THE ESSENTIAL FUNCTION OF YEAST ACETYLTRANSFERASE PROTEINS

by

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Abstract

The yeast proteins Sas3p (something about silencing 3) and Gcn5p (general control non-derepressible 5) are the catalytic subunits of the histone acetyltransferase (HAT) complexes NuA3 and SAGA, ADA, SLIK/SALSA, and HAT-A2 respectively. When incorporated into their respective complexes they gain the ability to acetylate nucleosomal histone H3 at the overlapping K14 residue. Previous work reveals \textit{gcn5\_\_sas3\_\_} strains are inviable, suggesting they have an essential function in the redundant acetylation of histone H3. Furthermore, researchers have shown that viability is dependent on the HAT domains of Sas3p and Gcn5p. We show that a H3 tail with an arginine substitution mutation at K14 but not K9,18,23,27R is synthetically lethal in a \textit{gcn5\_\_} background. Thus, NuA3's essential role in the cell is acetylating K14. To determine how Sas3p acetylation is targeted, we performed a yeast 2-hybrid screen against Sas3p's essential N-terminus. Hsl7p and Rlf2p are tentatively shown to interact with Sas3p but did not interact or share genetic interactions with NuA3. Previous research shows the synthetic lethality between loss of Gcn5p and loss of components of NuA3 is independent of the adaptor proteins Ada2p and Ada3p; thus, Gcn5p's essential function appears independent of known HAT complexes, suggesting it could be acetylating a non-histone protein. Consistent with this idea, we created a Gcn5p truncation that cannot interact with Ada2p or rescue a SAGA phenotype but rescues the synthetic lethality. Furthermore, we showed that histone H3 tail mutations in K9,14,18,23R or K9,18,23,27R are viable in a \textit{sas3\_\_} background. We attempted to find the essential subunit of Gcn5p and ruled out Sinlp and Htzlp as the sole substrate. In a synthetic lethality suppression screen, we identified several mutants that suppress the \textit{sas3\_\_gcn5\_\_} synthetic lethality. These mutants appear to have the characteristics of petites and/or prions. Thus, a possible role for these HATs in DNA damage repair is proposed.
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Nomenclature

Throughout the text, genetic symbols and gene products are denoted using conventional nomenclature. Italicized upper case letters symbolize wild type alleles of genetic loci (i.e. GCN5) while italicized lower case letters represent mutant genes (i.e. gcn5). A Greek ‘delta’ preceding italicized lower case letters denotes a gene deletion (i.e. gcn5Δ indicates deletion of the GCN5 locus). Double colons (::) indicate insertion mutations (i.e. gcn5::HISMX6 represents a HISMX6 gene inserted within the GCN5 locus). Capitalization of only the first letter of the protein name followed by a “p” postscript represents gene products (i.e. Gcn5p is the gene product of the GCN5 locus). Square brackets enclosing italicized upper case letters represents a prion mutation (i.e. [PSI+] is the prion mutant of the SUP35 gene).
Acknowledgements

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Chapter 1 – Introduction

1.1 Acetylation: a Common Protein Modification

Modifying proteins by either co-translational or post-translational acetylation is the most common form of protein modification in eukaryotes (1). Acetyltransferase proteins catalyze the transfer of an acetyl group from acetyl co-A onto the α-amino group of amino terminal residues or the ε-amino group of internal lysine residues. Co-translational amino-terminal acetylation is an irreversible modification that occurs on approximately 85% of proteins (1, 2). Post-translational acetylation, the focus of this study, occurs on the ε-lysine residues of a wide variety of substrates including high mobility group (HMG) proteins, transcription factors, nuclear receptors, α-tubulin, and histones (1, 3, 4). These modifications affect protein functions ranging from stability, DNA-binding, protein-protein interaction, peptide-receptor recognition, and enzymatic activity (1). Furthermore, acetylation is known to play a major role in transcription regulation (5).

The fact that histone acetylation is correlated with increased transcription has been known for many years (6). The packaging of DNA into condensed chromatin fibers is necessary for fitting a huge DNA molecule into the limited space of the nucleus. However, this extensive packaging of the genome acts to repress transcription (5). A means of regulating access to genomic DNA of transcription, DNA repair, and DNA replicating machinery is necessary to maintain life. Before addressing the role of acetylation in regulating gene expression, we must first discuss how DNA is packaged.
1.2 Chromatin – Structure and Function

1.2.1 The Nucleosome Core Particle

The basic building block of chromatin, or the “core particle,” is made up of 147 base pairs (bp) of DNA that is wound 1.7 times around a histone octamer core in a left-handed superhelix (7). The histone octamer core is made up of two copies each of the highly conserved histone proteins H2A, H2B, H3, and H4 (7-9). The (H3)_2 (H4)_2 tetramer forms the centre of the octamer, with the H2A-H2B dimers situated on either side (8). A histone fold motif present in each of the core histones is sufficient for forming both histone-histone and histone-DNA contacts (7). The association of the core histones together in a complex results in an overall left-handed protein superhelix structure similar to that of the DNA wrapped around the core particle (8). The histone-DNA interaction is limited to the DNA’s phosphodiester backbone and occurs along the inner surface of the super helix. Interaction of histones with only the backbone of DNA ensures that binding is universal and independent of sequence (8). Protruding out radially from the nucleosome are the N and C terminal histone tail domains (7). The histone tails contain numerous sites available for post-translational modifications. Researchers believe the tails form nucleosome-nucleosome bonds and interact with linker DNA, the 10-60 bp stretches of DNA located between nucleosomes. Together, the nucleosome and the linker DNA form the nucleosomal array, commonly referred to as “beads on a string.”

1.2.2 Higher Order Chromatin Structure

Addition of 1 to 2 mM divalent salt to nucleosomal arrays causes them to condense into a 30 nm fibre. Since cells have more than twice this divalent salt concentration (~5 mM Ca^{2+} and 3 mM Mg^{2+} during interphase), formation of the 30 nm fibre is expected to occur in vivo, as
well as considerable additional fibre-fibre interactions (7). The histone tails appear to be key players in facilitating fibre condensation since their deletion eliminates chromatin condensation. The tails are thought to form condensed fibres through contact with adjacent nucleosomes and/or manipulating linker DNA arrangement (8). Linker histones have also been shown to play a role in chromatin fibre formation (8). These histone proteins have sequences unrelated to the core histones. Nucleosomes associated with linker histones bind an additional 20 bp of DNA. Addition of the histone linker proteins H1, H5, or Hho1p (in yeast), to nucleosomal arrays stabilizes both fibre-fibre interaction and intramolecular folding (7). In general, linker histones associate with nucleosomes at a ratio of 1:1 (10). Hho1p, however, does not appear to have any clear ratio (8, 10, 11). Linker histones are believed to function downstream of the histone tails since deletion of the tails eliminates condensation despite the presence of linker histones (7).

Non-histone proteins also play a role in the formation of condensed chromatin. These proteins are classified on the basis of whether or not they interact with the core histone tails (10). Sir3p and Tup1p, yeast silencing proteins, are examples of proteins that interact with histone tails (10, 12, 13). Non-histone proteins bring nucleosomes together over long distances by cross-linking chromatin fibers together through the interaction of adjacent silencing proteins (10). Researchers have observed higher order structures of chromatin ranging in size from 30 nm to 300 nm in vivo due in part to the function of non-histone proteins (7). Such highly condensed regions of chromatin are referred to as heterochromatin and are usually gene poor, while less condensed regions are referred to as euchromatin, and are generally gene rich (14). Significantly, even in these highly condensed states, transcription has been shown to occur (7).
1.2.3 Regulating Chromatin Structure

Condensed chromatin restricts access of cellular machinery to genomic DNA and is thus repressive to gene transcription (15). For example, in vivo studies show that inhibiting histone synthesis causes loss of nucleosomes resulting in activation of previously silent genes (16). Transcriptional activity is further reduced by the formation of histone fold-DNA interactions in the core particle and fibre-fibre interactions between nucleosomes (7). Due to the repressive nature of chromatin, the cell must maintain mechanisms that allow transcription machinery to access promoters and ORFs.

The cell maintains several mechanisms to regulate gene expression. One such mechanism, the incorporation of histone variants into chromatin, plays a role in stimulating transcription (17). The most well studied histone variant is H2AZ, a histone H2A variant that contains substantially different N and C terminal tails from its canonical partner (18). Deposition of Htz1p, the yeast homolog of H2AZ, into the promoters of genes, destabilizes the nucleosome leading to localized loss of the core particle. Loss of nucleosomes frees the promoter for interaction with transcription binding factors, facilitating gene expression (17). Another class of complexes, the ATP-dependent SWI/SNF-like complexes, also regulate transcription by modifying chromatin structure (7). SWI/SNF has been shown to work primarily at the level of the nucleosome, destabilizing DNA-histone interactions (7). Another mechanism that influences chromatin structure is post-translational covalent histone modification. These modifications include lysine and arginine methylation, lysine acetylation, serine phosphorylation, and ubiquitination (19). Currently, the best characterized modification is lysine acetylation performed by histone acetyltransferases (HATs) (5).

HATs are specialized acetyltransferases that catalyze the transfer of an acetyl group onto histone proteins. Unlike chromatin remodeling complexes, HATs primarily target the histone
tails, which are outside of the core particle, and are unlikely to affect nucleosome structure (8). Recently, however, an acetylation site in the globular domain of histone H3 has been discovered at lysine 56 (see table 1.1) (20). An important quality of histone acetylation is that it is a reversible process. Histone deacetylases (HDACs) have been shown to catalyze the reverse reaction of HATs, removing acetyl groups from histone tails (21). In general, hyperacetylated regions of chromatin are linked with transcriptional activation and hypoacetylated regions are linked with repression (5). Thus, the opposing functions of HATs and HDACs work antagonistically to regulate the overall levels of histone acetylation and presumably control gene expression (22).

Table 1.1 Sites of Histone Acetylation in Yeast (20, 23, 24)

<table>
<thead>
<tr>
<th>Acetylation Sites</th>
<th>Histones</th>
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<tr>
<td></td>
<td>H2A</td>
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Classically, HATs and other histone modifying enzymes have been thought to promote decondensation of chromatin by neutralizing or adding a negative charge to the positively charged residues of the histone tail, weakening its interaction with negatively charged DNA, and/or affecting fibre-fibre interactions between nucleosomes (5, 25). The regulation of histone structure by chemically neutralizing or adding negative charges onto the histone tails is an attractive hypothesis due to its simplicity. However, a number of contradictions exist where the
same modification both increases and decreases gene expression (26). For example, serine 10 phosphorylation on histone H3 is required for chromatin condensation during mitosis, yet the same modification induces immediate-early gene transcription after mitogenic stimulation (26). An alternative explanation called the histone code hypothesis attempts to address these seemingly contradictory results.

1.2.4 The Histone Code Hypothesis

The histone code hypothesis states that the patterns of post-translational histone modifications could specify patterns of gene expression by providing docking sites for proteins recognizing specific modifications or combinations of modifications (19, 26). Much evidence supports the histone code hypothesis (27, 28). For example, RCAF (replication coupling assembly factor) specifically deposits histone H4 acetylated at lysine 5 and 12 into newly synthesized chromatin in *Tetrahymena*, flies and humans (but not in yeast) (29). This suggests the acetylation pattern present on H4 is specifically recognized and “decoded” by a component of a histone chaperone complex (26). Furthermore, the existence of the bromodomain, a motif that specifically recognizes acetylated lysine residues and promotes binding of proteins containing it to chromatin, supports the idea that proteins recognize specific modifications of the histone tail. For example, acetylated nucleosomes retain the bromodomain containing SWI/SNF and SAGA (Spt, Ada, and Gcn5 acetyltransferase) complexes longer than unacetylated nucleosomes. NuA4, a complex that acetylates histone H4 but lacks a bromodomain, is not retained on acetylated nucleosomes (26).

The histone code hypothesis suggests that modification of one residue can affect the modification of another. For example, histone H3 serine 10 phosphorylation promotes acetylation of histone H3 by SAGA (30). These modifications may occur within the same histone (CIS) or between different histones (TRANS). An example of a CIS acting modification
is methylation of K4 (lysine 4) strongly inhibits the purified methyltransferase SUV39H complex from methylating K9 \textit{in vitro} (19). K9 methylation is linked with heterochromatin due to the high affinity interaction between itself and HP1 (19). Thus, histone H3 K4 methylation inhibits HP1 binding at the H3 K9 residue. An example of a TRANS acting modification is ubiquitination of K123 of histone H2B’s C-terminus is required for H3 K4 methylation. Mutating this residue to arginine or alanine prevents ubiquitination and subsequently, H3 K4 methylation (19). Deleting \textit{RAD6}, one of the enzymes responsible for H2B K123 ubiquitination, also prevents H3 K4 methylation. Thus, modifications of histone H2B affect modifications of histone H3. The histone code hypothesis illustrates the broad range of effects possible as a result of specific modifications to histone residues.

\section*{1.3 Yeast HATs and Their Complexes}

The two general categories of HATs – type-A and type-B – describe where in the cell HATs perform their reactions. Type-A HATs are located in the nucleus while type-B HATs are found in the cytoplasm. Type-B HATs acetylate histones in preparation for their transport into the nucleus. Type-A HATs, the focus of this study, acetylate nucleosomal histones and are correlated with increased transcription (5). Two such HATs important to this study are Gcn5p and Sas3p.

The homolog of Gcn5p (general control non-derepressible 5), referred to as HAT A in \textit{Tetrahymena thermophila}, was the first type-A HAT identified (31). Gcn5p is the best characterized HAT and is a member of the GNAT (Gcn5p-related N-acetyltransferase) super family along with Hat1p, Elp3p, and Hpa2p (5). As the first transcriptional coactivator shown to display HAT activity (32, 33), Gcn5p provided the first link between histone acetylation and gene activation (34). Deletion of \textit{GCN5} results in a G2/M progression delay (35). This, taken
together with the observation that histone H3 acetylation levels increase following UV irradiation (36), suggests Gcn5p may have a role in DNA repair (37). Gcn5p contains several functional domains including a bromodomain, an Ada2p interaction domain, and a HAT domain (38). Disruption of the HAT domain effectively eliminates Gcn5p's ability to acetylate histones (35). Studies have shown that free histone H3 K14 residues are acetylated by Gcn5p in vitro (39). However, in vivo, Gcn5p is unable to acetylate nucleosomal substrates unless incorporated into a HAT complex (39, 40). In yeast, Gcn5p is the catalytic subunit of several complexes including SAGA, SLIK/SALSA, ADA, and HAT-A2 (40-44). Each of these complexes contains the trimeric module composed of Gcn5p and the adaptor proteins Ada2p and Ada3p (45). Not surprisingly, deletion of the genes encoding either one of these adaptor proteins disrupts Gcn5p catalyzed histone acetylation to the same extent as deletion of GCN5 itself in vivo (35). The other components of these complexes are listed in Table 1.2.

SAGA is the best characterized of the Gcn5p containing HAT complexes. SAGA is a 1.8 MDa complex with 18 known subunits. Several groups of proteins are contained in the SAGA complex, including the trimeric module proteins, a subset of Spt proteins, a subset of Taf11 proteins, and Tra1p (5). Deletion of ADA1, SPT7, SPT20, or TAF168 disrupts SAGA and impairs cell growth while the other components of SAGA do not appear vital for its formation (5, 46). SAGA is known to play a role in transcriptional activation of several genes including GAL1, TRP3, and HIS3 (47). Surprisingly, researches have found a role for SAGA in repressed transcription of the ArgR/Mcm1 complex (48). Incorporation of Gcn5p into its respective complexes not only allows nucleosomal acetylation but also expands the specificity of those modifications. For example, Gcn5p alone has been shown to acetylate H3 K14 in vitro while SAGA can acetylate lysine residues K9, K14, K18, and K23 on histone H3, and to a lesser extent, residues K11 and K16 on H2B.
Table 1.2 Components of the Gcn5p Containing HAT Complexes SAGA, SLIK/SALSA, and ADA (23) and the NuA3 Complex (49, 50)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>SAGA</th>
<th>SLIK/SALSA</th>
<th>ADA</th>
<th>HAT-A2</th>
<th>NuA3</th>
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Sas3p (something about silencing 3) was first identified in a screen looking for defects in epigenetic silencing in a sir1A background (51). In yeast, Sir1p, along with Sir2p-Sir4p, are involved in producing silent chromatin. A deletion of SIR1 eliminates mating in most cells due to silencing defects at the HM mating type loci but some cells can still mate. Deleting SAS3 in this background leads to a more severe phenotype (5). Paradoxically, Sas3p has a role in enhancing the silencing defect at the HML loci while restoring silencing to a derepressed HMR locus (51). Thus, depending on the gene loci, Sas3p can function as either a positive or negative regulator of transcription (50). Sas3p belongs to the MYST family, named for the components MOZ, Sas3p/Ybf2p, Sas2p, and Tip60p (5). Other members of the MYST family include Esa1p, HBO1, and MORF. Like Gcn5p, Sas3p possesses HAT activity but cannot acetylate nucleosomes in vitro unless incorporated into a complex.
Sas3p is incorporated into the NuA3 complex, a 400 KDa complex that contains the proteins Sas3p, Ynglp, Nto1p, Taf14p and Eaf6p (Howe, L. personal communication, 49, 50). Ynglp is necessary for NuA3 activity but not for structural integrity of the complex (49). Little is known about the other members of the complex. NuA3 has been shown to preferentially acetylate histone H3 at residues K14, and to a lesser extent K23 \textit{in vitro} (35).

1.3.1 The Synthetic Lethality of Gcn5p and Sas3p

The only essential HAT in yeast is Esa1p, a component of the H4 HAT complexes NuA4 and piccolo NuA4 (52, 53). Cells with single deletions of all other known yeast HATs, including Gcn5p and Sas3p, are viable. However, deletion of \textit{GCN5} in combination with \textit{SAS3} has been shown to halt cells at the G2/M border, resulting in a synthetic lethality (35). Deletion of two genes causing a lethal phenotype is called a "synthetic lethality" since a lethal phenotype is being "synthesized." Researchers have shown that the synthetic lethality is dependent on the previously defined HAT domains of Gcn5p and Sas3p (35). Therefore, the essential function of these previously characterized HATs is dependent on their ability to acetylate proteins. Interestingly, the synthetic lethality between \textit{gcn5A} and \textit{sas3A} is independent of the adaptor proteins Ada2p and/or Ada3p (35). Thus far, this is the only known case of mutations that are synthetically lethal with \textit{gcn5A} that are not also synthetically lethal with \textit{ada2A} or \textit{ada3A}. For example, \textit{swi1A} is synthetically lethal with \textit{gcn5A}, \textit{ada2A}, and \textit{ada3A} (54). Since Ada2p and Ada3p are essential for Gcn5p's SAGA, ADA, SLIK/SALSA, and HAT-A2 related functions (45) and the synthetic lethality is independent of Ada2p, it stands to reason that Gcn5p is performing an essential role independent of the Gcn5p dependent HAT complexes SAGA, ADA, SLIK/SALSA and HAT-A2. [Please note: throughout the text, the function of \textit{GCN5} that is essential in the absence of \textit{SAS3} will be referred to as \textit{Gcn5p's “essential function.”}]
It would seem unlikely that Gcn5p’s essential role in the cell is acetylating histones since Gcn5p cannot perform this role in vivo independent of the SAGA, ADA, SLIK/SALSA and HAT-A2 complexes. This leaves the question of what Gcn5p is acetylating to perform its essential role. The possibility that Gcn5p and/or Sas3p are acetylating non-histone proteins as their essential function is not without precedence. As previously mentioned, acetylation of proteins other than histones is a common occurrence in eukaryotic organisms. In fact, researchers implicate Gcn5p as the catalytic subunit involved in acetylating several non-histone components, including Sin1p and Htz1p (54-56).

Originally, Sin1p was identified in a genetic screen looking for suppressors of Ty and δ insertion mutations in the HIS4 promoter (57). SIN1 is a distant relative of mammalian HMG1, since it contains two HMG1-like domains and is likely to be a non-histone chromatin component (1, 58). Both HMG1 and Sin1p have been shown to bind 4-way junction DNA, which often is predictive of an affinity for binding DNA at the entry and exit points of the nucleosome (58). Acetylation of HMG1 has been shown to occur in the DNA binding domain, blocking it from association with chromatin (1). It is likely that acetylation of Sin1p by Gcn5p could have a similar effect. Significantly, a deletion of SIN1 has been shown to alleviate the synthetic lethality of GCN5 SWI1 deletions, establishing a genetic link between Sin1p and Gcn5p (54). Thus, this protein is a good candidate for the essential substrate of Gcn5p.

Htz1p, the only H2A variant in budding yeast, is a member of the highly conserved family of H2AZ variants (55). Htz1p is distinguished from H2A by three main features: a unique C-terminal tail that helps direct Htz1p deposition (59), an extended acidic patch forming an αC helix that has a possible role in compaction (60), and an internal loop that helps establish nucleosomes with either two Htz1p or two H2A molecules (17). Deletion of HTZ1 results in the misregulation of ~5% of genes. Htz1p, as previously discussed, is deposited into promoter regions of genes by the SWR-C complex, presumably priming promoters for activation (17).
Deletion of Gcn5p and to a lesser extent, Sas3p, reduces the occupancy of Htz1p at promoter regions of repressed loci (17). Therefore, Htz1p could potentially be Gcn5p and/or Sas3p’s essential substrate.

Thus, the overall goal of this project is to discover the essential function of the yeast acetyltransferases Gcn5p and Sas3p using genetic and proteomic approaches. We conclude that Gcn5p acetylates a non-histone protein that has a redundant role with Sas3p’s ability to acetylate histones.
Chapter 2 – Materials and Methods

2.1 Preparation of Yeast Strains and Plasmids

All yeast strain manipulations, including transformation, sporulation, tetrad dissection and screening were carried out using standard protocols (61). The yeast strains used in this thesis are listed in Table 2.1.

All $SAS3$ truncation mutants, including $pS4S3\text{FLAG} \ (1\text{-}831)$, $psas3\text{FLAG} \ (1\text{-}577)$, $psas3\text{FLAG} \ (241\text{-}831)$, $psas3\text{FLAG} \ (241\text{-}577)$, $pSAS3HA\text{A} \ (1\text{-}831)$, $psas3HA\text{B} \ (241\text{-}831)$, $psas3HA\text{C} \ (1\text{-}577)$, and $psas3HA\text{D} \ (241\text{-}577)$ were made previously in our lab. The plasmids contain $\sim1$ kb of upstream $SAS3$ sequence cloned as a BamHI/NcoI fragment, the appropriate ORF fragment cloned as a NcoI/SalI fragment, and the FLAG-CYC cassette (50) or the HA-CYC cassette (41) cloned into the YCplac33 vector (62).

The plasmid $pHHT2$, containing wild type $HHT2$ and $HHF2$ and only one BamHI site, was created from the plasmid pDM18 (63), which contains two BamHI sites. The plasmid pDM18 was digested with SpeI, producing a 4,812, a 1,825, and a 50 bp fragment. The largest fragment was purified using the Wizard SV Gel and PCR Clean-up system (Promega Corporation, Madison, WI) and dephosphorylated with CIP enzyme (New England BioLabs). This was then ligated to the gel purified 1,825 bp fragment, creating the complete plasmid. The plasmid $phht2\Delta3\text{-}29$ was created by phosphorylating the oligonucleotides ($5'\text{-GATCCAAGCAAACACTCCAAATGGCCAGACCATCTA-3'}$ and $5'\text{-CCGGTAGATGGTCTGGCCATTGTGGAGTGTTTGCTTG-3'}$) with T4 Polynucleotide Kinase (PNK enzyme) (Fermentas), annealing them together, and inserting the annealed product into the BamHI and AgeI sites of...
pHHT2. The pHHT2 K14R plasmid was created by site directed mutagenesis using Stratagene’s QuickChange Site-Directed Mutagenesis Kit (64).

The pH3modularsystem plasmid was created by digesting pHHT2 with BamHI and AgeI and annealing the phosphorylated oligonucleotides (5’-GATCCAGCAAAACGCTAGCGGCCA-3’ and 5’-CCGGTGGCCGCTAGCTGGT TTGGTTAGTCTGGCCATTGTGGAGTGTTTGCTTG-3’) into this site. The oligonucleotides contain an engineered silent mutation in the eighth amino acid (GCTAGA changed to GCTAGC) which inserts a NheI restriction site downstream of lysine 9. The plasmid thus has a gap between amino acid 8 and 31, with NheI and AgeI restriction sites flanking the gap. This allows for easy insertion of any number of histone tail mutants into the gap region. Using this plasmid, we created pHHT2-K9,14,18,23R and pHHT2-K9,18,23,27R. The oligos (5’-CTAGACGGTCCACTGGTGGTGGGCCCCAAGACGGCAATTAGCCTCCCGGGCTGCCAGAAATCCGCCCCCATCTA-3’ and 5’-CCGGTAGATGGGGCGGATTTTCTGGCAGCCC GGGAGGGTAATGGCGTCTTGGGGCCGCCAGCCACCAGTGACCAGT-3’) and (5’-CTAGACGGTCCACTGGTGGTAAAGCCCAAGACGGCAATTAGCCTCCCGGGCTGCCAGAC GGTCCGCCCCCATCTA-3’ and 5’-CCGGTAGATGGGGCGGACCGTCTGGCAGCCCGGG AGGGTAATTGGCGTCTTGGGGTTTGACCACCAGTGACCAGT-3’) were phosphorylated using PNK enzyme (Fermentas) and ligated into the NheI and AgeI restriction sites of pH3modularsystem. The NAPs unit (NAPs, UBC campus, Vancouver) performed all plasmid sequencing verifications.

The plasmid pgen5A262.314 was created by fusing the truncated GCN5 gene product into the vector pRS314 (65). This was accomplished by amplifying the gene from a genomic DNA template using primers with the engineered restriction sites SalI and KpnI that annealed in the GCN5 promoter and directly downstream of GCN5’s 261st amino acid (aa). The amplified product and vector pRS314 (65) were digested sequentially with KpnI and then SalI, gel
purified using the Wizard SV Gel and PCR Clean-up system (Promega Corporation, Madison, WI), and then ligated together. The plasmid pgcn5Δ262.424 was created by digesting pgcn5Δ262.314 and the vector pRS424 (66) sequentially with KpnI and then Sall, gel purifying, and ligating the products together.

The plasmids pgcn5Δ94N and pgcn5Δ169N were created by amplifying the gene from a genomic DNA template using primers with engineered BamHI and SacI restriction sites that anneal in the GCN5 ORF directly upstream of either GCN5’s 94 aa or 169 aa and downstream of GCN5’s ORF. The amplified products, along with the plasmid pGCN5HA.314 (65) were digested sequentially with BamHI and SacI, gel purified, and then ligated together.

The temperature sensitive plasmid psas3TS.ADE3 was made previously in our lab by digesting the plasmid pLP1364 (psas3-Δ638/URA3/CEN) (67) with KpnI and BamHI and the plasmid pPS709 (2μ URA3 ADE3) (68) with BamHI and Sall. These fragments were then cloned into the YCplac33 vector (62).
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<td>his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 ade2::TRP1 ade3::TRP1 gcn5::HIS3 sas3::KAN psas373::ADE3 URA3</td>
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</tr>
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Table 2.2 Plasmids Used in Study

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<td>Sikorski et al. 1989</td>
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<td>pRS424</td>
<td>TRP1 high copy vector</td>
<td>Sikorski et al. 1989</td>
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<td>pSAS3HA</td>
<td>SAS3 ORF on pRS314 fused to C-terminal triple HA cassette</td>
<td>L. Howe</td>
</tr>
<tr>
<td>pSAS3.416</td>
<td>SAS3 ORF on pRS316</td>
<td>L. Howe</td>
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<td>FLAG tagged SAS3 loci on pYCplac33 vector</td>
<td>John et al. 2000</td>
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<td>John et al. 2000</td>
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<td>hht2 mutant with gap between aa 8 and 31</td>
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<td>substitution mutant K9,14,18,23R of H3 on pH3modularsystem</td>
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<td>pHHT2 K-9,18,23,27-R</td>
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2.2 PCR Techniques

High efficiency transformation of PCR product was used for all gene knockouts and tags. A typical PCR reaction contained 77 μl of sdH2O, 10 μl of 10X Taq buffer, 8 μl of 2.5 mg/ml dNTPs, 0.5 μl of each 100 μM primer, 1 μl of template DNA, 1 μl of Taq enzyme (NOTE: Taq enzyme prepared in our lab). PCR was performed on the Eppendorf Mastercycler Gradient PCR machine (Eppendorf North America, NY) using the standard elongation protocol (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). PCR was also used when screening transformed cells for deletions or tags. Genomic preps of the strains were made and the genomic DNA produced was used as a template.
2.3 Plasmid Shuffle

Numerous plasmid shuffle experiments were performed in this project. In general, a yeast strain containing a URA3 based plasmid carrying a gene potentially essential to cell survival was simultaneously transformed with another TRP1 or LEU2 based plasmid carrying a gene of interest or vector alone. Simultaneously transformed cells were plated on synthetic dropout medium selecting for the plasmid of interest to ensure its presence. Tenfold serial dilutions were performed on each strain in sdH2O and spotted on synthetic complete (SC) media (control) and 5-FOA plates (0.1% of 5-flouroorotic acid) and incubated at 30°C for 4 days. Strains that required the URA3 based plasmid carrying the potentially essential gene for survival would not grow on 5-FOA, while those that did not shuffled this plasmid out.

2.4 Yeast 2-Hybrid Screen

PCR products encoding sas3 (1-577) or sas3 (1-241) were inserted into a GAL4 DNA-binding domain vector and were transformed into the yeast strain PJ69-4a [S. Fields] carrying an HO gene on a plasmid. These strains were mated against all transformants of the array (containing 6000 ORF’s fused to a GAL4 activation domain carried in the MATa strain PJ69-4a) by transferring 1 ml of an overnight culture of these strains to each of 16 OmniTrays. This was accomplished using a 384-pin High Density Replicating Tool (Beckman), followed by pinning of the MATa activation domain array transformants onto the same position. To select for diploids, the crosses were transferred with the replicating tool to synthetic dropout medium lacking leucine and tryptophan and incubated at 30°C for 2-3 days. To screen for 2-hybrid interactions, diploids were transferred to synthetic dropout medium lacking leucine, tryptophan,
and histidine and supplemented with 3 mM 3-AT. After two weeks, the plates were scored for growth at 30°C. 1

2.5 Preparation of Whole Cell Extracts for Various Calmodulin Pull-Down Assays

Strains were inoculated from freezer stock into 5 ml of yeast extract-peptone-dextrose (YPD) and incubated overnight at 30°C with shaking at 200 rpm. The cultures were then diluted into 50 ml YPD to an optical density of 0.5 at 600 nm (OD₆₀₀) and grown at 30°C to an OD₆₀₀ of 2.0. At this point, cells were spun down in the Sorvall Legend RT centrifuge (Thermo Electron Corporation, Walthum, MA) for 3 min at 4,000 rpm at 4°C, washed with 25 ml of distilled water and resuspended in 500 µl of 150 mM 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 CaCl₂, and 0.1% NP40 with 1 mM PMSF and 2 µg/ml pepstatin A). To the cell suspensions, equal volumes of glass beads were added, and samples were vortexed for 3 min. using the Vortex Genie (Scientific Industries, Bohemia, NY). Finally, the cell lysate was spun down for 5 min. at 14,000 rpm at 4°C, the supernatant was removed, and the cells were stored at -80°C.

2.6 Calmodulin Pull-Down Assays

Calmodulin pull-downs were performed for several experiments involving Hsl7TAP, Rlf2TAP, Sas3TAP, Nto1TAP, and Htb1TAP. Whole cell extracts were normalized for bulk proteins by Bradford assay. For each sample tested, 20 µl of calmodulin affinity resin (Stratagene, Cedar Creek, TX) was washed four times with cold calmodulin binding buffer and aliquoted into separate tubes. To each tube, 400 µl of normalized whole cell extract was added and samples were rotated for two hours at 4°C. Next, the calmodulin resin was washed four

1 All work done on the yeast 2-hybrid screen was performed by Stanley Fields lab.
times with 20 volumes of cold calmodulin binding buffer. The beads were then boiled at 100°C for 5 minutes in SDS sample buffer to elute the bound protein. Finally, western blot analysis was performed using anti-HA (Roche), and peroxidase anti-peroxidase (PAP) antibodies (Sigma). For the hsl7Δ SAS3HA HTB1TAP experiment, an additional antibody, the anti-acetyl H3 antibody (Biotech, Lake Placid, NY), was used to control for equivalent pull-down of chromatin.

2.7 Mating Assay

Mating assays were performed by spotting the strains of interest onto YPD and incubating at 30°C for three days. This plate was then replica plated onto a mating tester strain of opposite mating type (YLH101) that was previously spread onto YPD and allowed to dry on the bench for one hour. As a control, the plate was also replica plated onto YPD lacking a mating tester strain. After three days of growth at 30°C, replica plated both mating and non-mating conditions onto diploid selection media (ADE'TRP) and incubated these plates at 30°C for three days.

2.8 SAGA Defect Phenotype Assay

This assay was designed to test if strains exhibited a SAGA specific phenotype. To accomplish this, ten-fold serial dilutions of strains were spotted onto SC media (control) and 3-AT (50mM 3-Amino 1,2,4, Triazole) and incubated at 30°C for 4 days.

2.9 DNA Damage Repair Defect Phenotype Assay

This assay was designed to test if strains were sensitive to a DNA damage-inducing drug. To accomplish this, ten-fold serial dilutions of strains were spotted onto YPD media (control) and HU media (200 mM hydroxyurea) and incubated at 30°C for 4 days. [Please note:
HU sensitivity is also indicative of a DNA replication defect. In our experiment, we assayed for HU sensitivity, however, the high concentrations of HU required to see sensitivity suggests the phenotype may be caused by a repair defect and not a replication defect.

2.10 Petite Mutant Phenotype Assay

This assay was designed to test if strains had mutations in their mitochondrial DNA (mtDNA) causing petite mutants. To accomplish this, tenfold serial dilutions of strains were spotted onto YPD (control) and petite media (1% yeast extract, 0.03% dextrose, 2.4% glycerol, 2% agar) and incubated at 30°C for 4 days.

2.11 Preparation of Whole Cell Extracts for Anti-Acetyl Lysine Immunoprecipitation

Whole cell extracts were prepared as described for calmodulin bead pull-downs except 150 mM IP buffer (50 mM HEPES (7.4 pH), 1 mM EGTA, 0.5 mM DTT, 150 mM NaCl, 16 mM MgAc, 0.1% Nonident P-40, 10% glycerol) was used instead of 150 mM calmodulin binding buffer.

2.12 Performing Anti-Acetyl Lysine Immunoprecipitation

Whole cell extracts were normalized for bulk protein content using Bradford assay. For each sample tested, 20 µl of protein A sepharose beads (Amersham Biosciences, Piscataway, NJ) was washed four times with cold IPP buffer and aliquoted into separate tubes. To each tube, 400 µl of normalized whole cell extract was added and samples were rotated for one hour at 4°C. The beads were then centrifuged at 2000 rpm for 10 seconds and the cleared extract was transferred to a fresh tube. To the tube, 1 µl of anti-acetyl lysine antibody (Upstate Biotech) was
added and the tube was rotated at 4°C for two hours. At the same time, approximately 10 μl of protein A-Sepharose beads was washed four times with 20 volumes of cold IPP buffer. After rotating the WCE for two hours with the antibody, the mixture was added to the freshly washed protein A-Sepharose beads and they were rotated for one hour at 4°C. Then, the beads were centrifuged at 2000 rpm for 10 seconds and the supernatant was discarded. Next, the protein A sepharose beads were washed five times with 20 volumes of cold IPP buffer and then boiled at 100°C for 5 minutes in SDS sample buffer, eluting the bound protein. Finally, western blot analysis was performed using the primary anti-acetyl lysine (Sigma) antibody followed by the secondary anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ).

2.13 Synthetic Lethality Suppression Screen

In this screen, we looked for mutants that rescued the synthetic lethality of sas3Δgcn5Δ. To do this, we screened the strains YDG007 (MATa gcn5Δsas3Δade2Δade3Δpsas3TS.ADE3) and YDG008 (MATa gcn5Δsas3Δade2Δade3Δpsas3TS.ADE3) for loss of the URA3 based plasmid psas3TS.ADE3 which changed cells from pink to white. First, the parent strains were inoculated from freezer stock and grown in 10 ml of YPD media overnight at 30°C. Cells were grown to an OD600 of ~2.0 and then 5 ml of culture was spun down at 4,000 rpm for 3 min. Cells were washed once with 5 ml of sdH2O and then the pellet was transferred to a 1.5 ml microfuge tube and resuspended in 200 μl of sdH2O. The cell suspension was then plated on pre-warmed YPD medium and incubated at 37°C for 4-5 days. Any colonies that grew at the non-permissive temperature were then streaked out onto fresh YPD media and incubated again at 37°C. Those strains that remained viable were then subjected to tenfold dilutions, spotting on SC media and SC media with 5-FOA. Freezer stocks were made of all gss (gcn5 sas3 suppressor) mutant colonies that grew on 5-FOA and at 37°C.
Next, the mutants generated were characterized as either dominant or recessive. To do this, the mutants as well as their respective parent colonies, were crossed with either YLH112 (MATα GSS+ gcn5Δasas3ΔpSAS3) or YLH115 (MATa GSS+ gcn5Δasas3Δ pSAS3). YLH112 and YLH115 were transformed with the LEU2 vector (YCplacl11) then mixed with each mutant of opposite mating type in YPD media and incubated overnight at 30°C. To select for diploids, 50 μl of the mating mixture was streaked out onto synthetic dropout medium lacking leucine and tryptophan, and then incubated at 30°C for 4 days. Tenfold dilution assays of each resulting diploid were plated on YPD and SC with 5-FOA medium, and incubated at 30°C for 4 days.

Next, the gss mutants generated were examined to see if they were genetically inheritable. The diploids generated previously were transformed with a URA3 based plasmid carrying wild type GCN5, and were sporulated. Tetrad dissections were performed using a tetrad dissection microscope (Carl Zeiss Inc, Thornwood, NY) as described by standard protocol (61). Tetrads that produced four viable spores were then subjected to tenfold dilution assays spotted onto YPD, SC and SC with 5-FOA medium and incubated at 30°C for 4 days.
Chapter 3 – Results

3.1 The Essential Function of NuA3

The histone acetyltransferase complex NuA4 primarily acetylates histone H4 in nucleosomes (69). Esa1p, the catalytic subunit of piccolo NuA4, is the only HAT that is encoded by an essential gene (35). Previously, it was shown that deletion of the gene encoding the catalytic subunit of NuA3 – SAS3 – in conjunction with deletion of GCN5, which encodes another well known histone H3 HAT, results in cell death (35). Although the obvious interpretation of the esalA and gen5Asas3A lethalities is that histone acetylation is essential, this does not fit with the fact that neither the H4 or H3 tail, nor the lysines within them are essential (70, 71). Instead, we prefer the hypothesis that some HATs are acetylating non-histone substrates and it is these events that are essential.

3.1.1 Simultaneous Deletion of SAS3 and GCN5 is Synthetically Lethal

As the sas3Agcn5A synthetic lethality data forms the backbone of the current research, it was important to reproduce this result. First, GCN5 was knocked out of a sas3A strain (YHL106) transformed with a URA3 based plasmid carrying a wild type copy of SAS3. Then, the strain was simultaneously transformed with LEU2 based plasmids, including vector alone, pGCN5, or pSAS3, and plated on yeast extract-peptone-dextrose (YPD) (control) or SC with 5-fluroorotic acid (5-FOA) medium. This media effectively selects against cells harbouring URA3 based plasmids as described elsewhere (61). As seen in Figure 3.1A, strains without at least one copy of either SAS3 or GCN5 on a non-URA3 based plasmid were inviable. Thus, we have effectively reproduced the finding that deleting the two histone H3 HAT encoding genes, GCN5 and SAS3, results in a synthetically lethal phenotype.
Figure 3.1 SAS3 and NuA3 are Synthetically Lethal with GCN5

(A) SAS3 and GCN5 deletions are synthetically lethal. Tenfold dilutions of the yeast strain YDG080 (sas3Δ gcn5Δ) transformed with the indicated plasmids were plated on YPD medium (control), or SC medium with 5-FOA and incubated at 30°C for 4 days. (B) GCN5 deletion is synthetically lethal with components of NuA3. Tenfold dilutions of the yeast strain YLH101 (wild type), YLH105 (gcn5Δ), YLH117 (gcn5Δyng1Δ), YLH119 (gcn5Δnto1Δ), and YDG080 (sas3Δ gcn5Δ) transformed with the indicated plasmids were plated on YPD medium (control), or SC medium with 5-FOA and incubated at 30°C for 4 days.
3.1.2 Deleting Components of NuA3 is Synthetically Lethal with GCN5

Having verified the result that loss of SAS3 is synthetically lethal with loss of GCN5, the next step was to address whether this phenotype is unique to loss of Sas3p or if it applies to the disruption of the entire NuA3 complex. Previous research shows that loss of GCN5 is synthetically lethal with nto1Δ, yng1Δ as well as sas3Δ (Howe, L. personal communication, 49). To verify this data, the strains nto1Δgcncn5Δ (YLH119) and yng1Δgcncn5Δ (YLH117) transformed with a plasmid containing a URA3 marker carrying wild type GCN5, were plated on YPD medium (control) or SC with 5-FOA medium. As seen in Figure 3.1B, both nto1Δgcncn5Δ and yng1Δgcncn5Δ strains grew well on control media but could not survive on 5-FOA media, which selects against the pGCN5HA.416 plasmid. Thus, we reproduced the published data that loss of GCN5 is synthetically lethal with disruption of multiple components of NuA3. This data suggests that loss of NuA3 and not specifically Sas3p is responsible for the gcncn5Δsas3Δ synthetically lethal phenotype.

3.1.3 NuA3's Essential Function is Acetylating the Histone H3 Tail

Substantial evidence supports the observation that NuA3 acetylates the histone H3 tail in vitro, however, whether H3 is acetylated by NuA3 in vivo is unknown (35, 50). If the H3 tail were NuA3’s essential substrate, then deleting this substrate would be equivalent to deleting SAS3. Thus, we would expect deleting both GCN5 and the H3 tail would result in synthetic lethality. In fact, others have shown that deleting the HHT2 tail in combination with deleting GCN5 results in a synthetically lethal phenotype (34). However, it is possible to argue that the gcncn5Δhht2Δ3-29 synthetic lethality is a GCN5 specific phenotype that is independent of SAS3. Refuting this idea is the observation made previously in our lab that deleting ADA2 in combination with loss of the H3 tail is viable (64). As discussed in detail in Section 3.4, a
distinctive characteristic of the sas3Δgcna5Δ synthetic lethality is that it is independent of ADA2 and the SAGA, SLIK/SALSA, ADA, and HAT-A2 complexes. This is significant because, besides the present case, no other known proteins other than those encoding components of NuA3 are known to have genetic interactions with GCN5 but not ADA2 (49). Therefore, it is highly likely that the synthetic lethality between gcna5Δhht2Δa3-29 is a NuA3 phenotype.

We repeated the results described above by performing tenfold dilutions of the yeast strains YLH347 (hht2ΔpHHT2.URA3), YLH348 (gcna5Δhht2ΔpHHT2.URA3) and YLH349 (ada2Δhht2ΔpHHT2.URA3) transformed with the TRP1 based plasmids carrying either wild type HHT2 or hht2Δa3-29 and plating onto SC medium (control), synthetic histidine dropout medium with 50 mM 3-AT, or SC medium containing 5-FOA (see Figure 3.2A). Our data supports the previous assertion that deleting the H3 tail in combination with deletion of GCN5 is synthetically lethal. Furthermore, we also repeated the result that the synthetic lethality between gcna5Δ and hht2Δa3-29 is not shared with ada2Δ. Strains carrying ADA2 or GCN5 deletions are known to display poor growth on 3-AT media due to impaired HIS3 expression (see section 3.4.1 for more details) (32, 72). Both gcna5Δhht2Δ pDM18 and ada2Δhht2ΔpDM18 grew poorly on 3-AT media, thus demonstrating that the strains we used herein display previously published phenotypes. This suggests NuA3’s essential function in the cell is dependent on the H3 tail.

[Please Note: Yeast carry two copies of each histone encoding gene. Thus, to study the effect of histone mutations without interference from wild type histone gene products, both genomic copies of histone genes must be deleted. Furthermore, the HHT1/HHF1 and HHT2/HHF2 genes are expressed from divergent promoters. Consequently, deletion of one gene necessitates the deletion of the other. For this reason, the hht2Δ strains used herein have deletions in HHT1, HHT2, HHF1, and HHF2. The essential function of histone genes can be replaced by either copy of the gene expressed on a centromeric plasmid. The plasmids used herein contain the URA3 gene and thus can be used to shuffle in plasmids (non-URA3 plasmids) carrying
mutations of HHF2 or HHT2. For simplicity sake, the strain’s HHT1, HHF1, and HHF2 deletions, as well as the plasmid’s wild type copy of HHF2 will be omitted.

The possibility exists, however, that deleting the tail is not the same as loss of acetylation. For example, the tail itself could be repressing transcription through some unknown mechanism and deleting it may alleviate the repressive effect and consequently, the need for acetylation. To control for this possibility, we mutated histone H3’s lysine residues 9, 14, 18, and 23 to arginine, which were originally identified as the sites of H3 acetylation (4, 23). This was accomplished using the modular system previously described herein (see materials and methods), creating the TRP1 based plasmid phht2 K9,14,18,23R. We chose arginine as a lysine substitute since it has a similar charge but cannot be acetylated. The strain YLH348 (gcn5Δhht2ΔpDM18) was then transformed with either the mutant or a TRP1 based plasmid carrying wild type HHT2 and plated onto YPD medium or SC with 5-FOA. Figure 3.2B shows that, in a gcn5Δhht2Δ background, mutating HHT2 lysine residues 9, 14, 18, and 23 to arginine results in a synthetically lethal phenotype. This observation strongly suggests that the sas3Δgcn5Δ synthetic lethality is due to loss of acetylation of H3.

NuA3 acetylates primarily histone H3 K14 in vitro (35). If NuA3 were acetylating this residue as part of its essential function, we would expect to see a genetic relationship with loss of GCN5 combined with mutation of lysine 14 to arginine. To determine whether NuA3 is acetylating histone H3’s lysine 14 residue in vivo we transformed the strains YDG089 (hht2ΔpDM18), YLH348 (gcn5Δhht2ΔpDM18) and YLH349 (ada2Δhht2ΔpDM18) with the TRP1 based plasmid phht2 K14R. The resulting transformants were then plated onto SC medium (control), SC with 5-FOA or synthetic histidine dropout medium with 50 mM 3-AT and incubated at 30°C for 3 days. As seen in Figure 3.2C, mutating histone H3’s lysine 14 residue to arginine in a gcn5Δhht2Δ background results in a severe synthetic sickness. This result is consistent with previously published data (34). Again, we see that this result is specific for the
NuA3 pathway since mutating histone H3's lysine 14 residue to arginine in an *ada2Δhht2Δ* background is viable. This strongly suggests that NuA3 primarily acetylates H3 K14 *in vivo*.

To rule out the involvement of the other histone H3 residues we mutated all of the known histone H3 acetylation sites other than K14 and tested this mutant in a *gcn5Δ* background. We included K27R since recent data suggests that this site is also acetylated (24). Again, we used the modular system previously described herein (see materials and methods), to create the *TRP1* based plasmid *pht2* K9,18,23,27R. The strain YLH348 (*gcn5Δhht2ΔpDM18*) was then transformed with either the mutant or a *TRP1* based plasmid carrying wild type *HHT2* and plated onto YPD medium or SC with 5-FOA. As seen in Figure 3.2D, mutating *HHT2*'s lysine residues 9, 18, 23, and 27 to arginine does not impair cell growth (compare to wild type of the same background). Therefore, the other acetylated lysine residues of the histone H3 tail besides K14 are not significantly involved in the NuA3 pathway.

From these experiments, we were able to confirm the previous data showing a histone H3 tail deletion and a K14R mutation display strong genetic phenotypes with loss of *GCN5*. Furthermore, we showed that this genetic relationship is associated with NuA3 since it is also independent of *ADA2* and consequently, the SAGA, ADA, SALSA/SLIK, and HAT-A2 complexes. Lastly, we showed that mutating all the histone H3 tail lysine residues other than K14 to arginine does not produce a genetic phenotype in a *gcn5Δ* background, suggesting that these residues are not involved in the NuA3 pathway. Therefore, NuA3’s essential function in a *gcn5Δ* background appears to be the acetylation of the histone H3 lysine 14 residue.
Figure 3.2 NuA3’s Function is Acetylating the Histone H3 Tail

(A) Deletions of the H3 tail is synthetically lethal in a gcn5Δ background. Tenfold dilutions of the yeast strains YLH347 (hht2Δ), YLH348 (gcn5Δhht2Δ) and YLH349 (ada2Δhht2Δ) transformed with the indicated plasmids were plated on SC medium (control), SC medium with 5-FOA, or synthetic histidine dropout medium with 50 mM 3-AT and incubated at 30°C for 3 days. (B) The sas3Δgcn5Δ synthetic lethality is due to loss of H3 acetylation. Tenfold dilutions of the yeast strains YLH224 (hht2Δ) and YDG044 (gcn5Δhht2Δ) transformed with the indicated plasmids were plated on YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 4 days. (C) NuA3 acetylates primarily H3 K14 in vivo. Tenfold dilutions of the yeast strains YLH347 (hht2Δ), YLH348 (gcn5Δhht2Δ) and YLH349 (ada2Δhht2Δ) transformed with the indicated plasmids were plated as described in part A of this figure. (D) The sas3Δgcn5Δ synthetic lethality is independent of H3 K9, 18, 23, or 27. Tenfold dilutions of the yeast strains YLH224 (hht2Δ) and YDG044 (gcn5Δhht2Δ) transformed with the indicated plasmids were plated as described in part B of this figure.
3.2 Mapping the Essential Region of Sas3p

3.2.1 The C-Terminus of Sas3p is Dispensable

We have shown evidence that Sas3p acetylates K14 of histone H3 \textit{in vivo}. Studies from multiple labs have shown that this modification is localized to regions of transcriptional activity (73, 74). We were interested in determining how NuA3 is targeted to these regions by identifying potential Sas3p interaction partners. We rationalized that these proteins would interact with an essential region of Sas3p. Furthermore, Spt16p and Chk1p have been shown to interact with Sas3p’s C-terminus and it would be interesting to see whether this region of Sas3p is essential for rescuing the \textit{sas3Agcn5A} synthetic lethality. Deletion assays in which regions of the protein of interest are truncated and tested for rescuing a synthetic lethality has been used in the past with success (75). For these reasons, we decided to map the essential regions of Sas3p.

The Sas3p protein was cloned into a \textit{TRP1} based plasmid and truncated into three parts, roughly corresponding to the location of known domains and motifs, and tested to see which region could rescue the synthetic lethality (see Figure 3.3A). More specifically, Sas3p was split into an N-terminal domain deletion (241-831), a C-terminal domain deletion (1-577), and an N and C-terminal truncation (241-577). The N and C-terminal truncation maintains the zinc finger, HAT, and acetyl-CoA N-acetyltransferase domains (5, 50, 76). This region shares a high sequence homology to other members of the MYST family and is essential for the proteins ability to acetylate histones. Sas3p’s N-terminal deletion truncates a region that contains no known motifs, stopping short of the \textit{SAS} conserved domain, which begins at approximately aa 270 (76). Finally, the C-terminal deletion eliminates an acidic domain that stretches from aa 700-831 (50). This region has been shown to interact with Spt16p, a component of the FACT complex, and Chk1p, an evolutionarily conserved protein kinase that plays a role in regulating
the metaphase to anaphase transition (in budding yeast) via a yeast 2-hybrid (77, 78). These three truncation mutants, along with wild type Sas3p or vector alone, were transformed into a sas3Δagcn5Δ strain containing a URA3 plasmid carrying a wild type copy of SAS3. These stains were then plated onto YPD (control), synthetic tryptophan dropout medium and SC with 5-FOA medium and incubated at 30°C for 4 days. Strains with only a C-terminal truncation (1-577) show no growth defects compared to wild type (see Figure 3.3B). Truncating the N-terminus (241-577 and 241-831), however, results in a synthetic lethality comparable to vector alone. This data suggests that the C-terminus of Sas3p, and consequently, its association with Spt16p and Chk1p, does not play a role in Sas3p’s essential function.

With the in vivo data showing Sas3p’s C-terminus is dispensable, the next step was to back up this data biochemically. Specifically, we wanted to see if the C-terminal truncation of Sas3p could still interact with NuA3 in vivo. To this end, a strain with a deletion is SAS3 and an integrated NTO1TAP tag was transformed with vector alone, wild type pSAS3HA-A, or each of the three SAS3 truncations fused to HA tags. Whole cell extracts of the strains were then prepared and rotated with calmodulin beads according to protocol. Western blot analysis of these samples was then performed, using anti-HA antibodies to detect the presence of Sas3p. Strains missing the Sas3p N-terminus (241-577, and 241-831) show no interactions with the NuA3 subunit Nto1TAP (see Figure 3.3C). The C-terminal truncation (1-577), however, shows a strong interaction with Nto1TAP equivalent to that of wild type SAS3 (compare lanes 1 and 4). We cannot rule out the possibility that a Sas3p N-terminal truncation destabilizes the protein to such an extent that an interaction between it and Nto1TAP cannot be detected. However, this does not take away from the fact that deleting the C-terminus of Sas3p does not impede its interaction with Nto1TAP. Both the genetic and biochemical evidence supports the idea that the essential function of NuA3 does not depend significantly on Sas3p’s C-terminus. Thus, interactions with Spt16p and Chk1p are not involved in Sas3p’s essential function.
**Figure 3.3 NuA3 Function does not Require Sas3p’s C-terminus**

(A) A schematic diagram of Sas3p depicting the location, in terms of aa, and names of known structural motifs and domains (76). (B) C-terminal truncated Sas3p rescues the synthetic lethality. Tenfold dilutions of the yeast strain YDG081 (gcn5Δsas3Δ pSAS3.316) transformed with the indicated plasmids were plated on YPD medium (control), synthetic tryptophan dropout medium (TRP'), or SC medium with 5-FOA and incubated at 30°C for 4 days. (C) C-terminal truncated Sas3p interacts with Nto1p. Nto1TAP pull-down assays were performed with the yeast strain YLH182 (sas3ΔNTO1TAP) transformed with the indicated psas3HA plasmids. Western blot analysis with immunodetection by HA antibodies (SAS3HA) was performed with the resulting samples.
3.3 Yeast 2-Hybrid Screen Directed Against Sas3p's N-terminus

3.3.1 The 2-Hybrid Screen Reveals Many Potential Hits

Yeast 2-Hybrid screens aim to discover proteins that interact with a protein of interest (the bait). The interacting proteins (hits) may be associated with the bait as substrates, modifiers, or subunits of the same complex. Often, discovering which proteins or protein groups interact with the bait gives insight into the functional role of the protein of interest (79). For example, if the screen generates several hits involved with DNA damage repair, there is a very good possibility that the bait protein is also involved in this process. We performed a yeast 2-hybrid screen in collaboration with Stanley Fields’ lab in the hopes of discovering potential proteins that interact with essential regions of Sas3p.

The array we used contains approximately 6000 yeast ORF’s fused to a GAL4 transcription activation domain vector transformed individually into a reporter strain of a specific mating type carrying a HIS3 reporter vector (80). The array consists of 16 micro-assay plates with 384 colonies on each plate. The protein of interest’s corresponding gene is similarly fused to a GAL4 activation domain and transformed into a reporter strain of opposite mating type. The strain carrying the bait is then mated against the entire array of activation domain hybrids, selecting diploids by markers carried on the hybrid plasmids. Once mated, the diploids are transferred onto HIS+ medium. Colonies that contain an interacting pair of proteins will bring the activation and binding domains of GAL4 in close proximity to each other allowing transcription of the HIS3 gene to occur. Since the position of each ORF in the array is known, proteins that interact with the bait can be identified immediately simply by their position in the array (80).
Using the data that Sas3p's C-terminus is not significantly required for NuA3's essential function, we directed the screen against two Sas3p C-terminal truncation mutants (1-577 and 1-241). By using Sas3p's essential N-terminus as bait, we eliminate the possible hits that might interact with the nonessential C-terminus. Eliminating these hits means that more time and resources can be devoted to examining hits involved with the phenotype we are interested in studying. Table 3.2 displays the full list of hits generated from the yeast 2-hybrid screen. The presence of Nto1p, a protein known to interact with Sas3p (Howe, L. personal communication), shows that the screen is working correctly. Although not all of the hits have been analyzed, namely YOL164w and MMS22, preliminary evidence in our lab has narrowed down the list of potential hits to Hsl7p and Rlf2p.

Table 3.1 List of Hits Obtained from a Yeast 2-Hybrid Screen Directed Toward Sas3p's N-Terminus

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<th>Hit</th>
<th>Sas3 aa 1 to 577</th>
<th>Sas3 aa 1 to 241</th>
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<td>NTO1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>KIN3</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SPC25</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HSL7</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SWE1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>HEX3</td>
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</tr>
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<tr>
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<td>No</td>
</tr>
<tr>
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<td>Yes</td>
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<td>No</td>
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</tr>
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<td>RLF2</td>
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</tr>
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</table>
3.3.2 Hsl7p Interacts with Sas3TAP in vivo

Hsl7p (histone synthetic lethal 7) was initially picked out of a screen looking for mutants that were synthetically lethal with loss of the amino-terminus of histone H3 (81). During mitosis, Hsl7p is localized to the daughter side of the bud neck, specifically, to the septin ring (82). Deletion of HSL7 produces a G2/M delay in cell cycle progression (83). Furthermore, Hsl7p is a verified methyltransferase that has been shown to bind AdoMet and to methylate calf thymus histone H2A in vitro (84). Wild type human JBP (Jak2-binding protein), the homolog of Hsl7p, can rescue bud neck elongation in an hsl7Δ strain but JBP with a deletion in the methylation domain cannot (84). This suggests Hsl7p’s methyltransferase ability is important in rescuing the G2/M delay. What drew our attention to Hsl7p is that previous research demonstrated it is synthetically lethal with deletion of GCN5 (Pillus, L. unpublished data). Often, synthetic lethality between proteins means they are involved in cellular pathways that “buffer” each other biologically (85). The possibility that Sas3p and Hsl7p interact and are synthetically lethal with GCN5 deletion strongly suggests they would be involved in the same essential function.

To verify the interaction biochemically we TAP tagged Hsl7p on the C-terminus (YLH237) and transformed it with LEU2 based plasmids including vector alone or a wild type copy of SAS3 fused to a triple HA epitope tag (pSAS3HA.111). An advantage of using the TAP tag method to verify protein interactions is that all the tagged proteins are expressed from their endogenous promoters. This eliminates possible overexpression of the target protein, which often leads to the formation of nonspecific and/or unnatural protein interactions (86). As an added control, we also transformed a wild type strain (YLH101) with pSAS3HA.111. Whole cell extracts of the strains were prepared and normalized for overall protein levels using Bradford assay. Then, calmodulin pull-downs were performed specifically against the TAP epitope tag, as
outlined in materials and methods. Western blot analysis was then performed on the samples probing with antibodies targeted against Hsl7TAP (peroxidase anti-peroxidase (PAP)) or Sas3HA (αHA). Figure 3.4 shows that Hsl7TAP has an interaction with Sas3HA compared with the negative control (compare lanes 1 and 3). There was some concern that, due to the similar size of Sas3HA and Hsl7TAP, the αHA antibody was recognizing the TAP tag instead of HA, giving the appearance of an interaction. This cannot be the case, however, since Hsl7TAP alone (lane 2) registers a strong signal with PAP antibodies but no signal is seen when probed with αHA antibodies. Thus, we have putatively verified that Sas3p and Hsl7p are interacting with each other using the TAP tag pull-down.
Figure 3.4 Hsl7p Interacts with Sas3p in vivo

Performed Hsl7TAP pull-down assay with the yeast strains YDG039 (SAS3HA), YLH237 (HSL7TAP), and YDG040 (SAS3HA HSL7TAP). Western blot analysis with immunodetection by anti-HA (Sas3HA), or peroxidase-antiperoxidase (Hsl7TAP) antibodies was performed with the resulting samples. Whole cell extracts were normalized for bulk protein concentration by Bradford assay.
3.3.3 Hsl7p does not Regulate Sas3p Binding to Chromatin

Recently it has been shown that Sas3p binds chromatin and that this interaction is dependent on methylation of the H3 tail by Set1p and Set2p (64). As mentioned earlier, Hsl7p has been shown to methylate histone H2A (84). Thus, we wanted to test the possibility that methylation of chromatin by Hsl7p also regulates Sas3p’s ability to bind chromatin. To assay this, we monitored the effect an HSL7 deletion would have on NuA3’s ability to bind and acetylate histone H3. We expect that, if Hsl7p were indeed important in this pathway, we would see a reduction in Sas3p’s association with chromatin. To this end, we took a preexisting SAS3HA HTB1TAP (YLH251) strain and knocked out HSL7. With the strain in hand, we then prepared whole cell extracts as described in materials and methods. As controls, we also prepared whole cell extracts of the strains wild type (YLH101), wild type transformed with pSAS3HA (YLH101), and SAS3HA HTB1TAP (YLH251). Whole cell extracts were normalized for bulk protein by Bradford assay and then pull-downs were performed using calmodulin beads as described in materials and methods. Western blot analysis was performed on the samples and the blots were probed with PAP (anti-TAP), αHA (anti-HA) and anti-acetyl H3 antibodies. The anti-acetyl H3 antibodies control for the possibility that only the histone H2B/H2A dimer is being pulled down in the assay, instead of whole nucleosomes. Figure 3.5 shows that the amount of Sas3HA pulled down in hsl7Δ or wild type backgrounds were very similar (compare lanes 4 and 3). The PAP antibody verifies that the samples have been properly normalized (compare levels of Htb1TAP in lanes 3 and 4). In addition, the level of histone H3 acetylation is consistent for both of these strains and verifies that nucleosomes, and not just Htb1TAP, are being pulled down by the assay. We repeated this experiment three times with similar results. From this data, we can see that deleting HSL7 does not affect Sas3p’s ability to associate with the nucleosome; however, we cannot safely conclude that deleting HSL7 does not affect the levels of H3
Figure 3.5 Deletion of HSL7 does not Affect NuA3’s Interaction with Chromatin

Performed Htb1TAP pull-down assay with the yeast strains YLH101 (wild type), YDG039 (SAS3HA), YDM032 (SAS3HA HTB1TAP), and YDG068 (hsl7ΔSAS3HA HTB1TAP). Western blot analysis with immunodetection by anti-HA (Sas3HA), or peroxidase-antiperoxidase (Htb1TAP) antibodies was performed with the resulting samples. WCEs were normalized for bulk protein concentration by Bradford assay. In addition, samples were normalized for histone H3 levels by immunoblotting with histone H3 anti-carboxyl terminus antibodies (anti-acetyl H3).
acetylation since Gcn5p performs the bulk of the acetylation recognized by this antibody, and could potentially mask any changes seen here. It would be potentially interesting to perform this experiment again in a gcn5Δ strain background. Thus, from this assay, it does not appear that Hsl7p is required for NuA3 to bind chromatin.

3.3.4 Loss of Hsl7p does not Show Genetic Interaction with loss of NuA3 in vivo

The biochemical analysis showed that Hsl7p interacts with Sas3p but is not required for NuA3 to bind chromatin. We then attempted to assess whether HSL7 shares any genetic interactions with SAS3. Loss of Sas3p or other components of NuA3 rescues a mating silencing defect of the HMR locus caused by mutations in the Rap1p and Abf1p binding sites (49, 51). If Hsl7p is indeed required for NuA3’s function, it should also rescue the mating silencing defect. To test this, HSL7 was knocked out of a strain with a defect in the HMR locus (YDG111). A mating assay was performed as described in materials and methods for this strain, along with HMR-E-a** (YLH164), and sas3ΔHMR-e-a** (YLH165). Clearly, a strain with an HSL7 deletion does not restore the HMR silencing defect to any extent compared to the control (see Figure 3.6A). A deletion in SAS3, however, successfully restores mating efficiency. This result suggests that Hsl7p is not required for NuA3 function since an hsl7AHMR-e-a** mutant does not recapitulate the phenotype seen in the sas3ΔHMR-e-a** mutant. However, it is possible that the essential function of NuA3 in a gcn5Δ background is not involved in mating.

The above result was puzzling since loss of HSL7 is reported to be synthetically lethal with deletion of GCN5 (Pillus, L. unpublished data). To address this seeming discrepancy, we attempted to replicate the previous data. HSL7 was knocked out of a wild type strain (YLH101) and then mated against a gcn5Δ strain (YLH105). We then transformed the diploids with the plasmid pGCN5HA.416 and subjected them to random spore analysis. Using this technique, we managed to create the strain hsl7A gcnc5ΔpGCN5HA.416 (YDG67-A). This strain, along with
$gcn5\Delta$ (YDG084) and $gcn5\Delta sas3\Delta$ (YDG080) were spotted onto YPD (control) and SC with 5-FOA media and incubated at 30°C for 3 days. As seen in Figure 3.6B, we did not reproduce the previous findings that $hsl7\Delta gcn5\Delta$ is synthetically lethal since this strain is healthy on both control and 5-FOA media.

Before concluding that $HSL7$ deletion is viable in a $gcn5\Delta$ background, we created the $HSL7\ GCN5$ double deletion using an alternative approach. The strain YLH105 ($gcn5\Delta$) was transformed with pGCN5HA.416 and then $HSL7$ was knocked out of this strain. Repeating the above experiment with this newly made strain (YDG067-B), we reproduced our previous results (see Figure 3.6C). [Please note: the differences in growth seen between Figure 3.6B and C is due to different incubation periods of the plates (see figure legend for details)]. Thus, two independently made $hsl7\Delta gcn5\Delta$ strains are viable in our strain background, contrary to findings by L. Pillus. Therefore, although we were able to confirm an interaction between Sas3p and Hsl7p, the functional relevance of this interaction is not known since we were unable to show a requirement of $HSL7$ for NuA3 function in vivo using three independent assays.
Figure 3.6 HSL7 Deletion Does not Share Phenotypes with Loss of NuA3

(A) Deleting HSL7 does not silence mating defects in strains with HMRa-e** mutations. Tenfold dilutions of the yeast strains YLH164 (HMRa-e**), YLH165 (sas3ΔHMRa-e**), and YDG111 (hsl7ΔHMRa-e**) were spotted onto YPD medium and incubated at 30°C for three days. This plate was then replica plated onto a mating tester strain of opposite mating type (YLH101) that was previously spread onto YPD and allowed to dry on the bench for one hour. As a control, the plate was also replica plated onto YPD lacking a mating tester strain. After three days of growth at 30°C, replica plated both mating and non-mating conditions onto YPD (control), diploid selection media (ADE-TRP) and incubated these plates at 30°C for three days.

(B) HSL7 GCN5 deletions are viable. Tenfold dilutions of the yeast strains YDG084 (gcn5Δ), YDG080 (gcn5Δsas3Δ), and YDG067-A (gcn5Δhsl7Δ) or (C) YDG067-B (gcn5Δhsl7Δ) containing the plasmids indicated were plated onto YPD medium (control), or SC medium with 5-FOA and incubated at 30°C for (B) 3 days or (C) 4 days.
3.3.5 Rlf2p Shows Interaction with Sas3TAP but not NuA3 in vivo

Rlf2p (Rap1 localization factor 2) is the largest subunit of the highly conserved CAF-I complex (chromatin assembly factor I), a complex responsible for assembling newly synthesized histones into chromatin of recently replicated DNA (87). Rlf2p was picked out of a screen for mutations defective in Rap1p localization (88). Deleting RLF2 leads to silencing defects in the HMR loci and seriously disrupts inheritance of silent chromatin. Rlf2p is thought to bind diacetylated histone H3/H4 tetramers and position them into newly replicated silent loci (87, 89, 90).

The association of Rlf2p with chromatin made this 2-hybrid hit interesting considering the similar role it shares with NuA3 in the cell. The first step in verifying the hit was to reproduce the interaction between Rlf2p and Sas3p biochemically using the TAP tag pull-down technique. To this effect, a preexisting RLF2TAP (YLH240) strain was transformed with a LEU2 based plasmid carrying a copy of SAS3HA (pSAS3HA). Whole cell extracts of this strain, as well as the control strains RLF2TAP alone (YLH240) and wild type (YLH101) transformed with pSAS3HA, were then prepared as described in the materials and methods. We then performed pull-downs using calmodulin beads and western blot analysis probing with anti-TAP (PAP) and anti-HA (α-HA) antibodies, as described in materials and methods. Whole cell extracts were normalized for bulk protein concentration by Bradford assay. Figure 3.7A shows that Sas3HA interacts with Rlf2TAP in vivo compared to control (compare lanes 1 and 3). Again, due to the similar sizes of Rlf2p and Sas3p there was some concern that the anti-HA antibody was recognizing the TAP epitope tag and not Sas3HA. However, this is not the case, since an RLF2TAP strain lacking pSAS3HA fails to produce a signal at the proper location on the gel (see lane 2). Thus, we have verified the 2-hybrid data showing Rlf2p interacts with Sas3p in vivo. The next step was to examine if Rlf2p is necessary for proper function of NuA3.
In an attempt to explore the possibility that Rlf2p can interact with NuA3, we tested Rlf2p’s ability to interact with the NuA3 subunit Ntolp. The reasoning behind this experiment is that the subunits of NuA3, including Ynglp, Nto1p, and Sas3p, all interact with each other, and pulling down one protein ensures bringing down the rest (Howe, L. personal communication). Thus, we would expect Rlf2p to interact with Nto1p if it is indeed part of the essential pathway. To test this, the HA tag was integrated into the C-terminus of NTO1 in a wild type strain (YLH102), creating NTO1HA (YDG075). We then mated this strain against the preexisting strain RLF2TAP (YLH240). The diploids were subjected to random spore analysis and an NTO1HA RLF2TAP strain was isolated (YDG079). Whole cell extracts of the strain, as well as the control strains, wild type (YLH101), NTO1HA (YDG075), and NTO1HA SAS3TAP (YLH179) were prepared as described in materials and methods. We then performed pull-downs using calmodulin beads followed by western blots probing with anti-HA (αHA) and anti-TAP (PAP) antibodies, as described in materials and methods. As a further control, whole cell extracts were loaded directly onto the western. All whole cell extracts were normalized for bulk protein concentration using a Bradford assay. As expected, the positive control (Sas3TAP) shows a strong interaction with Nto1HA (see lane 4 of Figure 3.7B). Rlf2TAP, however, fails to pull-down any Nto1HA signal and is comparable to the negative control (compare lane 2 and 3). The anti-bodies failed to detect a presence of Nto1HA in the whole cell extracts. Thus, we cannot rule out the possibility that Nto1HA degraded in the Rlf2TAP strain and not the Sas3TAP strain. Furthermore, it is possible that the Nto1HA or the Rlf2TAP tags disrupt Nto1p-Rlf2p interaction. Therefore, it appears that Rlf2p does not interact with NuA3 but we cannot definitively rule out this interaction.
Figure 3.7 Rlf2p Interacts with Sas3HA but does not Interact with Other Components of NuA3 or Share Phenotypes with Loss of NuA3

(A) Rlf2TAP interacts with Sas3HA. An Rlf2TAP pull-down assay was performed with the yeast strains YDG039 (SAS3HA), YLH240 (RLF2TAP), and YDG042 (SAS3HA RLF2TAP). Western blot analysis with immunodetection by anti-HA (Sas3HA), or peroxidase-antiperoxidase (Rlf2TAP) antibodies was performed with the resulting samples. WCEs were normalized for bulk protein concentration by Bradford assay. (B) Rlf2TAP does not interact with Nto1HA. A Sas3TAP and NtolTAP pull-down assay was performed with the yeast strains YLH101 (wild type), YDG075 (NTO1HA), YDG079 (NTO1HA RLF2TAP), and YLH179 (NTO1HA SAS3TAP). Western blot analysis with immunodetection by anti-HA (Nto1HA), or peroxidase-antiperoxidase (Rlf2TAP and Sas3TAP) antibodies was performed with the resulting samples. WCEs were normalized for bulk protein concentration by Bradford assay. In addition, yeast whole cell extract was blotted for HA (Input). (C) RLF2 does not share genetic interactions with NuA3. Tenfold dilutions of the yeast strains YDG084 (gcn5Δ), YDG080 (gcn5Δsas3Δ), and YDG071 (gcn5Δrlf2Δ) containing the plasmids indicated were plated onto YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 4 days.
3.3.6 Loss of RLF2 does not Show Genetic Interaction with Loss of NuA3 in vivo

Having shown that Rlf2p interacts with Sas3p but not NuA3 in vivo, we now set out to see if loss of RLF2 shares any genetic phenotypes associated with loss of NuA3. As previously shown herein, loss of components of the NuA3 pathway is synthetically lethal with deletion of GCN5. If Rlf2p is part of the NuA3 pathway, it is predicted that deletion of RLF2 should also be synthetically lethal with deletion of GCN5. A gcn5Δrlf2ΔpGcn5HAA.416 (YDG071) mutant was created by knocking out RLF2 from a preexisting gcn5Δ mutant (YLH105) transformed with a URA3 based plasmid carrying wild type GCN5. Ten fold dilutions of this mutant, along with the controls gcn5ΔpGcn5HAA.416 (YLH105) and gcn5Δsas3ΔpSas3.416 (YLH112), were plated onto YPD (control) and SC with 5-FOA medium and incubated at 30°C for 4 days. Clearly, loss of RLF2 is not synthetically lethal with loss of GCN5, since it exhibits no growth problems on 5-FOA (see Figure 3.7C). As expected, SAS3 GCN5 double deletions show no growth on 5-FOA, indicating that the assay is working properly. Hence, although Rlf2p exhibited interaction with Sas3p using biochemical techniques, loss of RLF2 clearly does not share a genetic phenotype with loss of NuA3.

Thus, we performed a yeast 2-hybrid screen directed toward the region of Sas3p essential in rescuing the gcn5Δsas3Δ synthetic lethality and generated several potential hits including Hsl7p and Rlf2p. We confirmed that Hsl7p and Rlf2p interact with Sas3p in vivo using the TAP tag methodology described in materials and methods. We showed that Hsl7p’s reported methyltransferase ability was not required for NuA3’s interaction with chromatin and that Rlf2p was unable to interact with components of NuA3. In addition, loss of HSL7 or RLF2 was not shown to recapitulate any phenotypes associated with loss of NuA3. Thus, both biochemical and genetic data suggest that Rlf2p and Hsl7p interact with Sas3p in vivo but are not required for NuA3 function.
3.4 The Essential Function of Gcn5p

3.4.1 Gcn5p Exhibits Two Distinct Functions in the Cell

Gcn5p is the catalytic subunit of several yeast HAT complexes including SAGA, ADA, and SALSA and is the most well studied yeast HAT (40-43). Alone, Gcn5p lacks the ability to acetylate nucleosomes but can perform this function upon incorporation into a complex (39, 40). Disrupting the adapter proteins Ada2p or Ada3p effectively reduces acetylation levels of all residues known to be targeted by Gcn5p in the cell to the same extent as deleting GCN5 (35). Furthermore, mutations that are synthetically lethal with loss of GCN5 are also synthetically lethal with loss of ADA2 and ADA3 (54). These observations all suggest that Gcn5p’s function relies on its ability to incorporate into one of these HAT complexes.

Previously, an exception to this trend was discovered in the fact that loss of SAS3 is synthetically lethal with deletion of GCN5 but not ADA2 or ADA3 (91). This synthetic lethality is intriguing because it suggests that the synthetic lethality is independent of the HAT complexes SAGA, ADA, SLIK/SALSA, and HAT-A2. Due to the importance of this finding on the current research, it was necessary to reproduce this result. The yeast strains YLH170 (sas3Δ) and YLH146 (sas3Δada2Δ) were transformed with a URA3 based plasmid carrying a wild type copy of SAS3 (pSAS3.416). Tenfold dilutions of these strains, along with YLH356 (sas3Δgcn5ΔpSAS3.416), were then plated onto control (HIS'), 50 mM 3-AT (3-Amino 1,2,4, Triazole), and SC with 5-FOA medium and incubated at 30°C for 3 days. When plated on SC with 5-FOA, strains with deletions of SAS3 or double deletions of SAS3 and ADA2 exhibited growth, while SAS3 GCN5 double deletions were inviable (see Figure 3.8A). Thus, we reproduced the previous result showing a genetic interaction between SAS3 and GCN5 independent of ADA2, and by extension, SAGA. As a control, we show that yeast strains with
deletions in either \textit{ADA2} or \textit{GCN5} could not survive on 3-AT, ensuring that disrupting these proteins disrupts SAGA function equally. 3-AT acts to inhibit His3p leading to a decrease in overall histidine production. This medium is useful in studying SAGA related phenotypes since SAGA is required for activation of the \textit{HIS3} gene (92). Low levels of His3p production results from disrupting the SAGA complex leading to reduced levels of histidine production. Normally, these cells can still survive on histidine dropout medium, but the presence of 3-AT in the medium inhibits the already reduced levels of His3p, thus SAGA mutants fail to grow on 3-AT media (92). Taken together, these results suggest that Gcn5p has two functions in the cell, one reliant on SAGA, SLIK/SALSA, ADA, HAT-A2, and the adaptor proteins Ada2p and Ada3p, and the other function independent of these Gcn5p dependent HAT complexes.

Loss of Gcn5p does not appreciably disrupt the SAGA complex, yet it eliminates HAT activity completely (93). Thus, one possible explanation for why \textit{sas3A\textit{gen5A}} strains are synthetically lethal is that a HAT defective SAGA complex (and/or other Gcn5p dependent HAT complexes) may interfere with some other event in the cell and loss of this event is synthetically lethal with loss of \textit{SAS3}. For example, in a \textit{sas3A\textit{gen5A}} strain, residual SAGA may bind to the H3 tail, blocking other H3 HATs (i.e. mediator, and Elongator complexes) from acetylating the H3 tail lysine residues. This could potentially suppress transcription of vital genes, killing the cell. The SAGA complexes of \textit{sas3A\textit{ada2A}} or \textit{sas3A\textit{ada3A}} strains, on the other hand, may not be sufficiently intact to bind the H3 tail effectively, leaving it exposed for modification by other HAT complexes, resulting in normal growth. If this hypothesis were true, we would expect that deleting \textit{ADA2} or \textit{ADA3} in a \textit{sas3A\textit{gen5A}} background would eliminate residual SAGA complexes binding to H3 tails, and effectively restore growth. To create the \textit{ada3A\textit{gen5A}sas3A\textit{pSAS3.416}} triple knockout strain, we mated an \textit{ada3A} strain with YLH112 (\textit{gen5A}sas3A\textit{pSAS3.416}), and screened for diploids using replica plating and mating tester strains. Then, we subjected the diploids to random spore analysis and identified the strains using...
replica plating onto selective media followed by PCR screening. Tenfold dilutions of this strain, along with the controls YLH101 (wild type), YLH112 (gcn5Δasas3ΔpSAS3.416), YLH151 (ada3Δsas3ΔpSAS3.416), and YDG029 (ada3Δgcn5ΔpGCN5HA.416) were plated onto YPD (control) or SC with 5-FOA medium and incubated at 30°C for 4 days. Figure 3.8B shows that ada3Δgcn5Δsas3Δ strains display growth slightly greater than the negative control (compare to YLH112) and greatly reduced growth compared to an either ada3Δgcn5Δ or ada3Δsas3Δ. If the interference effect hypothesis were true, we would expect the ada3Δgcn5Δsas3Δ triple deletion strain to exhibit growth comparable to an ada3Δsas3Δ strain, since loss of Ada3p would effectively disrupt the SAGA complex, eliminating its interference effect with Sas3p. Clearly, this is not the case. Thus, the synthetic lethality does not occur due to an interference effect caused by a defective SAGA, SLIK/SALSA, ADA, or HAT-A2 complex.

To test the hypothesis that Gcn5p has multiple functional roles in the cell, we attempted to construct a Gcn5p mutant that could rescue the gcn5Δasas3Δ synthetic lethality but not a SAGA specific phenotype. Previously, it was shown that GCN5 mutants expressing amino acids 1 to 261 retain HAT activity comparable to wild type but do not co-precipitate with Ada2p, and thus, is not incorporated into any of the Gcn5p dependent HAT complexes (see Figure 3.9A) (38). Using this information, we cloned a GCN5 truncation mutant expressing only the first 262 amino acids and placed the mutant on a low copy plasmid (pgcn5A262). The strain DG081 (gcn5Δasas3ΔpSAS3.416) was transformed with the mutant plasmid or the controls, wild type GCN5 (pGCN5HA.314) or vector alone (pRS314). Tenfold dilution of the strains were plated onto control (HIS'), 50 mM 3-AT, or SC with 5-FOA and incubated at 30°C for 3 days. Figure 3.9B shows that strains transformed with either plasmids pGCN5HA.314 or pgcn5Δ262 could rescue growth on 5-FOA. We expected the slower growth of strains expressing the gcn5Δ262 truncation compared to wild type GCN5 seen here because loss of SAGA causes slow growth on minimal media. However, wild type pGCN5HA.314 rescues growth on 50 mM 3-AT, while
**Figure 3. 8 The sas3Δgcn5Δ synthetic lethality is independent of ADA2**

(A) Tenfold dilutions of the yeast strains YLH170 (sas3Δ), YLH356 (sas3Δgcn5Δ), and YLH146 (sas3Δada2Δ) transformed with the indicated plasmids were plated on synthetic histidine dropout medium, synthetic histidine dropout medium with 50 mM 3-AT, or SC medium with 5-FOA and incubated at 30°C for 3 days. (B) Interference by a defective SAGA complex does not cause sas3Δ synthetic lethality. Tenfold dilutions of the yeast strains YLH101 (wild type), YLH112 (gcn5Δ sas3Δ), YLH151 (ada3Δsas3Δ), YDG029 (ada3Δgcn5Δ), and YDG030 (ada3Δsas3Δgcn5Δ) transformed with the indicated plasmid were plated on YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 4 days.
pgcn5Δ262 cannot. This data seems to suggest that Gcn5p’s N-terminus is essential for performing the essential function shared with NuA3, while the C-terminus is essential for performing SAGA specific functions. However, it could be argued that rescuing the synthetic lethality requires less SAGA than overcoming the inhibiting effects of 3-AT, and that the Gcn5Δ262p truncation can partially rescue SAGA function. This would argue against a dual function of Gcn5p in favour of a single, dose dependant function.

To address this question, we subcloned the gcn5Δ262 mutation from the low copy plasmid (pRS314) into a high copy plasmid (pRS424), and repeated the above experiment. There are approximately 20 high copy plasmids per yeast cell (66) opposed to one low copy plasmid (65), ensuring higher levels of protein expression from the former. As seen in Figure 3.9C, the high copy plasmid gave comparable results to that of the low copy mutant on 5-FOA and 3-AT. A notable difference is the fact that the high copy Gcn5Δ262p mutant rescued growth on 5-FOA better than high copy Gcn5p while the low copy Gcn5Δ262p mutant rescued growth on 5-FOA worse than low copy Gcn5p. This discrepancy could be explained by a possible stability effect. If the truncation mutant is less stable than wild type, then strains carrying a low copy plasmid may not contain enough Gcn5Δ262p to rescue growth completely. Strains carrying high copy plasmids, however, would produce enough Gcn5Δ262p to overcome the limiting effects of protein instability and restore growth completely. The fact that the mutant restores growth better than wild type is interesting but difficult to explain. Perhaps the truncation is more efficient than wild type at performing its role, and/or, the C-terminus of Gcn5p is associated with a repressive functionality. Regardless, it is clear that increasing the plasmid copy number effectively restores growth on 5-FOA but not 3-AT, strengthening the argument that Gcn5p has two independent roles in the cell.

After we successfully constructed a GCN5 mutation that rescues the synthetic lethality but not the SAGA specific phenotype, we wished to create a GCN5 mutation that could perform
the opposite. Isolating a GCN5 mutant that rescues the SAGA specific phenotype but not the gen5Δsas3Δ synthetic lethality would further support the notion that Gcn5p has two distinct functions. Moreover, the existence of this mutant would strongly argue against the possibility that overcoming the 3-AT phenotype requires more intact Gcn5p than does the synthetic lethality. Previously, we showed that Gcn5p’s C-terminus is required for rescuing the SAGA specific phenotype. Using this information, we constructed two N-terminal truncations of Gcn5p and cloned them into TRP1 based plasmids. The first truncation expresses amino acids 94-439 (pgcn5Δ94N) and has previously been reported to interact with SAGA and exhibit normal growth in vivo (38, 45). The second truncation expresses amino acids 170-439 (pgcn5Δ169N), contains a deletion in the HAT I domain, and is reported to exhibit impaired growth and to not interact with SAGA (45). The strain YDG080 (gen5Δsas3ΔpSAS3.416), transformed with a URA3 based plasmid carrying wild type SAS3 was simultaneously transformed with TRP1 based plasmids carrying either of the GCN5 N-terminal truncations, vector alone (pRS314), or wild type GCN5. Tenfold dilutions of the strains were plated onto control (HIS'), 50 mM 3-AT, or 5-FOA and incubated at 30°C for 3 days. Strains carrying the pgcn5Δ94N plasmid exhibited growth similar to wild type on both 50 mM 3-AT and 5-FOA (see Figure 3.9D), suggesting that the region between aa 1-94 appears dispensable for both functions of SAGA. Strains expressing the pgcn5Δ169N plasmid, on the other hand, grew comparable to vector alone on both media. The fact that the pgcn5Δ169N did not mimic pgcn5Δ262’s phenotypes was interesting since neither associates with SAGA components but both retain their catalytic sites. This suggests that Gcn5p’s N-terminus plays an important role in the NuA3/Gcn5p pathway. However, it is possible that pgcn5Δ169N is not expressed in vivo since previous researcher shows that strains carrying this mutation display impaired growth, but we see almost zero growth (45). Regardless, a Gcn5p truncation mutant that rescues a SAGA specific phenotype and not the sas3Agcn5Δ synthetic lethality was not recovered using this method.
Figure 3.9 The Essential Function of Gcn5p is Independent of SAGA

(A) A schematic diagram of Gcn5p depicting the location, in terms of aa, and names of known structural motifs and domains (38). (B) Gcn5p lacking an Ada2p binding domain rescues the synthetically lethal phenotype but not a SAGA phenotype. Tenfold dilutions of the yeast strains YDG081 (sas3Δgcn5Δ), transformed with the indicated low copy (314) or (C) high copy (424) plasmids were plated on synthetic histidine dropout medium, synthetic histidine dropout medium with 50 mM 3-AT, or SC medium with 5-FOA and incubated at 30°C for 3 days. (D) A GCN5 mutation that rescues the SAGA specific phenotype but not the sas3Δgcn5Δ synthetically lethal phenotype could not be created. Tenfold dilutions of the yeast strain YDG080 (gcn5Δsas3Δ) transformed with the indicated plasmids were plated on synthetic histidine dropout medium (HIS'), synthetic histidine dropout medium with 50 mM 3-AT, or SC medium with 5-FOA and incubated at 30°C for 3 days.
3.4.2 Ada2p Functions Independent of Gcn5p

Previous research shows that gcn5A and yng2A strains are sensitive to the DNA damage inducing agents, such as UV, MMS, and high concentrations of hydroxyurea (52). Hydroxyurea is a potent inhibitor of ribonucleotide reductase, the enzyme responsible for de novo generation of deoxyribonucleotides (dNTPs). Hydroxyurea depletes the pool of available nucleotides inhibiting DNA synthesis but at higher concentrations leads to double strand breaks (94, 95). The researchers propose that H3 and H4 acetylation could have overlapping roles in DNA damage repair. Furthermore, in this study we tentatively show that mutations in mitochondrial DNA suppress the gcn5Asas3A synthetic lethality, suggesting that Gcn5p and/or Sas3p are involved in repairing DNA damage resulting from the production of free radicals (DNA damaging agents).

We wanted to establish whether the involvement of Gcn5p in DNA repair was dependent on the Gcn5p dependent HAT complexes. To this effect, tenfold dilution assays were then performed on YDG102 (wild type), YDG103 (ada2A), YDG100 (gcn5A), and YDG104 (gcn5A262) and plated on control medium (HIS'), 50 mM 3-AT, or 200 mM hydroxyurea (HU) and incubated at 30°C for 4 days. [Please note: in order for the results to be comparable on 3-AT, all of these strains were made HIS3+ either by integration of the HIS3 gene, or by using PCR and high efficiency transformation protocol to recover the HIS3 mutation engineered into our strain background]. We were unable to repeat the previously published observation that gcn5A strains exhibit impaired growth on 200 mM HU (see Figure 3.10) (52). Unexpectedly, we showed that ada2A strains display a growth defect on this medium. The gcn5Ada2A strain displays a slightly more severe growth defect than ada2A alone. The synergistic effect seen in gcn5Ada2A strains on HU supports the idea that Ada2p has a function independent of Gcn5p. Furthermore, the fact that a gcn5A262 mutant, (a mutation that disrupts Gcn5p-Ada2p
Figure 3.10 Ada2p has a function that is independent of Gcn5p

Tenfold dilution assay of yeast strains YDG102 (wild type), YDG103 (ada2Δ), YDG100 (gcn5Δ), YD098 (gcn5Δada2Δ), and YDG104 (gcn5Δ262) were plated on synthetic histidine dropout medium, synthetic histidine dropout medium with 50 mM 3-AT, or SC medium with 200 mM hydroxyurea (200 mM HU) and incubated at 30°C for 4 days.
interaction), grows normally on HU suggests that Ada2p does not require interaction with Gcn5p to perform this function. None of the mutants grew on 3-AT media, showing that they share the SAGA specific phenotype. These results are consistent with the previous observation that ADA2 deletions display severe growth defects when plated onto plates containing MMS, a DNA damaging agent that causes similar damage as hydroxyurea, while GCN5 deletions grow normally (96). Thus, the data seems to suggest Gcn5p and Ada2p have two different roles in response to DNA damage and that Ada2p has a function that is independent of Gcn5p.

3.4.3 Gcn5p’s Essential Function is not Acetylating Histone H3

Now that we have established that Gcn5p’s essential function in a sas3A background is independent of the SAGA, ADA, SLIK/SALSA, and HAT-A2 complexes, we wanted to address whether this function is also independent of Gcn5p’s ability to acetylate the histone H3 tail. Substantial evidence supports the observation that SAGA acetylates the histone H3 tail in vivo (34, 40, 41, 97). If the H3 tail were Gcn5p’s essential substrate, then deleting this substrate would be like deleting GCN5. Thus, it follows that a histone H3 tail with mutations in the lysine residues targeted by Gcn5p would be synthetically lethal in a sas3A background. The majority of researchers show that Gcn5p acetylates the histone H3 lysine residues 14, 9, 18, and 23 (in order of preference) (23, 34, 98). However, one researcher, using highly specific antibodies, shows Gcn5p acetylates the histone H3 lysine residues 9, 18, 23, and 27 (in order of preference) but not K14 (24). To account for both of these possibility, the plasmids phht2 K9,14,18,23R or phht2 K9,18,23,27R (described previously herein) were transformed into sas3Ahht2pDM18 (YDG045) containing a URA3 based plasmid carrying wild type HHT2. Figure 3.11 shows that, in a sas3Ahht2A background, mutating either histone H3’s lysine residues 9, 14, 18, and 23 or 9, 18, 23, and 27 to arginine does not impair cell growth (compare to wild type of the same background). This data clearly shows that Gcn5p’s essential role in a sas3A background is not
acetylation of histone H3. This observation does not suggest in any way that histone H3 is not a substrate of Gcn5p; only that Gcn5p must have another unknown function in the cell.

Thus, we showed that the sas3Δgcn5Δ synthetic lethality is independent of Ada2p, and consequently, the SAGA, ADA, SALSA/SLIK, and HAT-A2 complexes, and loss of H3 acetylation. First, we repeated previous results showing ada2Δsas3Δ strains are viable. We then expanded on this by showing a Gcn5Δ262p truncation mutant could rescue the sas3Δgcn5Δ synthetic lethality but not a SAGA specific phenotype. We then showed the different responses that ada2Δ and gcn5Δ strains exhibited on media containing high concentrations of HU, further supporting the idea that Gcn5p and Ada2p have separate functions. Lastly, we demonstrated that the H3 tail with mutations in all known sites of acetylation by Gcn5p is viable in a sas3Δ background. These results strongly suggest Gcn5p’s essential function in a sas3Δ background is not acetylating histone H3.
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**Figure 3.11 The $sas3\Delta gcn5\Delta$ Synthetic Lethality is not due to Loss of Histone Acetylation by Gcn5p**

Tenfold dilutions of the yeast strains YLH224 ($hht1\Delta hht2\Delta$) and YDG045 ($sas3\Delta hht1\Delta hht2\Delta$) transformed with the indicated plasmids were plated on YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 4 days.
3.5 Searching for a Potential Substrate of Gcn5p

3.5.1 Anti-Acetyl Western Blots Looking for a Novel Substrate of Gcn5p

Thus far, the evidence supports the idea that Gcn5p’s essential role in the cell is something other than acetylating histone H3. This leads to the inevitable question of what Gcn5p is acetylating. Current research has unveiled numerous and diverse substrates of acetyltransferases that are not histones; for example, transcription factors, α-tubulin, and importin-α (3). Thus, we attempted to discover a non-histone substrate of Gcn5p that functions in the essential pathway. One method for identifying novel components of a complex is to purify proteins using immunoprecipitation and perform mass spec analysis on the resulting samples (49).

We employed the method above using anti-acetyl lysine antibodies to find novel substrates of Gcn5p. To do this, whole cell extracts of the strains YLH101 (wild type), YLH105 (gcn5Δ), and YLH144 (ada2Δ) were prepared and subjected to immunoprecipitation with anti-acetyl lysine antibodies as detailed in materials and methods. We then performed western blot analysis on the resulting samples, probing with anti-acetyl lysine antibodies followed by anti-rabbit antibodies. As a control, we also performed western blot analysis on whole cell extracts. Figure 3.12A and B shows no distinct differences between immunoprecipitates or whole cell extracts of wild type strains compared to either ADA2 or GCN5 deletions. The whole cell extracts gave clear distinct bands while the bands in the immunoprecipitates were difficult to distinguish. Decreasing exposure time for the immunoprecipitate blot increased resolution slightly but effectively reduced the weaker band signals to nothing. Since this method failed to discover acetylation substrates unique to Gcn5p, we did not send purified samples for mass spectrometry analysis. Thus, we abandoned this method of discovering an alternative substrate.
Figure 3.12 Novel Substrates of Gcn5p could not be Isolated by Immunoprecipitation Assay

(A) Immunoprecipitation using anti-acetyl lysine antibodies performed on strains YLH101 (wild type), YLH105 (gcn5Δ), and YLH284 (ada2Δ). Western blot analysis with immunodetection by anti-acetyl-lysine followed by anti-rabbit antibodies was performed with the resulting samples. Normalized WCEs for bulk protein concentration by Bradford assay. (B) Western blot analysis of the WCE of the above samples was performed with immunodetection by anti-acetyl lysine followed by anti-rabbit antibodies. WCEs were normalized for bulk protein concentration by Bradford assay.
3.5.2 Testing known Substrates of Gcn5p for Genetic Interactions

Several studies have shown that Gcn5p has the ability to acetylate several non-histone proteins. These include the histone variant Htz1p (55), and the non-histone chromatin component Sin1p (54). Modification of one of these proteins could possibly be Gcn5p's essential function. Reasoning that if Gcn5p's essential function is acetylating Sin1p or Htz1p, then deleting these substrates would be equivalent to deleting Gcn5p. Thus, a SAS3 deletion combined with deletion of SIN1 or HTZ1 should also be lethal. Figure 3.13A shows that both a gcn5Δhtz1ΔpGCN5HA.416 (YDG093) and a sas3Δhtz1ΔpSAS3.416 (YDG91) strain are viable when plated on SC with 5-FOA medium. Likewise, sin1Δgcn5ΔpGCN5HA.416 (YDG109) and sin1Δsas3Δ pSAS3.316 (YDG108) strains are viable (See Figure 3.13B). Sin1p has been shown to have an antagonizing role in regards to Gcn5p that acetylation may alleviate. Thus, deleting SIN1 would bypass the need for Gcn5p's essential function. Therefore, we would expect a triple deletion of sin1Δsas3Δgcn5Δ [freezer stock not made] to rescue the synthetic lethality. However, the triple deletion failed to rescue the synthetic lethality (see Figure 3.13C). The positive and negative controls worked as expected, arguing against any problems with media used in this experiment. These results suggest that neither of these proteins is Gcn5p's essential substrate in a sas3Δ background.
Figure 3.13 Gcn5p's Essential Function is not Acetylating Htz1p or Sin1p

(A) Loss of HTZ1 does not share genetic interactions with loss of GCN5. Tenfold dilutions of the yeast strains YLH101 (wild type), YDG110 (htz1Δ), YDG091 (sas3Ahtz1Δ), and YDG093 (gcn5Ahtz1Δ) transformed with the indicated plasmids were plated on YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 3 days. (B) Loss of SIN1 does not share genetic interactions with loss of GCN5. Tenfold dilutions of the yeast strains YDG107 (sin1Δ), YDG109 (sin1Δgcn5Δ), YDG108 (sin1Δsas3Δ) transformed with the indicated plasmids were plated on YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 4 days. (C) Loss of SIN1 does not alleviate the gcn5Asas3Δ synthetic lethality. Tenfold dilutions of the yeast strains YLH112 (gcn5Δsas3Δ) and sin1Δgcn5Δsas3Δ [freezer stock not made] transformed with the indicated plasmids were plated on synthetic histidine dropout medium, synthetic histidine dropout medium with 50 mM 3-AT, or SC medium with 5-FOA and incubated at 30°C for 3 days.
3.6 Synthetic Lethality Suppression Screen

3.6.1 Several gss (gen5 sas3 Suppressor) Mutants Generated

Synthetic lethality between proteins is often indicative of their being involved in pathways that buffer each other (85). Often times the proteins are involved in redundant essential pathways but do not interact with each other directly. For this reason, a yeast 2-hybrid screen cannot identify genetic interactions. Our lab wanted to investigate the significance of the gen5Δsas3Δ synthetic lethality further. Previously, others attempted to perform a high copy plasmid suppressive screen looking for proteins that, when over expressed, suppress the synthetic lethality. However, only SAS3 and GCN5 were pulled out of this screen (Howe, L. personal communication). Therefore, we had to take a new approach. Valuable information concerning biological pathways can also be obtained by discovering proteins that suppress a synthetic lethality (85, 99). For example, Gcn5p's role in antagonizing histone and non-histone mediated transcriptional repression was elucidated in part through the discovery that loss of SIN1 suppresses the swilΔgcn5Δ synthetic lethality (54). To this effect, we designed a “suppression of synthetic lethality” screen looking for mutations that suppress the gen5Δsas3Δ lethality.

A psas3TS (temperature sensitive) plasmid was used in this assay as a way to keep cells with a gen5Δsas3Δ genotype alive and undergoing mutagenesis, while retaining the ability to eliminate Sas3p's function quickly. Strains with a GCN5 SAS3 double knockout transformed with psas3TS grew well at the permissive temperature (30°C) but were inviable at the non-permissive temperature (37°C). The plasmid also contains a URA3 marker, allowing selection against it on media containing 5-FOA. In addition, the plasmid contains a wild type copy of the ADE3 gene. As a means to visualize cells that contain the psas3TS ADE3 plasmid, the
gcnsas3A parent strain also has deletions in the adenine biosynthetic pathway genes ADE2 and ADE3. Cells with deletions in ADE2 build up the product phosphoribosylamino imidazole (AIR), which gives the cells a distinctive pink colouration. Deleting the upstream gene ADE3 effectively eliminates the production of AIR, restoring the cells natural colour (100). Thus, parent strains containing plasmids are genetically identical to ade2Δ strain and are pink, while strains that lose the plasmid are white.

For this assay, we created parent strains of both mating types, each possessing the genotype gcnsas3Aade2Aade3A psas3TS ADE3 (YDG007 MATa, YDG008 MATa). To do this, we knocked out ADE2 from a MATa, SAS3 deletion strain (YLH170) using PCR and high efficiency transformation of yeast. At the same time, ADE3 was knocked out of a MATa, GCN5 deletion strain (YLH105) using the same method. We then mated the two strains, transformed the resulting diploids with the psas3TS ADE3 plasmid, and then subjected them to random spore analysis. Colonies were screened by PCR, replica plating onto selective media, and visualization of pink, non-sectoring colonies.

With our strains created, the next step was to discover a method of inducing mutations. We chose spontaneous mutagenesis for several reasons. Primarily, the low mutation rate would decrease the chance of multiple mutations occurring. This would limit the amount of potential contributing mutations per mutant, simplifying analysis. Second, this method is economical and time efficient since no additional reagents need to be purchased or prepared. Lastly, spontaneous mutagenesis is non-toxic, eliminating exposure to harmful mutagens.

Once deciding on spontaneous mutagenesis, we performed the mutant screen using both mating type parent strains as described in methods and materials. Five gss mutants (repressors of synthetic lethality) of MATa mating type and seven gss mutants of MATα were isolated in this screen. As seen in Figure 3.14A and B, each mutant grows better than their respective parent strain on 5-FOA and at 37°C [Please note that the amounts of mutant cells plated is significantly
less compared to the parent strain]. Several mutants grow well under both conditions (gss5, gss9, gss10, gss11, and gss13), while others grow well only at 37°C (gss3, gss4, gss6, gss7, and gss12) or on 5-FOA (gss1 and gss8). It is possible that mutants that grow well only at 37°C are actually psas3<sup>TS</sup> reversion mutants. Reversion mutants could potentially stabilize the temperature sensitive copy of SAS3 on the plasmid allowing for growth at 37°C. In support of this is the fact that these mutants do not grow well on 5-FOA suggesting the strains still require their plasmid to grow. If this were the case, we would expect the strains to be red since the plasmid also contains the ADE3 gene. However, the mutant cells are white at 37°C, suggesting they have indeed lost their plasmid or have acquired a mutation that prevents accumulation of AIR. Those mutants that grow well on 5-FOA but not at 37°C are most likely genuine gss mutants since they can survive without the psas3<sup>TS</sup> plasmid. Furthermore, the strains grow well when streaked, rather than spotted, onto YPD at 37°C. If this were not the case, we would never have isolated these mutants in the first place. Thus, all the mutants, with the possible exception of those that only grow well at 37°C, appear to be bona fide suppressors of synthetic lethality.
**Figure 3.14 gss Mutants Grow at 37°C and on 5-FOA Compared to Parent Strain**

(A) Tenfold dilutions of the yeast strains YLH101 (wild type), the parent strain YDG007 (gcn5Δsas3Δade2Δade3Δ), and the subsequent repressors of synthetic lethality mutants (gss) YDG032 (gss1), YDG034 (gss3), YDG035 (gss4), YDG036 (gss5), and YDG037 (gss6) transformed with the indicated plasmids were plated on YPD medium (control), YPD medium at 37°C, or SC medium with 5-FOA and incubated at 30°C or 37°C for 5 days. (B) YLH101 (wild type), the parent strain YDG008 (gcn5Δ sas3Δade2Δade3Δ), and the subsequent gss mutants YDG046 (gss7), YDG047 (gss8), YDG048 (gss9), YDG049 (gss10), YDG050 (gss11), YDG051 (gss12), and YDG052 (gss13) transformed with the indicated plasmids were plated on YPD medium (control), YPD medium at 37°C, or SC medium with 5-FOA and incubated at 30°C or 37°C for 5 days.
3.6.2 All gss Mutants Isolated are Dominant Mutations

The next step of the screen was to determine which mutants were dominant or recessive. To ensure diploids were homozygous for the \textit{gcn5Asas3A} genotype, mutants were mated against \textit{gcn5Asas3A GSS} strains of the appropriate mating type and transformed with the \textit{URA3} based plasmid pSAS3.416 (YLH112 \textit{MATa}, and YLH115 \textit{MATa}). To select for diploids, we transformed the \textit{GSS} wild type strains with \textit{LEU2} plasmids (mutant strains are \textit{LEU2'} and \textit{TRP1'}, while \textit{GSS} strains are \textit{LEU2'} \textit{TRP1'}) and plated onto \textit{LEU TRP} media. The resulting colonies were verified as being diploid using mating assay tester strains (YLH162 and YLH163) as described in materials and methods. Recessive mutants will not survive on 5-FOA medium since Gcn5p and Sas3p are absent and a wild type copy of \textit{GSS} would suppress the effect of mutant \textit{gss}. Dominant mutations will grow on 5-FOA since the single copy of mutant \textit{gss} will overcome the effect of wild type \textit{GSS} and allow the cell to grow without Gcn5p or Sas3p. As seen in Figure 3.15A and B, all mutants characterized are dominant (compare to parent strains crossed with \textit{GSS} wild type).

Although identifying recessive mutants is much more straightforward, identifying dominant mutants is possible. A genetic library would have to be made out of the \textit{gss} mutant of choice, with gene fragments encoded on \textit{LEU2} carrying plasmids. We would then transform this plasmid library into a \textit{sas3Agcn5Asas3TS} strain and plate it onto \textit{LEU} media at the non-permissive temperature. Cells growing at this temperature must carry a plasmid with a dominant \textit{gss} mutant, suppressing the synthetic lethality. Finally, we would purify the plasmid from yeast and sequence the insert. One serious drawback to this method is the time involved per mutant analyzed. Also, since the mutations are dominant, it is impossible to perform complementation assays on the mutants to eliminate potential mutations in the same genes. However, before beginning such a lengthy procedure, we must verify that the mutations are genetically inherited.
Figure 3.15 All gss Mutants Characterized are Dominant

(A) The parent YDG007 (gcn5Δsas3Δade2Δade3Δ) and gss mutants (YDG032, YDG034, YDG035, YDG036, and YDG037) were mated against wild type YLH112 (gcn5Δsas3ΔpSAS3.416). Tenfold dilutions of the diploid yeast strains were plated onto SC medium (control) or YPD medium with 5-FOA and incubated at 30°C for 4 days. (B) The parent (YDG008) and gss mutants (YDG046, YDG047, YDG048, YDG049, YDG050, YDG051, and YDG052) were mated against wild type YLH115 (gcn5Δsas3ΔpSAS3.416). Tenfold dilutions of the diploid yeast strains were plated onto YPD medium (control) or SC medium with 5-FOA and incubated at 30°C for 5 days.
3.6.3 Two gss Mutants Tested were not Inherited Genetically

The standard method for analyzing whether mutations are genetically inherited is tetrad dissection (61). Tetrad dissections were performed as described in materials and methods. Briefly, two mutants, gss11 and gss12, were selected for tetrad dissections. The diploids of gss11 and gss12 created previously were used here (YDG050 and YDG051, respectively). Because GCN5 deletions have sporulation defects (92, 101), a URA3 based pGCN5HA.416 plasmid was transformed into the diploid strains. Spores were generated and then the tetrads were dissected onto YPD medium. Of those tetrads that produced four competent spores, each spore was subjected to tenfold dilution assays plating on YPD medium (control), synthetic uracil dropout medium, and 5-FOA medium. As controls, wild type (YLH101) and parent strains (YDG008) were also plated. Figure 3.16A and B shows that for both gss11 and gss12, all the tetrads segregated 4:0, which is indicative of non-Mendelian inheritance. Since the mutations are not genetically inheritable, it impossible to characterize the mutation by the genomic library method outlined earlier.

One possible explanation for this phenomenon is the mutation of mitochondrial genes creating petite mutants. This idea is supported by several studies which show that mutations in mitochondrial genes suppress the formation of red pigment in ADE2 deletion strains (100, 102). Furthermore, the strain background we use in our lab contains a deletion in his3d200, which disrupts the adjacent gene PET56. This additional gene disruption results in an elevated frequency of spontaneous petite formation (103). Thus, we explored the possibility that our gss mutants are petites.
Figure 3.16 Two gss Dominant Mutations are not Genetically Inheritable.

(A) YDG050 (gss11) and (B) YDG051 (gss12) were mated against YLH115 (gcn5Δsas3Δ) and the resulting diploids were sporulated and the tetrads were dissected. Tenfold dilutions of three sets of tetrads were plated on YPD medium (control), synthetic uracil dropout medium, or SC medium with 5-FOA and incubated at 30°C for 4 days.
3.6.4 The gss Mutants Isolated Behave like Petite Mutants

Since several of the gss mutants studied segregated 4:0, it is apparent that at least a portion of our mutants are inherited non-genetically. As discussed above, one possible explanation for this result is petite formation. Wild type mitochondria in yeast segregate biparentally, meaning that they segregate approximately 2:2. However, certain petite mutants, when crossed with diploids, "suppress" wild type mitochondria. In these crosses, approximately 85% to >95% of the resulting diploids contain predominately the petite mitochondrial genome (104). Those petites that have >95% suppressivity are termed hypersuppressors (HS). Thus, certain petite mutations, like our gss mutants, are dominant mutations that segregate ~4:0.

To test this possibility, we plated the gss mutants generated onto YPD and petite media (2.4% glycerol). Petite media is a special media that contains no fermentable carbon source, limiting cells to respiratory growth. Petite mutants lack functional mitochondria and cannot perform cellular respiration, and thus, cannot grow on this media. We show that all of the gss mutants generated grow poorly on petite media compared to the parent strain (see Figure 3.17A). This data strongly suggests that our gss mutants are indeed petite mutants.

An alternative explanation for the above finding is that the parent strain contains the plasmid psas3TS.ADE3, while the gss mutants do not. Thus, slow growth on petite media may be derived from lack of Sas3p or Ade3p rather than due to a mtDNA mutation. To address this concern, we transformed each YDG007 and YDG008 derived gss mutant with psas3TS.ADE3, to test for growth on petite media. Immediately apparent was the fact that several of the mutants turned pink after transformation (gss3, gss6, gss7, gss11, and gss12) while others remained purely white. This argues against the fact that gss 3, 6, 7, 11, and 12 are petite mutants since oxidative metabolism is required for AIR production and red colony colour. The transformants,
Figure 3.17 gss Mutants Exhibit Poor Growth on Petite Media

(A) Tenfold dilutions of the yeast strains YLH101 (wild type), the parent strain YDG007 (gcn5Δsas3Δade2Δade3Δ) and the gss mutants YDG032 (gss1), YDG034 (gss3), YDG035 (gss4), YDG036 (gss5), and YDG037 (gss6) or (B), YDG46 (gss7), YDG047 (gss8), YDG048 (gss9), YDG049 (gss10), YDG050 (gss11), or YDG051 (gss12) were plated on YPD medium (control) or synthetic glycerol based medium (petite) and incubated at 30°C for 5 days.
along with the parent strain YDG008, were plated onto YPD (control) and petite media. As seen in Figure 3.18A, those strains that turned red after transformation with $\text{psas}^\text{TS} . ADE3$, with the exception of $\text{gss}7$, grew well on petite media, while those strains that remained purely white after transformation, with the exception of $\text{gss}9$, did not. The fact that the amount of cells plated for $\text{gss}7$ was far less than that of the other strains (see control plate) could explain why this strain appears not to grow on petite media. The latter exception is more difficult to explain since equal amounts of $\text{gss}9$ cells were plated compared to the other strains. Furthermore, none of the other white $\text{gss}$ mutants could grow on petite media as predicted, suggesting that $\text{gss}9$ is not a true petite mutant. Thus, for the most part, white coloured colonies containing the $\text{psas}^\text{TS} . ADE3$ plasmid cannot grow on petite media while red coloured colonies can. We therefore conclude that $\text{gss} \ 8, \ 10, \ and \ 13$ are bona fide petites, whereas $\text{gss} \ 7, \ 9, \ 11, \ and \ 12$ do not appear to be so.

The fact that $\text{gss} \ 7, \ 11, \ and \ 12$ can grow on petite media when transformed with $\text{psas}^\text{TS} . ADE3$ is consistent with their red colouration, indicating that they are not petite mutants. However, this data seems contradictory to the initial observation that all the $\text{gss}$ mutants could not grow on petite media if they are lacking $\text{psas}^\text{TS} . ADE3$ (see Figure 3.17A). It is possible that deleting both $\text{SAS3}$ and $\text{ADE3}$ or even $\text{SAS3}$ and $\text{GCN5}$ results in a glycerol auxotrophy phenotype. However, it seems highly coincidental that both true petites and glycerol auxotrophs can rescue the $\text{sas}3\Delta\text{gcn}5\Delta$ synthetic lethality. Furthermore, the idea that $\text{gss} \ 7, \ 9, \ 11, \ and \ 12$ are glycerol auxotrophs might be true, but it does not address the difference between these strains and their parent strains that allows one to grow in a $\text{sas}3\Delta\text{gcn}5\Delta$ background but not the other. Perhaps these strains rescue the synthetic lethality via an alternative non-Mendelian, dominant mutation. One possibility is prions, infectious protein mutants that propagate an alternative protein folding conformation in the cell (see Discussion for details).
The fact that a portion of our gss mutants are true petites raises the intriguing question of whether a petite mutant can suppress the sas3Δgcn5Δ synthetic lethality. To test this hypothesis, we create a petite mutant from the parent strain YDG008 using an alternative method. Our parent strain was streaked out onto YPD media with 20 μl of ethidium bromide (EtBr) solution spread on top. EtBr is known to stimulate the occurrence of petite mutants (102). Colonies that appeared less red were restreaked onto fresh EtBr media until a white strain was generated. This petite mutant, alongside YDG008 parent and gss9, were plated onto 5-FOA and petite media. [Please note: we chose gss9 as a control before obtaining the conflicting results previously mentioned.] Interestingly, the newly made mutant cannot grow on the glycerol based medium suggesting it is a petite; however, it also could not grow on 5-FOA (see Figure 3.18B). This would seem to suggest that petite mutations do not suppress the synthetic lethality. Thus, we were not able to show definitively that petite mutations alone can rescue the synthetic lethality.

Therefore, we designed and executed a synthetic lethality suppression screen looking for mutations that rescue the sas3Δgcn5Δ synthetic lethality. From this screen, we identified twelve dominant mutations that could survive without a functional copy of either Sas3p or Gcn5p. Two of these mutants were subjected to tetrad analysis (gss11 and gss12) and both mutants were not genetically inheritable. The fact that all the mutants were dominant and at least a portion of which segregated in a non-Mendelian fashion led to the idea that they may be petite mutants. All of the gss mutants grew poorly on glycerol based media, strengthening the petite hypothesis. However, of these twelve gss mutants, seven remained white after transformation with psas3TS.ADE3 (gss 1, 4, 5, 8, 9, 10, 13), while five turned red (gss 3, 6, 7, 11, 12). For the most part, those strains that stayed white after transformation could not grow on petite media, while those that turned red could grow on petite media. Lastly, the petite mutant we created via sequential streaking of the parent strain onto media containing EtBr was a verified petite but could not rescue the synthetic lethality. From these results, we conclude that several of our gss
mutants are petite mutants, but loss of a functional copy of mtDNA alone does not necessarily confer suppression of the \textit{sas3Agen5A} synthetic lethality.
Figure 3.18 Several gss Mutants are Bona Fide Petite Mutants

(A) The mutant strains gss 8, 10, and 13 are bona fide petite mutants. Tenfold dilutions of the strains YLH101 (wild type), the parent strain YDG008 (gcn5Δsas3Δade2Δade3Δpsas3TS.ADE3) and the gss mutants YDG046 (gss7), YDG047 (gss8), YDG048 (gss9), YDG049 (gss10), YDG050 (gss11), YDG051 (gss12), or YDG052 (gss13) were transformed with the indicated plasmid and plated on YPD medium (control) or synthetic glycerol based medium (petite) and incubated at 30°C for 4 days. (B) Petite mutations alone cannot account for suppression of the sas3Agcn5Δ synthetic lethality. A petite mutant derived from the parent strain (YDG008) was created by sequentially streaking the parent strain onto medium containing ethidium bromide and selecting for white colonies. Tenfold dilutions of the yeast strains YDG008 (parent), YDG048 (gss9), and the petite mutant created by sequential streaking (petite parent) were plated onto YPD medium (control), SC medium with 5-FOA, and synthetic glycerol based medium (petite) and incubated at 30°C for 4 days.
Chapter 4 – Discussion

The overall goal of this study was to discover the essential function of two known HATs, Gcn5p and Sas3p. One strong possibility for this function is the modification of the H3 tail, since both Gcn5p and Sas3p have been shown to acetylate this substrate \textit{in vivo} (64, 67). Modifications to the histone tail have been linked to increased transcription and is thought to play a major role in transcriptional regulation (25). The fact that Esa1p, the main histone H4 HAT, is encoded by an essential gene, sets a precedence for the importance of histone modification for cell viability (105, 106). All of these observations suggest that the essential function of Sas3p and Gcn5p is histone modification. However, this does not fit with the fact that neither the H3 nor H4 histone tails nor the lysine residues within them are essential (70, 71).

Another possibility is that these acetyltransferases are acetylating a non-histone substrate and that modification of this substrate is essential for cell viability. Evidence supporting this theory revolves around the fact that deleting \textit{SAS3} in combination with components of SAGA (i.e. \textit{ADA2} and \textit{ADA3}) or other HATs is not synthetically lethal (35, 67). Since the HAT activity of Gcn5p \textit{in vivo} is dependent on its incorporation into the Gcn5p dependent HAT complexes (98), this data strongly suggests that Gcn5p is acetylating a non-histone protein as its essential function (67). The observation that Gcn5p can acetylate non-histone proteins like Sin1p (35) in yeast, and p53 in humans (107, 108) gives validity to this possibility. In this study, we explore the essential function of these acetyltransferase proteins in two parts, the first dealing with Sas3p and NuA3, and the second dealing with Gcn5p.
4.1 The NuA3 Pathway

In this thesis, we have presented several lines of evidence suggesting that NuA3, the H3 tail, and K14 of the H3 tail function in the same essential pathway as Sas3p. First, we have reproduced previously published results showing that disruption of Gcn5p is synthetically lethal with loss of the NuA3 components Ynglp, Nto1p, and Sas3p. This data strongly suggests that the essential function of Sas3p in a gcn5Δ background is dependent on the NuA3 complex as a whole. Next, we repeated the previous observation that loss of the histone H3 tail is synthetically lethal with gcn5Δ (64, 98). We also show that this lethality is specific to the NuA3 pathway, since ada2Δhht2Δ3-29 strains are viable. To eliminate the possibility that loss of the tail was not the same as loss of acetylation, we showed that strains with arginine substitutions in the originally identified acetylatable H3 lysine residues 9, 14, 18, and 23 (4, 23) are inviable in a gcn5Δ background. We then showed an H3 K14R mutation alone was lethal in a gcn5Δ background but viable in an ada2Δ background, suggesting that NuA3's essential function is acetylating H3 K14. This is consistent with previously published work showing that lysine 14 is the predominant substrate of NuA3 in vivo (35, 109). Furthermore, this data is supported by previously published evidence showing a lysine 14 to arginine mutation alone is enough to cause a synthetic lethal phenotype with loss of Gcn5p (64). Recent evidence shows that K27 of histone H3 can also be acetylated (24). Thus, to eliminate the possibility that the other histone H3 residues play a role in the sas3Δgcn5Δ synthetic lethality, we showed that mutation of K9, 18, 23, and 27 is not lethal in a gcn5Δ background. From this, we show that maintaining the ability of NuA3 to acetylate K14, and no other residue, is essential to cell function in the absence of GCN5. It could be argued that other H3 acetyltransferase complexes like Elongator complex also play a role in histone H3 K14 acetylation. However, it seems unlikely that they are acetylating this particular residue in vivo (110, 111) since they cannot compensate for NuA3
loss in a $\textit{GCN5}$ deletion background. Therefore, components of NuA3, the H3 tail, and H3 K14 all function in the same pathway which may have a redundant role with Gcn5p in the cell.

4.2 Yeast 2-Hybrid

Proteins rarely function by themselves; instead, they interact with other proteins to perform essential functions in the cell (79). Discovering an overall protein interaction network is important in understanding how the cell performs its various functions. We wanted to find out more about the NuA3 pathway in hopes of better understanding the greater ramifications of its function in the cell. One method of doing this is discovering novel protein interactions that share similar phenotypes with the NuA3 pathway. We chose to use a yeast 2-hybrid screen because of its rapid and systematic ability to screen a protein of interest against every known ORF (112). The major disadvantage of this method is the occurrence of false positives and negatives.

The screen we used generates an average of 3.3 positives and 1-30 false positives per screen (80). False positives are a relatively minor concern since hits can be verified quickly using biochemical techniques. Furthermore, picking those hits that have a similar role in the cell, shared interactions, or homology with the protein of interest quickly generates a selection of high priority hits. Regardless, screening several false positives to generate even a single bona fide interacting protein is extremely efficient, given the alternatives. False negatives, on the other hand, are more problematic since there is no way to double check if these non-hits are actual interacting proteins. Thus, ruling out potentially useful protein interactions without the researchers’ knowledge.

Before performing our screen, we mapped the essential region of Sas3p to its N-terminus. The truncation sas3p (1-577) rescues the synthetic lethality of $\textit{sas3A}\textit{gcen5A}$ and shows interaction with chromatin. This effectively rules out a role of Spt16p or Chk1p in the essential function. Spt16p is an essential protein that is a component of the CP (Cdc68/Pob3) and FACT
(facilitates chromatin transcriptions) complexes and interacts with the carboxyl-terminal 174 amino acids of Sas3p (49). Chk1p is an evolutionarily conserved protein kinase that plays a role in regulating the metaphase to anaphase transition (in budding yeast) and also interacts with Sas3p’s (77, 78). By using the truncation in our 2-hybrid screen, we limited our search to only those proteins immediately involved in NuA3’s essential function, and avoided interactions like Spt16p, that bind to Sas3p’s non-essential residues. Stanley Fields’ lab performed the yeast 2-hybrid and generated several hits. None of these hits was found to associate with NuA3’s essential pathway by either biochemical or genetic methods. The two hits we focused our research on were Rlf2p and Hsl7p.

4.2.1 **HSL7 may Interact with Sas3p but does not Interact with NuA3**

The fact that the yeast 2-hybrid screen implicated Hsl7p as interacting with Sas3p’s essential terminus was promising because it shares similar phenotypes with Sas3p. For instance, Hsl7p is a verified methyltransferase that has been shown to methylate calf thymus histone H2A in vitro (84). Thus, like Sas3p, Hsl7p potentially modifies histones. However, researchers have not been able to show in vivo methylation of yeast histones or non-histone proteins by Hsl7p, but stipulate that methylation could be occurring at undetectable amounts in the cell (113). Most interesting, however, is the observation that a gcn5Δhsl7Δ strain is synthetically lethal (Pillus, L. unpublished data). This strongly implicates Hsl7p as being part of the NuA3 pathway since it potentially shares genetic phenotypes with every other component of the pathway. Thus, **HSL7** appears to modify histone proteins and share genetic interactions with **SAS3**. Taken together, Hsl7p is a strong candidate for being involved in the NuA3 essential pathway.

Our data shows that Hsl7p was able to interact with Sas3p in vivo but not with any other component of NuA3. We verified the 2-hybrid data showing that Hsl7p and Sas3p interact using the TAP tag methodology. However, we could not show that Hsl7p was required for recruitment
of NuA3 to chromatin. As mentioned previously, the methylation of histone H3 K4 and K36 by Set1p and Set2p, respectively, is essential for Sas3p’s association with chromatin (64). The deletion of HSL7, however, did not affect Sas3p’s ability to bind nucleosomes, ruling out a possible methylation signal catalyzed by Hsl7p that promotes NuA3 binding. Nevertheless, we cannot rule out the possibility that deleting HSL7 affects the actual acetylation of histone H3. We did show that bulk histone acetylation remained the same in hsl7Δ strains compared to wild type; yet, the majority of histone H3 acetylation that is recognized by the antibody used is performed by Gcn5p. Thus, any change in the H3 acetylation due to loss of Hsl7p would be effectively masked by this stronger signal (35). Hence, we cannot conclude definitively that Hsl7p does not affect histone H3 acetylation by Sas3p. In addition, we cannot rule out the possibility that Hsl7p functions downstream of NuA3 binding and H3 acetylation. If this were the case, deleting HSL7 would have no noticeable effect in this assay. Thus, it appears unlikely that Hsl7p functions in the NuA3 pathway, but we cannot rule out this possibility using these biochemical techniques.

Our data shows that an hsl7Δ strain does not share any known phenotypes with disruption of Sas3p or the NuA3 complex. Firstly, an HSL7 deletion, unlike a SAS3 deletion, does not restore silencing to derepressed HMR loci. Other components of the NuA3 pathway have been shown to share this phenotype with sas3Δ, for example set2Δ (64). However, this assay is not a definitive test since other components of the NuA3 pathway, when disrupted, do not share this phenotype, for example set1Δ (64). Secondly, and more importantly, HSL7 deletion is not synthetically lethal with deletion of GCN5 in our strain background. Every other component of the NuA3 pathway studied thus far is synthetically lethal with GCN5, strongly suggesting that HSL7 is not functioning in the NuA3 pathway in an essential way. Although this result conflicts with previous findings (Pillus, L. unpublished results) it is consistent with the finding that HSL7 is synthetically lethal with the histone H3 tail (81). Since one function of the
NuA3 pathway is to acetylate the histone tail, we would not expect to see a synergistic effect when deleting components of NuA3 in combination with the H3 tail. Thus, the fact that \textit{HSL7} deletion is synthetically lethal with the H3 tail deletion argues against its involvement in this pathway. Therefore, we propose that Hsl7p can interact with Sas3p but does not function in the same essential pathway as NuA3.

4.2.2 \textbf{Rlf2p does not Function in the NuA3 Pathway}

Rlf2p/Cac1p (Rap1 localization factor 2/ chromatin assembly factor 1) was a promising candidate for functioning in NuA3's essential pathway. Like \textit{SAS3}, deleting \textit{RLF2} leads to silencing defects in the \textit{HML} loci (87). Furthermore, Rlf2p directly interacts with diacetylated histone H3/H4 tetramers and positions them into newly replicated silent loci (87, 89, 90). Thus, both Sas3p and Rlf2p share the same substrate. These similarities between Sas3p and Rlf2p made Rlf2p a strong candidate for being part of the NuA3 pathway.

We were able to verify the yeast 2-hybrid hit between Rlf2p and Sas3p's essential region but could not show an association between Rlf2p and NuA3. This strongly argues against Rlf2p being part of the NuA3 pathway since every other component of this pathway is dependent on components of NuA3 (49). Furthermore, \textit{RLF2} did not share genetic interactions with \textit{SAS3} since \textit{gcn5\textunderscore A\textunderscore rlf2\textunderscore A} strains are viable. These two lines of evidence argue against Rlf2p's involvement in the essential pathway.

Thus, our yeast 2-hybrid screen did not identify any novel components of the NuA3 pathway. However, we did show that Sas3p alone interacts with Rlf2p and Hsl7p. It would be premature to rule these results out as false positives simply because they failed to interact with NuA3. Sas3p may have a function unrelated to the NuA3 pathway that encompasses Hsl7p and/or Rlf2p. Indeed, a component of FACT, Spt16p, has also been shown to interact with Sas3p but
not with other components of NuA3 (49). Therefore, Hsl7p and Rlf2p interact with Sas3p but not NuA3.

4.3 Gcn5p’s Function is Independent of SAGA

As previously mentioned, numerous multiprotein complexes modify histones in the cell. Two well-characterized modifiers of histone H3 are Gcn5p and Sas3p. In vitro, recombinant Gcn5p alone has been shown to acetylate K14 of free histone H3, however, it lacks the ability to acetylate nucleosomes (39, 40, 44). Incorporation of Gcn5p into a HAT complex, like SAGA or ADA, allows it to acetylate nucleosomes and expands the lysine acetylation specificity of the complex (38, 98, 114). Substantial evidence shows that Gcn5p containing HAT complexes preferentially acetylate lysine residues on the histone H3 tail in vivo (34, 40, 41, 97). Ada2p and Ada3p are two adapter proteins vital in the formation and function of active Gcn5p dependent HAT complexes (32, 38, 45). Indeed, deleting ADA2 or ADA3 is equivalent to deleting GCN5 itself, in terms of the cells ability to acetylate histones (35). The only known exception to this is Gcn5p’s reported role as a type B HAT which acetylates newly synthesized histone H3 and targets it for deposition into DNA (115). However, even this function is dependent on Ada3p. Furthermore, mutants that are synthetically lethal with loss of Gcn5p are also synthetically lethal with loss of Ada2p and Ada3p, for example, Swi1p (54). These observations all suggest that Gcn5p’s ability to acetylate nucleosomes in vivo relies on its incorporation into complexes such as SAGA, SLIK/SALSA, ADA, and HAT-A2.

Important to this study is the possible role of Gcn5p in acetylating a non-histone substrate independent of SAGA or any other Gcn5p dependent HAT complex. Evidence for this role comes from the observation that \textit{gcn5Asas3A} strains are synthetically lethal but \textit{ada2Asas3A} and \textit{ada3Asas3A} strains are viable (35). These results were reproduced in the current study. Since Ada2p is essential for Gcn5p’s association with SAGA and for performing SAGA related...
functions in the cell (45) and the synthetic lethality is independent of Ada2p, it stands to reason
that Gcn5p is performing an essential role independent of SAGA. It is possible to argue that an
interference effect causes the synthetic lethality between loss of GCN5 and SAS3. In other
words, a HAT defective SAGA complex (or ADA, SLIK/SALSA, HAT-A2 complexes) may
interfere with some other event in the cell and loss of this event is synthetically lethal with loss
of SAS3. In strains with an ada2Asas3A or ada3Asas3A background, SAGA would be
sufficiently disrupted to no longer interfere with the proposed crucial event. If this model holds,
an ada3Agcn5Asas3A strain should eliminate the interference of a HAT defective SAGA and the
cell should be viable. However, we showed that disrupting ADA3 along with GCN5 and SAS3
did not significantly restore growth, as predicted by the interference effect model. Therefore,
Gcn5p is performing another role in the cell independent of Ada2p related complexes.

The gcnc5Asas3A synthetic lethality is dependent on the HAT domains of Sas3p and
Gcn5p (35). Therefore, Gcn5p’s essential function is dependent on its acetyltransferase ability.
As mentioned, Gcn5p’s ability to acetylate nucleosomes in vivo is dependent on its interaction
with Ada2p associated complexes (45). This seeming contradiction can be resolved if Gcn5p’s
essential function is acetylating a non-histone protein. Indeed, we were able to create a Gcn5p
truncation (pgcn5A262) that retained HAT activity but could not interact with SAGA. This
truncation mutant rescues the synthetically lethal phenotype but not a SAGA specific phenotype.
Thus, we managed to differentiate the two functions of Gcn5p physically using this mutant. It is
possible that the pgcn5A262 mutant retains a residual level of SAGA association and that this
small amount of functional SAGA is sufficient to rescue the synthetically lethal phenotype but
not the SAGA specific phenotype. If this were true, then increasing the amount of pgcn5A262 in
the cell should also increase the amount of functional SAGA, and consequently, should rescue
the SAGA specific phenotype as well. However, we showed that expressing the pgcn5A262
from a high copy plasmid did not rescue the SAGA specific phenotype. Thus, it seems unlikely
that a dosage effect is occurring here. To address this question further, we attempted to create a GCN5 mutant that rescues the SAGA specific phenotype and not the synthetic lethality phenotype. Finding such a mutant would further differentiate Gcn5p's two functions and definitively prove the dosage effect was not occurring. The fact that we could not isolate this mutant does not necessarily mean a dosage effect is occurring and that Gcn5p function is always related to SAGA. More than likely, this data suggests that both functions of Gcn5p require a full-length copy of the conserved HAT domain. Thus, the HAT domain is required for both functions of Gcn5p while the Ada2p binding domain is necessary for only the SAGA function.

The fact that an ada2Δ strain displays susceptibility to HU media while gcn5Δ and gcn5Δ262 mutants do not (seen in our study) was unexpected since others have shown Gcn5p is susceptible to HU media (52). One possible explanation for this discrepancy is that our plates were incubated at 30°C while their plates were incubated at 25°C. Although gcn5Δ strains do not have any appreciable phenotype at 25°C compared to 30°C, it is possible that the combination of temperature change and the presence of HU causes the reduced growth seen previously. Supporting our data is the observation that ada2Δ strains display reduced growth on MMS media, a DNA damaging agent that causes similar damage as hydroxyurea, while gcn5Δ strains grow normally (96). Furthermore, another group of researchers show that loss of SPT20, a gene encoding a component of SAGA, results in a severe growth defect on HU media while loss of GCN5 does not (93). Thus, this data suggests ADA2 and SAGA are required for DNA damage repair independently of GCN5. Although the above data does not prove Gcn5p has two separate functions, it does provide evidence that Gcn5p is not always functionally linked with Ada2p or the Gcn5p dependent HAT complexes.

Before concluding that Gcn5p has two distinct functions in the cell we must first show evidence that its essential function is not acetylating the histone H3 tail. The bulk of Gcn5p acetylation is proposed to be directed toward the histone H3 lysine residues 14, 9, 18, and 23 (in
order of preference) by the majority of researchers (23, 34, 98). However, one researcher, using highly specific antibodies, showed that Gcn5p does not acetylate the K14 residue, and instead, acetylates residues 9, 18, 23, and 27 (in order of preference). If Gcn5p’s essential role in the cell is acetylating histone H3 on any combination of these residues, it follows that mutating these residues should be synthetically lethal with a SAS3 deletion. We addressed this question by showing a SAS3 deletion is viable in combination with mutating either histone H3’s lysine residues 9, 14, 18, 23 or 9, 18, 23, 27 to arginine. Thus, mutations in all of the proposed H3 residues that Gcn5p acetylates is not synthetically lethal with a SAS3 deletion, strongly suggesting that Gcn5p’s essential function is not acetylating histones. Taken together with previous data showing the synthetic lethality is dependent on the ability of Gcn5p and Sas3p to perform acetylation (35), and with the evidence showing Gcn5p is operating independently of SAGA, ADA, SLIK/SALSA, and HAT-A2, Gcn5p’s essential function is likely the acetylation of a non-histone protein.

4.3 Finding Gcn5p’s Essential Substrate

Since we could not see any difference in acetylation patterns between GCN5 and ADA2 deletion strains via an immunoprecipitation assay using anti-acetyl lysine antibodies, we needed to discover a new method for finding a non-histone subunit of Gcn5p. From researching the literature, two potential candidates shown to be acetylated by Gcn5p in yeast were discovered: Sin1p and Htz1p (54-56).

Sin1p is an HMG1-like protein that can bind four-way junction DNA often found at the entry and exit point of nucleosomes (58). Sin1p is physically associated with the coding regions of actively transcribed genes and not the promoters. Deletion of this non-histone chromatin component results in a decrease of histone H3 associated with the coding regions of genes (58).
This effect only becomes apparent at actively transcribing genes, arguing for a role in maintenance of the chromatin structure of actively transcribed genes. Furthermore, deletion of SINI alleviates the synthetic lethality between ada2Δswi1Δ, ada3Δswi1Δ, and gcn5Δswi1Δ, suggesting a role in antagonizing the activating effects of SAGA and SWI/SNF (54). In other words, Sin1p plays a role in repressing transcription of a gene through reassembling silent chromatin.

As mentioned above, Gcn5p has the ability to acetylate Sin1p in vitro (54). It is possible that acetylation of Sin1p by Gcn5p could block or inhibit Sin1p’s ability to stabilize or reassemble nucleosomes. If Gcn5p’s essential function in the cell is acetylating Sin1p, we would expect a deletion of Sin1p would alleviate the need for Gcn5p. Accordingly, the triple deletion sin1Δsas3Δgen5Δ should be viable since the SINI GCN5 deletions would cancel each other out, leaving the cell with a viable sas3Δ genotype. We show, however, that the SINI deletion does not rescue the sas3Δgen5Δ synthetic lethality. To discount the idea that Gcn5p and Sin1p interact in the same pathway we also prepared the double deletion strains sin1Δsas3Δ and sin1Δgen5Δ. Both of these double deletions were healthy, strongly arguing against a role of Sin1p functioning in concert with Gcn5p to perform an essential function.

Htz1p is the only H2A histone variant in yeast and is deposited into chromatin by the SWR-C complex (116). Current research implicates a role for Htz1p in binding repressed promoters of inducible genes, priming them for activation through subsequent loss of nucleosomes, facilitating the binding of transcription factors (17). Supporting this is the fact that deletion of HTZ1 results in 5% of genes being misregulated (55) and causes a decrease in transcription at repressed promoters which control heat shock genes normally containing Htz1p. This suggests that Htz1p occupancy poises the gene for transcription (17). Recently, a role for acetylation of Htz1p in restricting the spread of silent chromatin has also been discovered. The researchers propose a role for acetylated Htz1 tails, rather than interactions between octamer
cores, in preventing the spread of silent chromatin through the formation of chromatin structure incompatible with Sir protein binding (56). Gcn5p and Esa1p are reported to catalyze nearly all acetylation of Htz1p \textit{in vitro} and \textit{in vivo} (55, 56). Thus, Gcn5p's essential role in the cell could be the acetylation of Htz1p.

Interestingly, Htz1p occupancy is negatively correlated with histone H3 acetylation of K9, K18, and K27 and positively correlated with K14 and K23 acetylation (17). The residues that promote Htz1p occupancy are substrates of NuA3, while those that inhibit Htz1p occupancy are targeted by SAGA. Thus, it seems unlikely that Gcn5p's function is the acetylation of histone H3 to promote Htz1p binding, as proposed in the literature (17). Furthermore, a role for Gcn5p in acetylation of Htz1p to promote its deposition into chromatin by the SWR-C complex has been ruled out by the observation that Htz1p lacks significant acetylation in a \textit{swr1A} mutant (55, 116). This suggests that modification of Htz1p takes place after it is deposited by SWR1 and not before. If acetylating Htz1p were Gcn5p's essential function in the cell, we would expect an \textit{HTZ1 SAS3} double deletion to be inviable. However, neither a \textit{HTZ1 SAS3} nor a \textit{HTZ1 GCN5} deletion strain showed any sickness when we performed this experiment. One explanation for this result is the possibility that loss of Htz1p is not the same as loss of Htz1p acetylation. It could be possible that Htz1p has a repressive effect alleviated by acetylation. If this were the case, we would expect loss of Htz1p to be viable in a sas3Δ background but an unacetylatable Htz1p to be lethal. Thus, we cannot rule out the possibility that loss of Htz1p acetylation is toxic in a sas3Δ strain and that loss of Htz1p is not the same as loss of K14 acetylation.

The fact that we did not discover a genetic relationship between \textit{HTZ1} or \textit{SIN1} with \textit{SAS3} or \textit{GCN5} argues against their being the essential substrate of Gcn5p. However, we cannot rule out the possibility that loss of Htz1p is not the same as loss of acetylation of Htz1p. Furthermore, it is highly possible that Gcn5p acetylates multiple non-histone substrates \textit{in vivo}.
that cumulatively or redundantly perform the essential function. For example, Gcn5p may acetylate both Htz1p and Sin1p and the combined effect of this acetylation increases Htz1p deposition at the promoter and decreases nucleosome reassembly in the coding region, enhancing the expression of an essential gene or several non-essential genes. Therefore, we cannot rule out the possibility that Gcn5p’s essential role involves the acetylation of several non-histone subunits.

4.4 Synthetic Lethality Suppression Screen

Our synthetic lethality suppression screen yielded 12 mutants that could suppress the gcn5Δsas3Δ synthetic lethality, each of which was dominant. Since all of the mutants were dominant, we could not perform complementation grouping analysis to eliminate repeat mutants from the screen. Tetr dissections of two of the mutants revealed that the mutations segregated in a non-Mendelian fashion. All progeny from these dissections displayed the mutant phenotype; therefore, the mutations segregated 4:0. We only performed two tetr dissections since it appeared likely that all of the mutations were the same because they were all dominant mutations. Two possible types of mutations exist that can explain dominant mutations that segregate 4:0 - petites and prions.

4.4.1 gss Mutants are Potentially Petites

Petite mutants have mutations in the mitochondrial genome that disrupt mitochondrial function, namely cellular respiration, and are characterized by their small colony size and white colouration, even in cells with ADE2 deletions (104). Three types of petites exist, those that lack a functional mitochondria (rho⁺), those that have extensive deletions in mitochondria DNA (rho⁻), and those with no mitochondria at all (rho⁰) (102). The mitochondrial genome in yeast is circular and contains ~75 kb of DNA, about five fold larger than that of animal mitochondria.
About half of the genome contains long stretches of short alternating AT:AT and non-alternating A:T sequences (the AT spacer). The GC content of wild type mitochondrial DNA (mtDNA) is less than 5%. Only two genes are present in the mitochondrial genome, COX1 (cytochrome oxidase subunit 1) and CYTB (apocytochrome B), which contain several introns that generate several protein products including maturases, reverse transcriptases, site-specific endonucleases, and the 21S RNA subunit (117). Eight ori sites (origin of replication) dispersed throughout the genome initiate mitochondrial DNA replication. Petite mutants occur frequently (~1% of cells) because of illegitimate, unequal recombination events originating from the high degree of repetitive nucleotide sequences (104, 117). The make up of a petite mutant’s mitochondrial genome consists of tandem repeats of a DNA segment derived from any region of the parental wild type genome. In most cases, the genome contains only an ori and AT spacer DNA due to extensive deletions (117).

Yeast progeny inherit mitochondria bi-parentally, in a non-Mendelian fashion (104). Thus, most cells contain an ~equal distribution of mitochondria from both parents. The mitochondria of petite mutants, however, often have a replicative advantage over wild type mitochondria since they have genomes formed by very short repeat units that retain a functional origin of replication (ori) sequence (104, 117). These petites are termed suppressive since, when they are crossed against wild type strains, their mitochondrial genomes are inherited by progeny at a rate of 85% to >95% (102, 104, 117). The petite’s degree of suppressiveness depends greatly on which ori sequence is contained in its genome. Certain petites with highly efficient ori and very short repeating units (400-900 bp) are called hypersuppressive petites since over 95% of progeny from wild type crosses contain only the petite genome (104, 117). Hypersuppressive petites are the extreme and not the norm. In some cases, cells maintain both rho- and wild type mitochondrial genomes for up to eight generations after crosses against wild type (104). The characteristics of our mutants derived from our synthetic lethality suppression
screen match well with that of petite mutants. Thus, petites share similar characteristics to our gss mutants since they are dominant, and segregate in a non-Mendelian fashion.

Several of our gss mutants displayed different growth rates on 5-FOA media and at 37°C, yet, when crossed with GSS⁺ gcns₅ Δsas₃Δ strains, the diploid progeny all grew well on 5-FOA. If the mutation was in a nuclear gene, it would seem highly unlikely that the mutant would be sick on SC with 5-FOA media in haploid strains, but healthy in diploid strains. Diploids would have a wild type copy of the gene that would be working antagonistically to the suppressive effect of the mutant gene, thus, we would expect diploids to be sicker, or at the very least, exhibit the same level of growth as the haploid. However, if the mutation is in mitochondria, suppressive petites can become the predominant genome in the cell in less than one generation. Thus, a diploid could potentially have the same or higher proportion of petite: wild type genomes as a haploid strain. The growth advantage could also come from wild type copies of ADE2 and ADE3 being present in the genome (100). Therefore, although we did not perform tetrad analysis on all of our mutants, it seems highly unlikely that these mutations are genetically inheritable.

Petite mutants are characterized by the inability to grow on non-fermentable media, such as glycerol, due to dysfunction in aerobic respiration. All gss mutants have severe growth defects compared to their respective parent strain when plated on glycerol-based media. However, the parent strains possess a growth advantage since they contain a functional copy of psas₃ts ADE3. When we transformed our gss mutants with psas₃ts ADE3 to eliminate this growth advantage, a fraction of the mutants generated transformants with red colonies while the rest remained purely white. When plated on glycerol media, the red strains can grow similar to wild type while the white strains grow poorly. It seems unlikely that the mutants are reverting to wild type since this occurred at far too high a frequency. It could be possible that the original mutants harboured both rho⁻ and wild type mitochondrial DNA and the presence of Sas3p
allowed the wild type to out-compete the rho⁻ somehow. This idea is supported by the observation that some cells maintain both rho⁻ and wild-type mitochondrial genomes for up to eight generations after crosses against wild type (104). Furthermore, with the addition of Sas3p to the genome, cells would potentially no longer require petite mutations to suppress the synthetic lethality for survival. Therefore, the presence of Sas3p could alleviate the selective pressure for maintaining the petite genome in the cell. One inconsistency was seen in our results; gss9 is a purely white mutant that exhibits strong growth on petite media when transformed with psas3TS ADE3. This mutant is the only exception to the trend discussed above. This mutant is difficult to explain in terms of petite or nuclear mutations. Regardless, the white gss mutants, for the most part, do not grow on petite media in the absence or presence of psas3TS ADE3. This argues that those gss mutants that remained white after transformation with psas3TS ADE3 may be petites.

The data would seem to argue that petite mutations suppress the gcn5Δsas3Δ synthetic lethality. This makes some sense since Gcn5p has been linked to DNA damage repair (36, 37, 118) and respiration is the main source of free radicals that can cause extensive DNA damage (102). For example, respiration produces superoxide (O₂⁻), peroxide (H₂O₂), and the hydroxyl radical (OH) (102). Thus, in the absence of Gcn5p and Sas3p, it is possible that free radicals produced by respiration damage the DNA to such an extent that cells cannot survive. This idea is supported by the fact that gcn5Δsas3Δ deletions halt at the G2/M border (35). It follows that DNA damage checkpoint proteins could halt cells permanently at G2/M in the absence of these proteins due to accumulation of DNA damage. Elimination of respiration through petite formation would thus stop the production of free radicals and the associated DNA damage and allow gcn5Δsas3Δ strains to proceed through the G2/M border.

To test the above theory, we created a petite mutant through an alternative method and tested it for the ability to rescue gcn5Δsas3Δ strains. One method of creating petites is using
ethidium bromide, which causes immense degradation of mitochondria DNA in addition to sequence rearrangements (117). We created a petite mutant derived from the YDG008 parent strain using ethidium bromide. This petite could not grow on glycerol medium but neither could it suppress the synthetic lethality. This argues against petite mutations alone recovering the synthetic lethality. However, we cannot rule out the possibility that the gss mutants generated in our screen have different mitochondrial DNA mutations than that of the EtBr derived petite mutants and only certain petites can rescue the synthetic lethality. Supporting this is the fact that EtBr causes massive degradation of mitochondrial DNA in addition to sequence rearrangements, producing petite mutants that do not genomically resemble spontaneous petites (117). Creating several spontaneous mutants derived from the parent strain and testing them for the ability to rescue the \textit{gcn5Asas3A} synthetic lethality would be a logical next step. However, differentiating these spontaneous mutants from an gss mutant is impossible, since the screening techniques employed for both would be essentially the same.

Thus, the gss mutants are white, cannot grow on petite media, and are dominant. Furthermore, two of the gss mutants tested segregate in a non-Mendelian fashion. These are all characteristics shared with petite mutants. Although the gss mutants appear to be petites, some inconsistencies must be sorted out before drawing any firm conclusions. For instance, we should distinguish between rho$^-$ and rho$^0$ petite mutants using the DAPI staining microscopy technique (102).

\subsection*{4.4.2 gss Mutants are Potentially Prions}

Another possible way to explain the characteristics of our gss mutants is prions. Prions are stable and inheritable alternate protein confirmations that are deleterious to the normal function of the properly folded protein (119, 120). Prions have the ability to direct the folding of additional proteins, ensuring that they adopt the altered confirmation. Prions are dominant
mutations as a result of their ability to influence protein folding without changes in genetics (119). Furthermore, inheritance of prions is non-Mendelian since the proteins are present in the cytoplasm, and segregate to both mother and daughter cells. As a result, prions often segregate 4:0 (119). Two known prions exist in yeast, they are \([PSI^+]\) and \([URE3]\), controlled from the genes \(SUP35\) and \(URE2\), respectively. Both of these prions are dominant and are inherited through the cytoplasm to all of their progeny, segregating 4:0 (119, 121). \([PSI^+]\) mutants can promote read-through of UAG and UGA nonsense codons as well as UAA codons, while \([URE3]\) mutants allow yeast to take up ureidosuccinate (USA) on ammonia-containing media (119).

Three criteria have been outlined for describing mutations as prions, including: (a) reversible curing, (b) overexpression of the protein increases the frequency of prion formation and (c) phenotypic relation of the prion and mutations in the gene expressing the protein (119). The first criterion, reversible curing, occurs when the prion spontaneously changes back to its normal form. Growth on millimolar concentrations of guanidine aids this process. In our studies, we see that a subsection of our \(gss\) mutants revert to red colonies and exhibit growth on petite media when transformed with \(psas3^{TS}.ADE3\), which could qualify as reversible curing. However, the prion theory cannot explain why the same mutants revert to red each time instead of reversion occurring randomly, as would be expected. It is possible that only these small subsets of proteins are prions. Regardless, there is a small possibility that the \(gss\) mutants are an undiscovered yeast prion mutation. The other two criteria distinguishing a prion mutation are not possible to examine unless the protein or gene in question is isolated. Based on the current evidence, the possibility that the \(gss\) mutants are prions rather than petites is slim.

4.5 Concluding Statement
In this study, we explored the essential function of two acetyltransferases, Gcn5p and Sas3p. We showed that Sas3p’s essential function is dependent on the NuA3 complex and its ability to acetylate histone H3’s K14 residue. We also showed that Gcn5p has a function that is independent of the SAGA complex and its role as a HAT protein. These results suggest a very different view of the role yeast HATs play in the cell currently outlined in the literature. Gcn5p’s function in the cell has long been linked with SAGA and its ability to acetylate histone H3, predominantly at K14 (5, 97, 98). Our data, on the other hand, supports the model proposed by Suka, et al. which proposes SAGA acetylates primarily H3 K9, 18, 23, and 27 and not K14 (24). Furthermore, much of the literature states that Gcn5p’s role is primarily acetylating histones (5, 36, 122, 123). However, we give strong evidence showing a distinct function of Gcn5p unrelated to histone acetylation. We attempted to discover the non-histone substrate of Gcn5p by immunoprecipitation assays of *gcn5A* and *ada2A* strains, however, no significant differences between these strains could be seen in the subsequent western blots. Furthermore, we tested loss of Sin1p and Htz1p, two proteins previously shown to be acetylated by Gcn5p, for genetic interactions with loss of *GCN5* or *SAS3*. However, no genetic relationship was discovered, suggesting these proteins are not Gcn5p’s exclusive, non-histone substrate. Another possible substrate of Gcn5p acetylation is Fet4p, an iron transport protein involved in respiration that has been shown to interact with Gcn5p via a yeast 2-hybrid screen (124). In preparation for future research in this line, we have already successfully cloned *FET4* into an IPTG inducible bacteria expression vector and purified the recombinant protein for subsequent HAT assays.

We also attempted to expand our knowledge of the *GCN5 SAS3* essential pathway using a genomic and a proteomic approach. We showed that Hsl7p and Rlf2p interact with Sas3p but not NuA3. Regardless, Rlf2p and especially Hsl7p share similar properties to Sas3p, warranting their further study as Sas3p interactors independent of NuA3. In addition, we devised our own genetic screen looking for suppressors of synthetic lethality. The isolation of petite mutants as
suppressors seems to suggest a role of \textit{GCN5} and \textit{SAS3} in the DNA damage repair pathway. However, we must sort out several inconsistencies before concluding this definitively. Nevertheless, the genetic screen was a novel way to isolate mutants that interact in the essential pathway and could be useful as a technique with further tweaking.
Chapter 5 – References


