, we created wild type and *spt16Δ922* strains which expressed HA-tagged H2B and TAP-

tagged Pob3p. TAP pull-downs followed by HA western blot analysis were used to examine the interaction of FACT with chromatin with the wild type and *spt16Δ922* strains. As Figure 3.9 shows, no difference in the association of FACT with the nucleosome was observed in the *spt16Δ922* mutant. This argued that the partial N-terminal truncation of this Spt16p mutant was not interfering with normal FACT-nucleosome interactions. Although it is still formally possible that FACT is able to bind but not modify chromatin structure in the *spt16Δ922* strain, we were intrigued by the possibility that the mutant Spt16p in the *spt16Δ922* strain was interfering with a FACT independent function.

3.2.2 The *spt16Δ922* Mutant Shows a Loss in H3 Acetylation

Structural predictions of the *spt16Δ922* mutant suggest that the partial NTD in the Spt16p polypeptide is misfolded, possibly to an extent that interferes with other nearby functions (83). We proposed that this NTD fragment is interfering with the acetylation of nucleosomal histones, thus affecting normal chromatin maintenance and causing mutant phenotypes such as the *spt* phenotype. There are several lines of evidence that suggested histone acetylation might be affected by mutation of Spt16. First, the FACT complex is present at the nucleosome, as seen in its ability to bind nucleosomal H2A/H2B dimers (89) and would likely be in close proximity to any histone tail modifications, including acetylation. Second, it has been shown that certain *spt16* mutants acquire serious synthetic growth defects when combined with *sas3Δ* or *gcn5Δ* (91), and thus interference of histone acetylation by mutant Spt16p may explain the *sas3Δgcn5Δ* synthetic lethality. Finally the phenotypes of certain *spt16* mutants can be rescued by the deletion of the gene encoding the histone deacetylase Rpd3p (86). These data

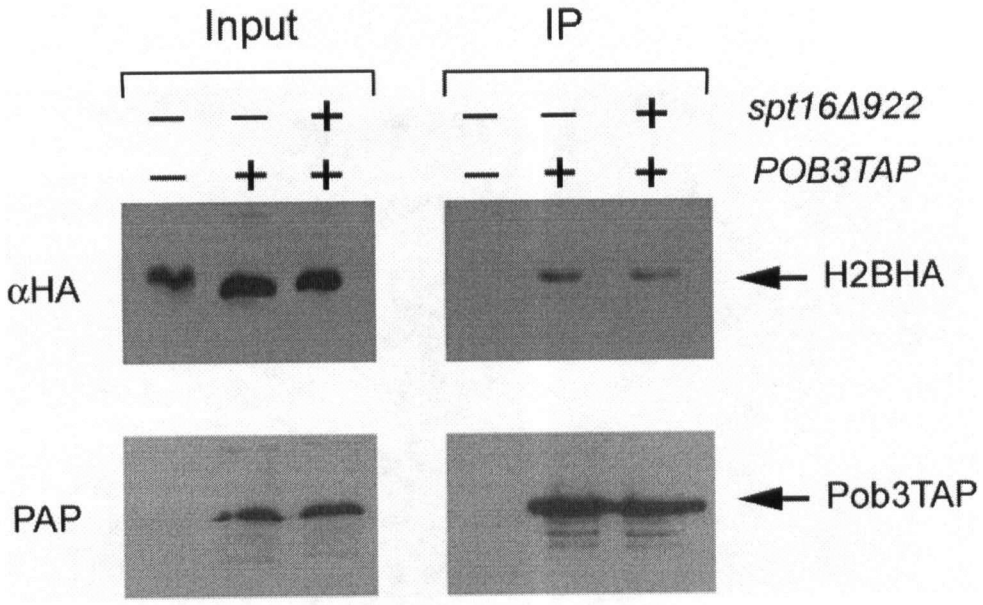

Figure 3.9 FACT association with the nucleosome is not altered in the *spt16Δ922* mutant.

Strains expressing Pob3TAP with *SPT16* and *spt16Δ922* alleles were subjected to TAP-tag pull-down. Following co-precipitation, anti-HA western blot analysis was used to visualize levels of $H2BHA_3$ associating with Pob3TAP. PAP western blot analysis was used to ensure equal precipitation of Pob3TAP.

demonstrate a possible connection between histone acetylation and FACT function.

To determine whether histone acetylation was affected in the *spt16Δ922* mutant, whole cell extracts were prepared from *SPT16* and *spt16Δ922* strains, normalized for protein concentration using a Bradford assay, and subjected to anti-acetylated H3 western blot analysis. As Figure 3.10A shows, the *spt16Δ922* strain showed a marked decrease in H3 acetylation, confirming the possibility that the partial NTD truncation of Spt16p interferes with histone tail acetylation. Although the PAP western blot of H2BTAP suggested that each lane contained equal loadings of histones, the samples are expected to contain newly synthesized H2B, which is non-nucleosomal, and thus the H2B signal seen may not accurately represent the level of histone H3 present in the cell. Thus it is conceivable that mutating Spt16p caused an alteration in the levels of nucleosomal histone H3, and not actual levels of histone acetylation.

To resolve this problem, we chose to combine the H3 acetylation assay with an H2BTAP-tagged protein purification. This allowed us to purify significant quantities of nucleosomal histone H3 which could be normalized by coomassie staining. To this end, large scale calmodulin affinity purifications of nucleosomal histones from both *SPT16* and *spt16Δ922* strains were done using a TAP-tagged histone H2B. The histones were resolved by SDS PAGE and stained with coomassie blue (Figure 3.10B lower panel) which demonstrated equal levels of histone H3 in the lanes corresponding to *SPT16* and *spt16Δ922*. As shown before, levels of H3 acetylation are noticeably lower in the truncated NTD mutant, indicated by anti-acetylated H3 western blot analysis. A PAP western blot showed proper normalization of H2BTAP, demonstrating that although H3 acetylation is decreased in the *spt16Δ922* mutant, presence of H2BTAP is equal in both strains (Figure 3.10). These results confirmed that spt16Δ922 mutants exhibited decreased levels of histone acetylation.

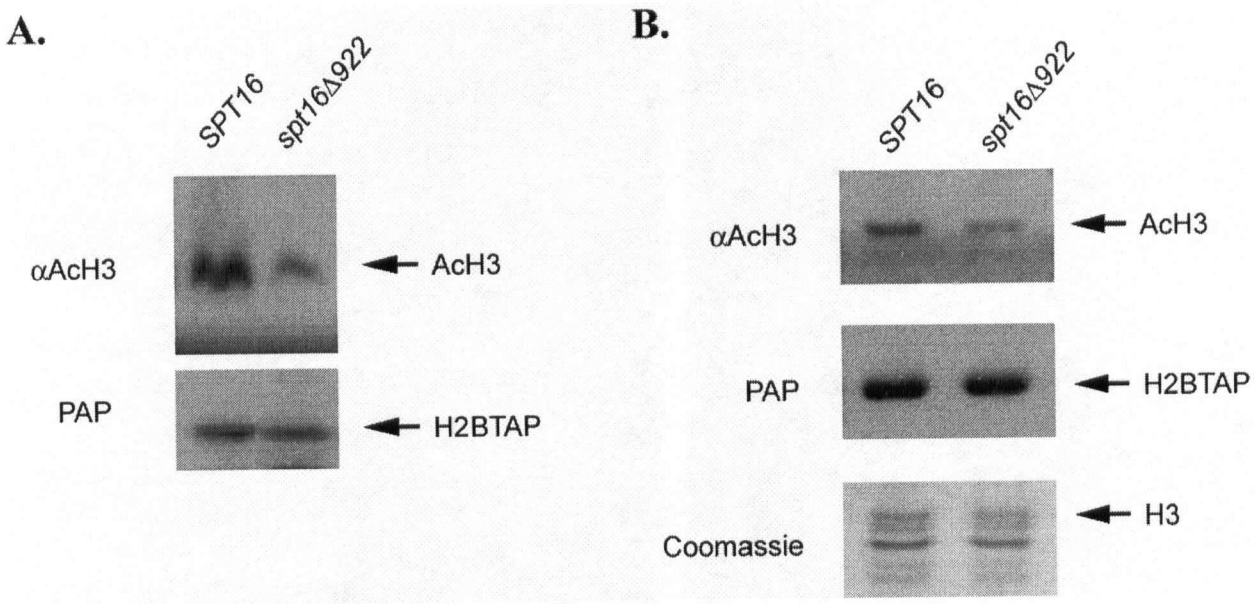

Figure 3.10 *spt16Δ922* mutant shows a loss of H3 acetylation.

(A) Whole cell extracts from *spt16Δ* strains carrying plasmids for *SPT16* or *spt16Δ922* were normalized for bulk protein content using Bradford assay (10 mg/mL), and levels of H3 acetylation were probed by anti-acetylated H3 western blot analysis. Sample normalization was verified by PAP detection of H2BTAP. (B) Histones from wild type *SPT16* and *spt16Δ922* were purified by calmodulin affinity chromatography of H2BTAP and subjected to anti-acetylated H3 western blot analysis. PAP western blot analysis was used to verify normalization of H2BTAP present.

We had previously shown that in an *spt16ΔNTD* mutant, lacking the entire N-terminal domain, there was no change in H3 acetylation (3.2.2). However, in the *spt16Δ922* mutant, which has 182 more residues than *spt16ΔNTD*, there was a loss in H3 acetylation. Although the idea of having more of the wild type Spt16p present, and less than wild type H3 acetylation at first seems unusual, it does agree with the model of *spt16Δ922* interference. It suggests that the partial N-terminal truncation is actually gaining a function, albeit one that interferes with normal H3 acetylation, through the extra 182 residues. It also suggests that the FACT complex is in a position at the nucleosome to affect histone modifications, supporting our hypothesis. Finally, this data may indicate that the *spt* phenotype seen in the *spt16Δ922* strain is a result of loss of histone acetylation as opposed to disruption of FACT function.

3.2.3 Histone H3 Acetylation is Altered in Multiple *spt16* Mutants

To further examine the relationship between histone H3 acetylation and Spt16p function, we chose to investigate whether other *spt16* mutants caused the same loss of histone acetylation as *spt16Δ922*. If the *spt16Δ922* mutant was expressing a misfolded polypeptide that interferes with histone acetylation, we would expect there to be other *spt16* mutants with a similar effect, possibly due to mutation of residues important in folding. For that reason, the level of histone H3 acetylation was examined in four other *spt16* mutants, and compared to the wild type, *spt16ΔNTD,* and *spt16Δ922* strains. Figure 3.11 shows a western blot analysis for levels of histone H3 acetylation in these strains. All four previously untested *spt16* mutants, *spt16NM2(4), spt16NM3(4), spt16M3(12),* and *spt16M3(19)* are point mutations in various regions of the gene (see Table 2.2 for detailed description). As seen before (in Figures 3.8 and 3.10 respectively), *spt16ΔNTD* showed wild type levels of H3 acetylation, and *spt16Δ922* showed a loss in H3 acetylation. In the four point mutation *spt16* mutants, *spt16M3(19)* showed

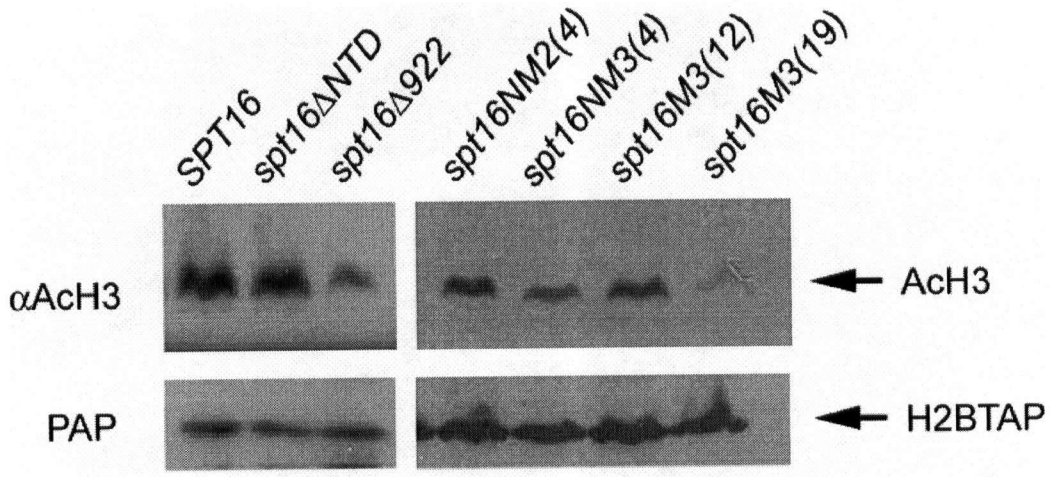

Figure 3.11 *spt16* mutants display altered levels of histone H3 acetylation.

Whole cell extracts from *spt16Δ* strains carrying plasmids for mutant *spt16ΔNTD, spt16Δ922, spt16NM2(4), spt16NM3(4), spt16M3(12),* and *spt16M3(19)* were normalized for bulk protein content using Bradford assay (10 mg/mL), and levels of H3 acetylation were probed by anti-acetylated H3 western blot analysis. Sample normalization was verified by PAP western blot analysis of H2BTAP.

a significant loss in acetylation, and *spt16NM2(4)* showed a slight loss. Both *spt16NM2(3)* and *spt16M3(12)* appeared to show near wild type levels of H3 acetylation. Although the underlying reasons for the loss of H3 acetylation in each of the *spt16* mutants is not known, the fact that altered acetylation was observed lended support to the theory of an *spt16Δ922* mutant interfering with normal histone acetylation. It was now clear that FACT is in a position to affect histone modifications, as mutants of *spt16* were shown to alter histone H3 acetylation in Figure 3.11. In order to understand the importance of the *spt16Δ922* mutant's interference of H3 acetylation, our goal now shifted towards relating this biochemical data to an observable genetic phenotype. In this way, we aimed to not only understand *spt16Δ922,* but also further elucidate the role of histone modifications in chromatin structure and function.

3.2.4 The *spt* Phenotype of *spt16* Mutants Correlates with the Loss in H3 Acetylation

The first published data of the *spt16Δ922* mutant shows that the deletion of the N-terminal 300 residues decreases the need for Swi/Snf chromatin remodeling in transcription, and confers an *spt* phenotype (98). Both of these results suggest that the *spt16Δ922* mutation is in some way compromising chromatin structure, which is well known in itself to be an important negative regulator of gene expression (110, 111). Our data showed that the *spt16Δ922* mutation also resulted in a significant loss in histone H3 acetylation (Figure 3.10A and B). We therefore wished to examine a possible connection between this altered acetylation and chromatin mediated gene repression. To do this, all six *spt16* mutant strains, containing the *lys2-128δ* allele, were screened for an *spt* phenotype through ability to transcribe the mutant *lys2* transcript on lysine drop-out media.

The results this far (Figure 3.12) confirmed the *spt* phenotype of *spt16Δ922* and also showed that *spt16ΔNTD* did not confer such a phenotype. This paralleled the H3 acetylation

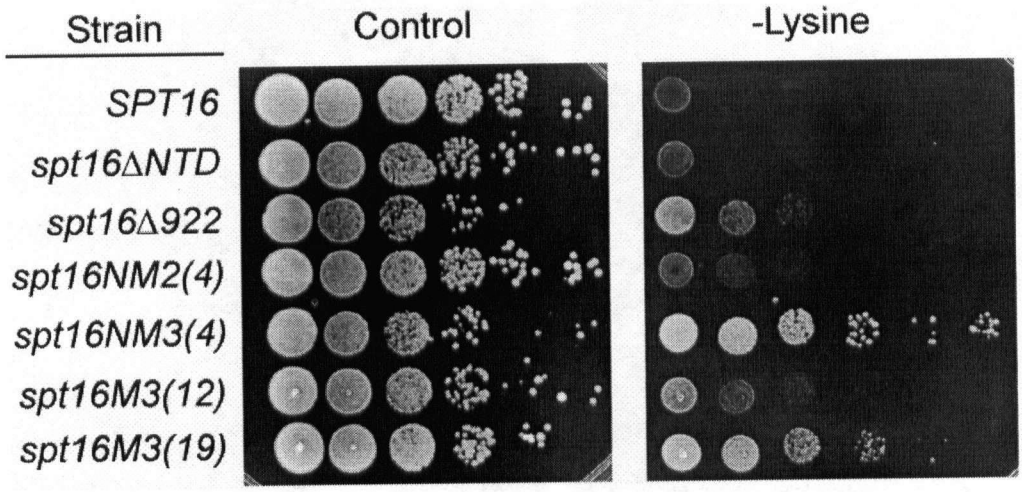

Figure 3.12 *spt16* mutants confer an SPT phenotype.

lys2-128δ strains containing the wild type *SPT16,* six *spt16* mutants and the were serially diluted ten-fold and spotted on YPD (control) and lysine drop out media to screen for the *spt* phenotype. Plates were incubated at 30°C for 3 days.

data of these two strains, and again argued that the extra residues on the *spt16Δ922* expressed polypeptide were interfering with some aspect of chromatin structure regulation. Amongst the four other *spt16* mutants, *spt16NM3(4)* conferred the greatest *spt* phenotype, slightly stronger than that of *spt16M3(19)*. Of the two remaining mutants, *spt16M3(12)* showed a noticeable level of growth indicative of an *spt* phenotype, though lesser than that of *spt16Δ922,* especially when the amount of cells on the control plate are taken into account. The *spt16NM2(4)* mutant displayed growth on lysine drop-out media marginally better than the wild type and *spt16ΔNTD* strains, though not enough to indicate an *spt* phenotype.

When the phenotype data of the various *spt16* mutants was compared with the results of the histone H3 acetylation westerns seen in Figure 3.11, a certain degree of correlation was observed between those mutants that showed loss of histone H3 acetylation, and those conferring an *spt* phenotype. Compared to the wild type, the mutants *spt16Δ922, spt16NM3(4),* and *spt16M3(19)* all showed decreased histone H3 acetylation (Figure 3.11). These same mutant strains also demonstrated the greatest *spt* phenotypes. The fact that the *spt* phenotype is indicative of a defect in chromatin-mediated repression suggested to us that histone acetylation may have a novel role in proper transcription repression. Although this is contrary to the general view of acetylation and transcription, it is not entirely without precedent (41, 68). The correlation that we see between histone acetylation and *spt* phenotype, though, is not exact. For example, *spt16M3(19)* showed the lowest level of H3 acetylation, whereas *spt16NM3(4)* had the most prevalent *spt* phenotype. It can also be argued that the slight *spt* phenotype in the *spt16M3(12)* mutant was not accompanied by any noticeable loss in H3 acetylation. These discrepancies are not surprising considering FACT is a multi-domain and apparently multi-functional complex. It is conceivable that the *spt* phenotype is due to loss of histone acetylation in addition to disruption of another unrelated FACT function. However, it is also possible that the *spt* phenotype and loss in histone H3 acetylation seen in some of the *spt16* mutants is a result

of disruption of two independent FACT functions. Therefore, we chose to further investigate a possible role for histone acetylation in the *spt* phenotype. We rationalized that if the *spt16Δ922* mutant was interfering with the interaction of HAT complexes with histones, then loss of HAT-nucleosome interactions in this mutant may be observed. In addition, strains harbouring mutations in the genes encoding HATs should also have an *spt* phenotype since blocking HAT activity and not having any HAT activity would be expected cause the same effect.

3.2.5 The Association of HATs with the Nucleosomes is not Affected in *spt16Δ922*

To investigate the effect of the *spt16Δ922* mutation on the association of HAT complexes with nucleosomes we generated wild type and *spt16Δ922* strains which expressed Sas3HA$_3$, Ahc1HA$_3$, or Spt8HA$_3$ along with H2BTAP. These strains were subjected to TAP pull-downs, and western blot analysis was used to visualize the association of Sas3HA$_3$ (NuA3), Ahc1HA$_3$ (ADA), and Spt8HA$_3$ (SAGA) with nucleosomes. These HATs were tested as they all show specificity toward histone H3 *in vitro*. Regrettably, none of the HATs tested showed any observable difference in nucleosomal associations between the wild type *SPT16* and mutant *spt16Δ922* strains (Figure 3.13), suggesting that the N-terminal fragment of the *spt16Δ922* mutant was not interfering with the interaction of these HATs with chromatin. However, it is still possible that although mutant Spt16p does not interfere with the interaction of these HATs with chromatin, it still blocks their ability to acetylate histones. We therefore decided to test strains lacking these HATs for an *spt* phenotype.

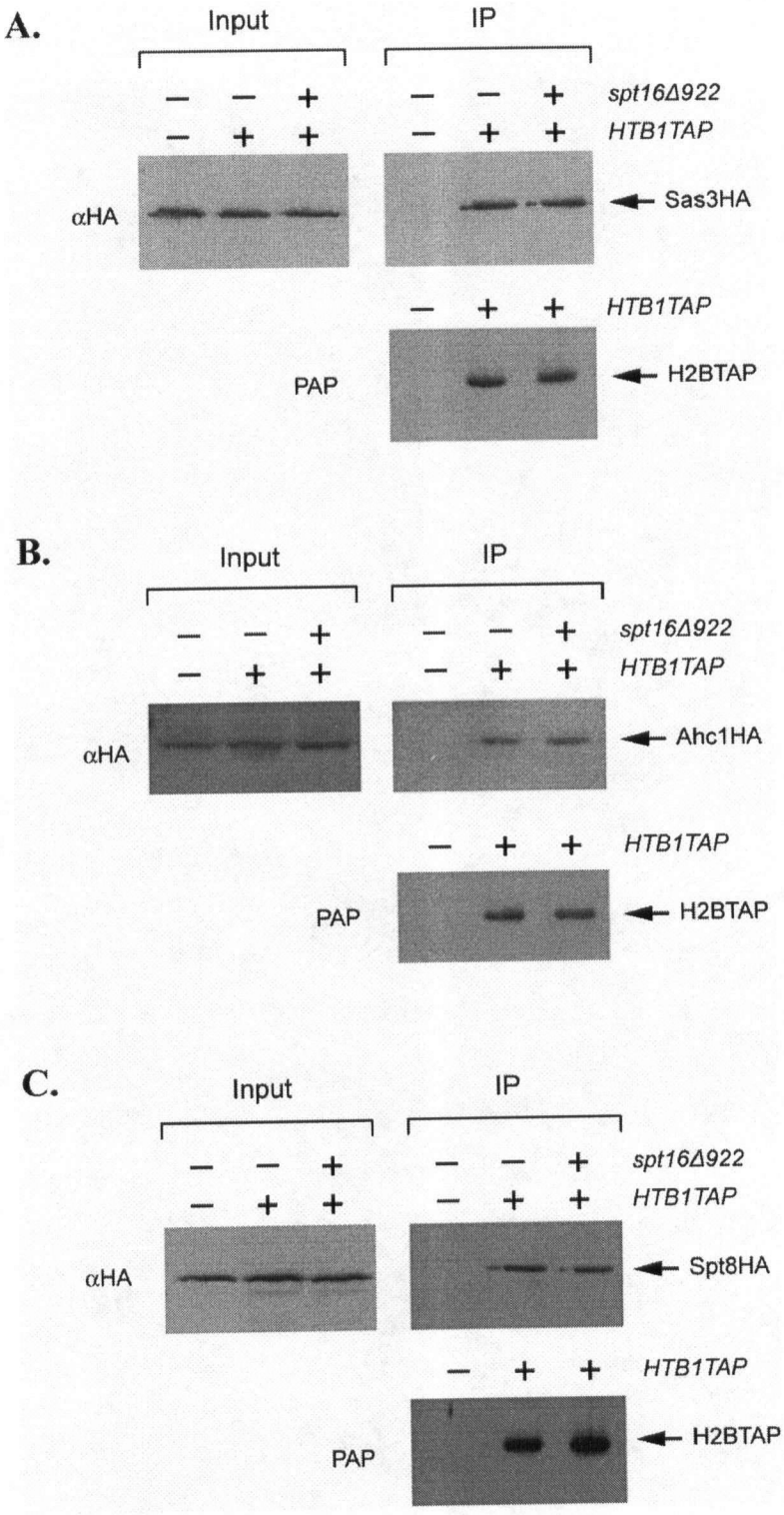

Figure 3.13 Loss of NTD does not affect NuA3 and ADA association with the nucleosome.

Chromatin pull-down (using H2BTAP) of Sas3HA$_3$ (A), Ahc1HA$_3$ (B), and Spt8HA$_3$ (C) in *SPT16* and *spt16Δ922* mutant strains. Anti-HA western blot analysis was used to visualize levels of Sas3HA$_3$ pulled-down. PAP western blot analysis of IP samples was done to verify equal levels of H2BTAP in precipitates.

3.2.6 Deletion of the Histone H3 Specific HATs Does not Confer an *spt* Phenotype

To test whether HAT mutants have an *spt* phenotype, the genes encoding several subunits of SAGA, ADA, and NuA3 were deleted from a strain containing the *lys2-128δ* allele and screened for an *spt* phenotype. These genes included *GCN5* which encodes the catalytic subunit of SAGA, SALSA, and ADA, as well as *SAS3,* which encodes the catalytic subunit of NuA3. The loss of either gene results in a loss of HAT activity for the respective complexes (59, 63). In addition, *ADA2*, important for Gcn5p HAT activity (61), and *AHC1,* essential for ADA complex stability (58), were deleted and screened. The results (Figure 3.14) revealed no *spt* phenotype in any of the null mutant strains. We also tested mutant combinations of both *sas3Δada2Δ* and *sas3Δahc1Δ* to rule out the possibility of functional redundancies between these HATs. Unfortunately, this screen was limited in its capacity to thoroughly test all combinations of HAT mutants. For example, a *sas3Δgcn5Δ* mutant would have been ideal to test the effect of loss of the combined HAT activity of NuA3, ADA, and SAGA, however, such a mutant is synthetically lethal (64). We therefore concluded that, although the screen of H3 HAT mutants could not confirm the link between loss of histone acetylation and the generation of an *spt* phenotype, it did not eliminate the possibility.

Currently, there is still much to be understood in regards to HAT complexes, their specific acetylation targets, and their overall downstream functions. It is thus inherently challenging to derive conclusions from our experiments involving the HAT complexes themselves. Therefore, we decided to adapt our approach in examining the *spt16Δ922* mutant interference hypothesis. Instead of focusing on the HATs themselves, a genetic strategy would

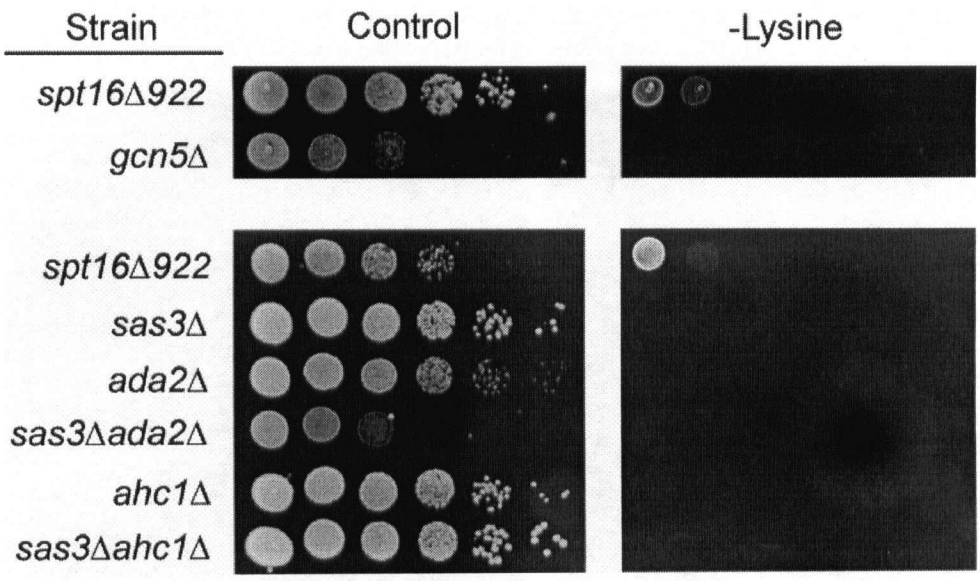

Figure 3.14 HAT Mutants do not confer an *spt* Phenotype.

lys2-128δ strains containing HAT mutants *gcn5Δ, sas3Δ), ada2Δ, sas3Δada2Δ, ahc1Δ,* and *sas3Δahc1Δ* were serially diluted ten-fold and spotted on YPD (control) and lysine drop out media to screen for the *spt* phenotype. Plates were incubated at 30°C for 3 days.

be used to investigate the modulators of histone acetylation, and the histone targets of acetylation directly.

3.2.7 Genetic Interactions Suggest that the *spt* Phenotype of *spt16Δ922* is Due to Loss of Histone Acetylation

The *spt16Δ922* mutant encodes an N-terminal domain partial truncation that is hypothesized to interfere with the access of histone modifying complexes with chromatin (83). In this study, we have so far shown that the *spt16Δ922* mutant did indeed show a loss of histone H3 acetylation, and that this altered histone modification showed a general correlation with the *spt* phenotype in other *spt16* mutants. To this point, attempts to confirm any functional relevance between these two observations from the perspective of HAT complexes failed to generate conclusive results. Here, we shifted our work from the modifier being interfered with, to the modification in question, histone acetylation.

Histone acetylation of chromatin is counteracted by the activity of histone deacetylase complexes (HDACs) (112). If indeed the *spt* phenotype was a result of loss of histone acetylation then we rationalized that removal of deacetylase activity in the cell might alleviate it. The *RPD3* gene encodes the catalytic subunit of the Rpd3 histone deacetylase complexes (113, 114), and has been implicated in the suppression of other FACT mutant phenotypes (103). We created *rpd3Δ* strains containing the *SPT16, spt16ΔNTD,* and *spt16Δ922* alleles and screened them for an *spt* phenotype alongside *RPD3* strains of the same *spt16* mutants (Figure 3.15). Results show the *spt* phenotype of *spt16Δ922* was significantly alleviated by the loss of the Rpd3 HDACs. As seen before, no *spt* phenotype was conferred in the *spt16ΔNTD* mutant. These

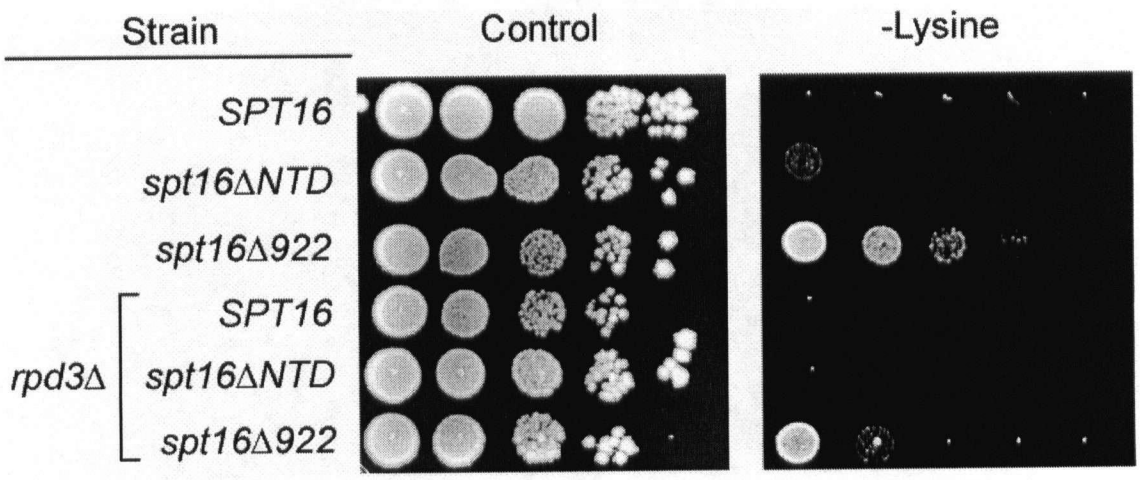

Figure 3.15 Deletion of *RPD3* suppresses the *spt* phenotype of *spt16Δ922*.

RPD3 deletion strains carrying the *SPT16, spt16ΔNTD,* and *spt16Δ922* alleles were serially diluted ten-fold and spotted on YPD (control) and lysine drop out media to screen for the *spt* phenotype. The same *spt16* mutants containing the wild type *RPD3* were plated alongside for comparison. Plates were incubated at 30°C for 3 days.

results suggest that the loss of acetylation observed in the *spt16Δ922* mutant, caused by the hypothesized interference of histone interactions, was in fact responsible for the development of an *spt* phenotype. The suppression of this phenotype through deletion of one of the HDACs, though, argued that this interference was not complete, and perhaps acetylation is merely being slowed down. If acetylation was completely lost, deletion of *RPD3* would have no effect.

The most well understood targets of histone acetylation are on the N-terminal tails of histones. If, as suggested, histone H3 acetylation is important for chromatin mediated repression and preventing the *spt* phenotype, one would expect the mutation of acetylated residues to have a similar effect. Previous work in our lab has produced a number of histone H3 tail mutants (101). To generate these strains, a parent strain in which the genomic copies of both histoneH3 and H4 encoding genes, *HHT1-HHF1* and *HHT2-HHF2,* are deleted was created, and maintained by an *HHT2-HHF2* locus on a plasmid (p*HHT2*). Plasmids carrying mutant *HHT2* were then introduced into this strain using a "plasmid shuffle". To further examine the *spt16Δ922* interference theory, we tested a variety of histone H3 mutants for an *spt* phenotype. We found that the mutation of lysine 14 and 23 to arginine conferred an *spt* phenotype (Figure 3.16). Several HATs are thought to acetylate residues K14 and K23, including NuA3, ADA, and SAGA (61, 64, 115). This result further supports the idea that histone acetylation is important for maintaining proper chromatin structure, and preventing an *spt* phenotype.

Interestingly, the wild type plasmid also seemed to confer a slight *spt* phenotype in a certain population of cells, while no *spt* phenotype was observed in mutants of lysine 4 and 36. The *spt* phenotype observed in the wild type histone H3 strain is most likely due to altered stoichiometry of histones resulting from expression of histone H3 and H4 from a plasmid. However, why the K4R and K36R mutants do not show a similar phenotype is unknown.

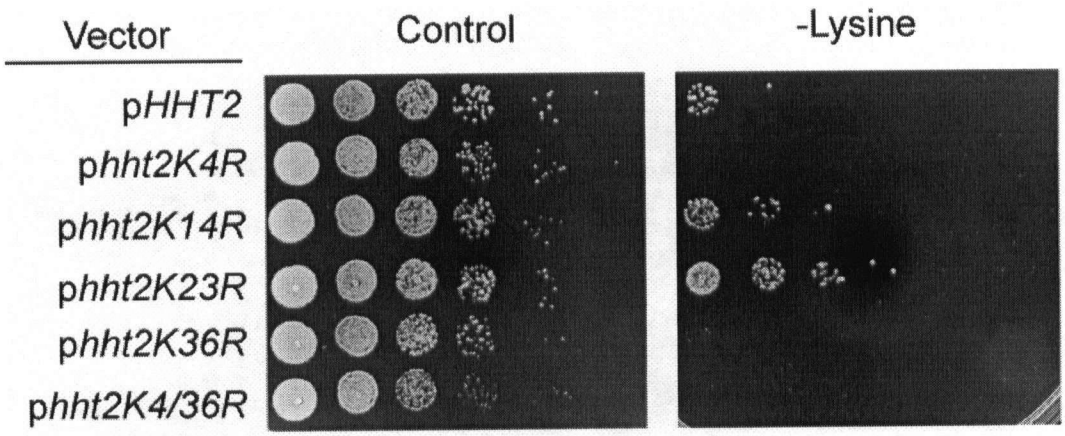

Figure 3.16 Histone H3 mutants confer an SPT phenotype.

Strains with both the *HHT1-HHF1* and *HHT2-HHF2* loci deleted and transformed with the indicated *hht2-hhf2* mutant locus carried on a plasmid were serially diluted ten-fold and spotted on YPD and lysine drop-out media to test transcription from the *lys2-128δ* allele. Plates were incubated at 30°C for 3 days.

Recent work has shown that K36 methylation acts in a pathway with Rpd3p in histone deacetylation (29, 30), and mutation of lysine 36 to arginine results in increased histone acetylation at specific loci. Thus the fact that mutation of K36 can suppress an *spt* phenotype is consistent with the fact that the *spt* phenotype is due to loss of histone acetylation.

In conclusion, our results support the hypothesis that loss of histone acetylation may be partially responsible for the *spt* phenotype of the *spt16Δ922* mutant strain. The fact that the *spt* phenotype is indicative of loss of chromatin mediated repression suggests that in addition to facilitating transcription activation, histone acetylation may also be linked to the proper repression of specific loci.

Chapter 4 – Discussion

4.1 The Interaction of FACT with NuA3 and ADA

This study began with an investigation into the interaction of the FACT complex with histone acetyltransferases. Given that both FACT and histone acetylation target the nucleosome (35, 88) and affect chromatin structure (1, 92), such a relationship is not at all surprising. Initial evidence of an interaction between Spt16p of FACT and Sas3p of the NuA3 HAT complex came from a yeast two-hybrid screen (96) and co-precipitation experiments using GST-Spt16p and anti-Spt16p polysera (63). Interestingly, while FACT mutants display a plethora of phenotypes, NuA3 mutants share none of these, suggesting the possibility that other HATs are interacting with FACT in a redundant role. In the first part of this study, we demonstrated that FACT interacts with both the NuA3 and ADA HAT complexes through the previously described N-terminal domain of Spt16p (83). We then attempted to investigate the relevance of this interaction by characterizing an *spt16* mutant lacking NuA3 and ADA interactions.

4.1.1 The Importance of the HAT Interaction Domain of Spt16p

In our original search to identify a HAT interacting with FACT and serving a redundant role as NuA3, we focused on Gcn5p dependent HATs in part because of a *sas3Δ gcn5Δ* synthetic lethality. It was reasoned that the shared essential function of Sas3p and Gcn5p may be related to the interaction between a Gcn5p dependent HAT, NuA3, and FACT. However, it has since been shown that this assumption was flawed, as the synthetic lethality of Sas3p and Gcn5p is caused by non-HAT related activities (D. Grimes and L.J. Howe unpublished data). In

addition, the observation that the HATs are interacting with a non-essential region of Spt16p rules out the possibility that interaction with HATs is required for FACT's essential function. This would again argue that the function of NuA3 and ADA with FACT is either non-essential or redundant to some other process.

To determine the functional relevance of the FACT-HAT interactions, it was predicted that a loss of the HAT interaction domain, and consequent loss of NuA3 and ADA interaction, would produce a mutant phenotype to study. Unfortunately, screening of the *spt16ΔNTD* mutant produced no such phenotypes. Initially, it was somewhat surprising that an entire domain of the essential Spt16p could be removed without serious effect, however this is not without explanation. It is possible that, in the context of FACT-mediated HAT complex recruitment, the NTD of Spt16p is redundant with other mechanisms for the recruitment of HAT activity to chromatin. For example, the histone H3 specific HAT SAGA has been shown to interact with a variety of transcriptional activators (105), and could be compensating for a loss in NuA3 and ADA recruitment in an *spt16ΔNTD* mutant. Consistent with this, deletion of *GCN5*, which encodes the catalytic subunit of multiple histone H3 specific HATs, including SAGA, results in severe growth defects in specific *spt16* mutants. However whether the *spt16ΔNTD* mutant shows a similar genetic interaction has not been determined.

Despite the lack of any *spt16ΔNTD* associated phenotype, it was still possible to investigate the effect a loss in FACT-HAT interaction would have on NuA3 and ADA association with the nucleosome. Our results using a chromatin pull-down assay, however, failed to show a loss in the association of NuA3 or ADA with nucleosomes in the *spt16ΔNTD* mutant. Again, this result, although negative, is not without explanation. First, the ability to see a loss in NuA3 and ADA association is based on the assumption that the NTD of Spt16p has a role in if not all, then a significant portion of NuA3 and ADA interactions with the nucleosome. As mentioned above, it is possible that there are alternative pathways in the recruitment of these

HATs. The interaction of the NuA3 complex with chromatin has been recently shown to be dependent on methylation of lysines 4 and 36 within histone H3. It is therefore possible that loss of Spt16p-NuA3 interaction does not result in a net loss of NuA3 from chromatin due to a redundant interaction of NuA3 with these methyl marks. Using multiple mechanisms to mediate the interaction of HAT complexes with various regions of the genome may be one way for a cell to fine tune histone acetylation levels to regulate transcription.

Although the interaction between ADA and NuA3 was not required for these complexes to interact with histones, it was still formally possible that these complexes require FACT to access their nucleosomal substrates. To test this, the levels of histone H3 acetylation in the *spt16ΔNTD* mutant was analyzed using anti-acetylated H3 antibodies raised against peptides acetylated at lysines 9 and 14. Although results did not show any loss in histone acetylation, this did not discount the possibility of a requirement for FACT in mediating NuA3 and ADA activity for many of the same reasons mentioned already. The assessment of this result is also made difficult by the fact that the actual lysines acetylated by NuA3 and ADA *in vivo* are still not known. NuA3 has been shown to acetylate K14, and to a lesser extent K23 *in vitro* (64), while the ADA complex acetylates lysines 14 and 18 *in vitro* (115). However, recent data has shown that deletion of *GCN5* does not result in a loss of histone H3 lysine 14 acetylation, suggesting that ADA is not acetylating this site *in vivo*. In addition, it has recently been suggested that the anti-acetylated H3 antibody used in this study preferentially recognizes acetylated K9 (D. Martin and L.J. Howe unpublished data). This could either be a result of the antibody showing stronger affinity toward acetylated K9, or a significantly higher proportion of acetylated K9 than K14 in the cell. In either case, it is possible that we failed to detect a loss of NuA3 or ADA acetylation in the *spt16ΔNTD* mutant because the antibody used was not be suited for the assay.

We conclude that although FACT is interacting with the histone H3 HATs NuA3 and ADA, the hypothesis that FACT is recruiting these HATs to the nucleosome cannot be confirmed or denied based on our evidence. With no observable *spt16ΔNTD* phenotype, and no available technique to test for NuA3 and ADA function, we can only speculate as to what the relevance of this interaction may be.

4.1.2 The Relevance of NuA3 and ADA Interaction with FACT – Alternative Models

Since we were unable to provide support for the hypothesis of FACT recruiting NuA3 and ADA to the nucleosome, we discuss here alternative models. One such model is that the HATs NuA3 and ADA recruit FACT to the nucleosome either directly or through their HAT activity. Precedence for such a model exists as nucleosome binding of the SWI/SNF and SAGA complexes has been shown to be dependent on histone acetylation (28, 116). Also, recent work in our lab has demonstrated a role for histone modifications, specifically histone methylation, in the recruitment of NuA3 to the nucleosome (101). Results in this study, though, fail to support this model as no change was observed in the association of FACT with the nucleosome in either *spt16ΔNTD*, or *sas3Δ* and *gcn5Δ* null mutants. This does not discount the possibility of the NuA3 and ADA dependent recruitment of FACT acting in parallel with some other pathway.

The work on *spt16ΔNTD* and FACT's role with NuA3 and ADA in this study focused on functions relating to the nucleosome. This was the obvious choice since the only targets of acetylation yet to be identified for the NuA3 and ADA HAT complexes are histone residues. However, there is a growing field of research indicating HATs can acetylate non-histone targets (117). In regards to the *sas3Δ gcn5Δ* synthetic lethality, recent work has suggested that this phenotype is due to loss of acetylation of a non-histone protein, yet to be identified (D. Grimes and L.J. Howe unpublished data). It is possible that FACT's association with NuA3 and ADA

may be required for acetylation of this substrate. As for non-histone targets of acetylation, the FACT associated DNA binding protein, Nhp6p, is an interesting candidate. Nhp6p shares a DNA binding motif with the HMGB-1 and 2 proteins (86), which are known to preferentially bend and bind distorted DNA (118). Furthermore, in vertebrates, acetylation of lysine residues in HMGB-1 by CBP enhances its binding affinity to bent DNA (119). The association of NuA3 and ADA with FACT, then, may serve to modulate Nhp6p function. It should be noted, however, that although Nhp6p is not essential, loss off the protein does cause a number of severe phenotypes (87, 120). No such phenotypes were observed in an *spt16ΔNTD* mutant, suggesting that if NuA3 and ADA are acetylating Nhp6p, this activity is not required for Nhp6p function. It should also be noted that, to this date, no evidence of NuA3 or ADA acetylating non-histone residues has ever been published.

The function of the N-terminal domain of Spt16p remains largely unknown. Besides an interaction with HATs, the only other previously described interaction is with the catalytic subunit of the protein kinase CK2, shown *in vitro* with human Spt16p (95). CK2 acts to phosphorylate p53 upon replication stress (121), however the significance of FACT involvement in this process is currently unclear. In the research described here, we characterized the interaction between FACT and two H3 HATs, NuA3 and ADA. Attempts to examine the relevance of this interaction, though, provided no conclusive results. Until such time as the downstream functions of NuA3 and ADA are elucidated, it will remain difficult to learn more about the importance of their interaction with the FACT complex.

4.2 The *spt16Δ922* Mutant and the Interference of Histone Acetylation

The second part of this study focused on another Spt16p N-terminal domain truncation mutant, *spt16Δ922.* The mutant Spt16p expressed by this strain has 182 more residues than *spt16ΔNTD,* yet causes a number of mutant phenotypes, suggesting that the extra residues are interfering with normal functions (83). Because of FACT's role in mediating chromatin structure, and the importance of histone modifications in a number of processes, our hypothesis was that the *spt16Δ922* mutant was interfering with normal histone modifications. Results showed that histone acetylation was indeed lost in the *spt16Δ922,* and not *spt16ΔNTD,* supporting our hypothesis. In addition, deletion of the *RPD3* histone deacetylase suppressed the *spt* phenotype of the *spt16Δ922* mutant and mutation of modified residues within the H3 tail conferred a similar *spt* phenotype further supporting a model of histone acetylation interference.

Although the above evidence supported the hypothesis that aberrant Spt16p in the *spt16Δ922* strain was interfering with histone acetylation, biochemical co-precipitation experiments failed to demonstrate this interference with respect to the association of the H3 HATs, NuA3, ADA, and SAGA, with the nucleosome. However, in retrospect, the chromatin pull-down assay was not an ideal system to test this. First of all, the *spt16Δ922* mutant may be interfering with HAT activity, and not HAT association, something we were not able to test directly. Secondly, it is possible that a variety of HATs are subject to a small amount of interference undetectable when considering the complexes individually. The loss in histone H3 acetylation observed would then be a result of the net loss in HAT activity of multiple complexes. A final consideration is that not all H3 HATs were tested in this study. Previous evidence showing *spt16* mutants having synthetic defects with *sas3Δ* and *gcn5Δ* (91) directed our focus on the Sas3p and Gcn5p dependent HATs. Additionally, the anti-acetylated H3 antibody used was designed to recognize acetylated lysines 9 and 14. Both of these points

prompted the testing of NuA3, ADA, and SAGA. However, evidence suggesting the anti-acetylated H3 antibody primarily recognizes lysine 9 suggests interference of NuA3 and ADA are not as involved in the observed loss in H3 acetylation as both preferentially target K14 *in vitro* (61, 64).

In the end, results did support the hypothesis that the *spt16Δ922* mutant was interfering with normal histone modifications, notably acetylation. The functional relevance of the loss in histone acetylation will be discussed in the following section.

4.2.1 Histone Acetylation and Cryptic Initiation

Suppression of the *lys2-128* allele is due to a failure to properly repress cryptic initiation, presumably because of a defect in establishing repressive chromatin structure following transcription (75, 76). The current model of FACT function involves facilitating the passage of RNA polymerase II during elongation by disrupting nucleosomal structure (82, 86, 89). The failure to properly repress transcription in FACT mutants, then, may be the result of improper nucleosome reassembly or maintenance following elongation (92).

Our data demonstrates a correlation between loss of histone acetylation in *spt16* mutants and an *spt* phenotype, suggesting that histone acetylation may be involved in reestablishing repressive chromatin following transcription. However, the question becomes whether or not there is a direct relationship between these two observations or whether these may simply be two effects with the same root cause, interference of normal histone related processes. In argument for the latter, the correlation between histone acetylation and the *spt* phenotype that we saw was not exact. In addition, null mutants of several histone acetyltransferases failed to confer an *spt* phenotype, although this negative result can be explained by the inability to screen all possible combinations of HAT mutants. However, the *spt* phenotype of the *spt16Δ922*

mutant was suppressed by deletion of *RPD3* suggesting that this phenotype was a result of histone acetylation loss. Moreover, although histone acetylation is generally associated with increased levels of transcription (31, 122), there is precedence for acetylation causing a repression of transcription. Hos2p, another histone deacetylase in the same class as Rpd3p, is required for the expression of certain genes (68). It is proposed that following elongation, the permissive state of chromatin may be disrupted by histone acetylation, preventing further rounds of transcription. It is possible that histone acetylation, in this case, may serve as a marker for other factors important in repression to bind chromatin. Following this model, lysines 14 and 23 may be important in conjunction with histone acetylation for the association of these repressive elements.

4.2.2 The Possibilities of an Interference Model

Besides acetylation, histones are post-translationally modified by methylation, ubiquitination, and phosphorylation (28). Recent studies have implicated methylation of lysine 36 on histone H3 in directing histone deacetylation by an Rpd3p complex known as RPD3S (29, 30). Mutation of two RPD3S complex subunits resulted in the appearance of aberrant transcripts, corresponding to an increase in acetylation in the *FLO8* and *STE11* open reading frames (29). This pathway would seem to be an obvious candidate to be disrupted in the *spt16Δ922* mutant, especially with its link to the prevention of aberrant transcription. Results from this study, however, do not seem to agree. In our experiments, the deletion of *RPD3* alleviated the *spt* phenotype, whereas the Set2p-RPD3S model describes Rpd3p as serving a repressive function. Additionally, the lysine 36 mutant failed to show an *spt* phenotype, however it is possible that the *spt* phenotype represents a more severe case of cryptic initiation, requiring the disturbance of several repressive mechanisms. Indeed, recent work with histone

modifications suggests complexity in the number and combination of histone residues important in the expression and repression of genes (41). In any case, the presence of pathways functioning to repress aberrant transcription does demonstrate that there are possible pathways to be interfered with.

Another possible model of the *spt16Δ922* mutant is that the misfolded protein is interfering with other general factors important in maintaining transcriptional repression. These factors may be chromatin remodelers themselves, or possibly binding factors that inhibit further transcription after RNA polymerase II passage. Likely candidates would include other *SPT* genes that show similar mutant phenotypes as *SPT16*, such as *SPT4, SPT5,* and *SPT6,* all of which are in the same category of *SPT* genes. The proteins Spt4p and Spt5p form the DRB sensitivity-inducing factor (DSIF), and are involved in both transcriptional repression and elongation (123, 124). This is thought to occur in a mechanism involving negative elongation factor (NELF) and interactions with RNA polymerase II, regulated in part by methylation of Spt5p (125). It is possible that *spt16Δ922* may interfere with any stage of transcriptional regulation by DSIF and/or NELF, or affect the modification of Spt5p and its elongation activation role.

The possibility of Spt6p interference is more intriguing. Past research has shown that Spt6p functions to repress transcription from cryptic sites (74), while more recent work has paralleled initial data regarding the function of Spt16p as a negative regulator of gene expression (126). The absence of Spt6p alleviates the need for transcriptional activators at certain genes, much like initial studies showed *spt16* mutants alleviated the need for binding sequences for such activators (127). In addition, the genes identified as requiring Spt6p for repression include *PHO5, ADH2,* and *SUC2* (126), the latter of which has also been shown to be repressed by Spt16p (78), suggesting the two *SPT* genes may act similarly for the same set of genes. Finally, Spt6p mediates these repressive effects by nucleosome reassembly at the

promoter following activation. These similarities to Spt16p and FACT may indicate a functional association between the two *SPT* genes. Spt6p could be recruited to the nucleosome following FACT mediated elongation to counteract nucleosomal disruption and prevent aberrant transcription. FACT mutants that cause cryptic initiation, such as *spt16Δ922*, may not be directly affecting FACT function, but preventing the activities of Spt6p. This may be through a loss of association between FACT and Spt6p, or perhaps prevention of Spt6p-nuclesome association. Spt6p may also require association with specific modified histone residues, for example, lysines 14 and 23 may act as nucleosome docking site for Spt6p.

The failure to repress aberrant transcription in *spt16* mutants has often been interpreted as a loss in FACT function (75, 78, 98). Our data supports the hypothesis that the *spt16Δ922* mutant may be interfering with normal histone modifications, specifically, histone acetylation, and that this interference is in part causing an *spt* phenotype. FACT, then, may not be directly responsible for preventing aberrant gene expression. Instead, FACT mutants may be interfering with the normal processes and pathways required for proper nucleosome restructuring following transcription elongation.

4.3 Concluding Statement

The first part of this study focused on the interaction between FACT and two H3 specific HAT complexes, NuA3 and ADA. After confirming the putative interaction between Sas3p of NuA3 and Spt16p, we went on to demonstrate both *in vitro* and *in vivo* that FACT interacts with the ADA complex as well. The HAT interaction domain was then mapped to the N-terminal 482 residues of Spt16p. The functional relevance of this interaction, however, remains unknown as

attempts to implicate FACT's interaction with ADA and NuA3 with known FACT functions proved unsuccessful.

In the second part of our research, we demonstrated that the partial N-terminal domain truncation encoded by *spt16Δ922* interfered with histone H3 acetylation. Further work showed that this loss in acetylation was partially responsible for improper repression, resulting in cryptic initiation from the *lys2-128δ* allele. Although the molecular basis of this interference and its downstream effects still require further elucidation, our results supported the hypothesis of histone modification interference in the *spt16Δ922* mutant.

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