THE ROLE OF ING HOMOLOGS IN THE YEAST SACCAROMYCES CEREVISIAE

by

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Abstract

The yeast inhibitor of growth (ING) homolog, Yng1p, was identified based on sequence similarity of its highly conserved plant homeodomain (PHD) finger with other ING family members. Yng1p has been shown to be a stable subunit of the NuA3 histone acetyltransferase (HAT) complex. While previous work has indicated the requirement of Yng1p for NuA3 function, little was known regarding its role within the NuA3 complex. The SAS3-dependent HAT NuA3 was characterized through in vitro studies as having specificity towards histone H3. Whether NuA3 acetylates histones in vivo or what factors influence the binding of NuA3 with the nucleosome was also unknown. Using a chromatin pull-down assay, in conjunction with genetic studies, we show that Yng1p is required by NuA3 for its interaction with the nucleosome, and this interaction occurs within the histone H3 tail. Additionally, we show that mutation of lysine 14, the preferred site of NuA3 acetylation in vitro, shows the same phenotype as a sas3A specific phenotype indicating that modification of this residue is important for NuA3 function. The interaction of NuA3 is dependent upon Setlp and Set2p methyltransferases, as well as their substrates, histone H3 lysine residues 4 and 36 respectively. These results indicate that NuA3 is functioning as a histone acetyltransferase in vivo, and that methylation serves as a mark for the recruitment of NuA3 to the nucleosome. Through the use of additional chromatin pull-down experiments, we extend our investigation to assess whether two other yeast ING homologs, Yng2p and Pho23p, (found in the NuA4 HAT and Rpd3-Sin3 histone deacetylase complexes respectively) perform similar functions to Yng1p within their respective complexes. Finally, we show that over-expression of human ING2, like YNG1, is toxic within yeast. Having shown that Yng1p interacts with the nucleosome, we discuss the possibility of human ING proteins functioning in a similar manner.
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Nomenclature

The text follows the conventional method for designating genetic symbols and protein products. Dominant alleles of wild type genetic loci are designated by italicized upper case letters (e.g. \textit{SAS3}) while mutant genes are designated with italicized lower case letters (e.g. \textit{sas3}). Gene deletions are written as a mutation with a Greek ‘delta’ following the designation (e.g. \textit{sas3}Δ indicates deletion of the \textit{SAS3} locus). Insertion mutations are indicated with the symbol ::. For example \textit{sas3}::\textit{HISMX6} indicates that the \textit{HISMX6} gene is inserted within the \textit{SAS3} locus. Gene products are not italicized and only the first letter is capitalized followed by a ‘p’ postscript (e.g. Sas3p is the gene product of the \textit{SAS3} locus).
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The influence of my family, both my parents and my brothers, is evident in my work. They provide me with the confidence and endurance to endeavor projects such as these. Your support does not go unnoticed, and will not be forgotten.

I cannot tell how the truth may be;
I say the tale as 'twas said to me.

Sir Walter Scott
Chapter 1 – Introduction

1.1 Chromatin – Structure and Function

1.1.1 Chromatin Basics

For any individual cell to remain autonomous, it must carry with it all the genetic information which defines it. Due to the overwhelming amount of DNA that is required to store this essential information, mechanisms must exist which permit the cells to properly package their DNA. This packaging process must not only be efficient, but must also facilitate access to the DNA in order that cellular processes such as transcription and DNA repair and replication may occur. This problem is resolved in eukaryotic cells through the packaging of DNA, with proteins, into a highly condensed form known as chromatin (1).

The basic unit of chromatin is the nucleosome core particle, which consists of four highly conserved core histone proteins H2A, H2B, H3 and H4, in addition to the DNA (2). These four core histone proteins interact to form an octamer comprised of an H3-H4 tetramer and two H2A-H2B dimers (3, 4). Assembly of the nucleosome requires the association of two H3-H4 heterodimers resulting in the H3-H4 tetramer, around which two revolutions of DNA is wrapped, an interaction which typically involves 100-120 base pairs (bp) of DNA (5, 6). Following the formation of this preliminary complex, two H2A-H2B heterodimers bind to either side of the H3-H4 tetramer expanding the length of DNA involved in each core particle to ~147 bp (5, 7). When viewed as a whole, the core particles give rise to the so called 10-nm beads on a string structure (or the nucleosome) comprised of repeating units of core particles separated by connecting DNA, referred to as linker DNA (1). This linker DNA varies in length depending on
a number of factors including the presence of DNA binding proteins, as well as the thermodynamic properties of the DNA (1).

The folding of nucleosomes is accomplished through histone – histone interactions as well as with the aid of additional proteins, resulting in the formation of higher ordered chromatin structures (6). The four core histone proteins consist of a structured three-helix histone fold domain, necessary for interaction with other core histones in the formation of the histone octamer, in addition to a largely unstructured, highly basic, N-terminal histone tail (2). The histone tails protrude outwards from the core particle and appear to mediate nucleosomal folding through several mechanisms inclusive of an electrostatic component (charge neutralization of the DNA) as well as protein – protein interactions (such as intra-nucleosomal tail-tail interactions) (8). One additional histone protein, the linker histone, is found on the periphery of the core particle and is thought to impart stability to the core complex (8). Linker histones, including H1, H5 and Hho1p (in yeast), typically associate with nucleosome core particles at a ratio of 1:1, with the exception of Hho1p, for which no clear ratio has yet been defined (8, 9). While not essential in yeast and other lower organisms, linker histones are essential in vertebrates, and have been shown to promote stability within the nucleosome in addition to folded and oligomeric states of nucleosomes (8, 10, 11). Indeed, linker histones appear to facilitate additional folding of nucleosomes, and this additional folding is predicted to give rise to a maximally folded chromatin structure referred to as the 30 nm chromatin fiber (8).

The folding of the canonical 30-nm chromatin fiber into higher ordered structures also involves the use of non-histone proteins which are grouped into two different classes based on whether or not these proteins interact with the core histone tail domains (8). Examples of proteins that interact with the histone tail domains are the yeast silencing proteins Sir3p and Tup1p (8, 12, 13). These proteins function by cross-linking chromatin fibers through the interaction of two or more adjacent silencing proteins. Ultimately, these proteins bridge
nucleosomes together over long distances, resulting in highly ordered chromatin structures (8). In essence, regions of the genome that contain condensed or highly ordered chromatin, known as heterochromatin, are usually low in gene density and hence transcriptionally inert, while euchromatic regions, consisting of more relaxed chromatin structures, are generally gene rich and thus correlated to transcriptional activity (14).

The presence of nucleosomes within promoter regions of genes has been shown to repress gene transcription (1). In vitro studies have shown that promoters that were packaged with nucleosomes were deficient in initiating transcription with recombinant RNA polymerase enzymes (15). Additionally, in vivo studies performed in yeast have shown that inhibiting histone synthesis, causing subsequent nucleosomal loss, results in activation of genes that were inactive prior to nucleosomal loss (16). These results along with other studies have led to the current belief that histones have a general repressive effect on genes. The repressive nature of histones is thought to arise through two independent interactions including histone fold – DNA interactions within the core particle and histone tail interactions with the chromatin fiber (1). Alleviation of the repressive effects brought on by the histone fold domains is though to be achieved by chromatin remodeling complexes while evidence suggests that the repressive effects of the histone tails are opposed by post-translational modifications of the histone tail itself (1).

A number of post-translational modifications have been shown to occur on the histone proteins, within the histone octamer. The bulk of these post-translational modifications take place on the amino-terminal (N-terminal) tails of the core histones. Acetylation and methylation are the predominant modifications shown occur within the histone tails (17). There are known to be, however, a few exceptions where acetylation and methylation modifications occur within the globular domains of histone proteins (17-19). Additional histone modifications include phosphorylation and ubiquitination. While phosphorylation has been observed on either N- or
carboxy-terminal (C-terminal) regions of histone proteins, ubiquitination is confined to the C-terminal regions of histones H2A and H2B (17, 20).

The current model of the nucleosome was first predicted almost 30 years ago, and it was at that time hypothesized that post-translational modification, of the histone tails, played a role in regulating chromatin structure (21). Since this early prediction, much work has been done on identifying the modifications present on the histone tails, the complexes responsible for carrying out these modifications, and the subsequent effects that these modifications have on chromatin structure. The result of these studies is the emergence of a proposed model, known as the histone code hypothesis, that suggest these modifications serve as a signal to regulate access to DNA through remodeling of the chromatin structure (22, 23).

1.1.2 The Histone Code Hypothesis

Initially it was accepted that histone modifications regulate chromatin structure by directly altering DNA – histone and histone – histone interactions (24). In the case of modifications such as acetylation and phosphorylation this explanation was easy to rationalize, as addition of either of these groups would neutralize the positive charge associated with the histone tails, while addition of phosphoryl groups would add an additional negative charge. This alteration likely weakens the DNA – histone interaction resulting in a more open chromatin structure and in turn facilitating transcription (24). Indeed, correlations were shown to exist between histone acetylation and gene transcription (25). Similarly phosphorylation of serine 10 of histone H3 has been shown to be correlated with gene induction (24, 26). However, due to the complexity of the modifications that are now know to take place on the histone tails, as well as the presence of certain discrepancies between the aforementioned correlations, this initial
hypothesis appears to be inadequate. For example, while histone acetylation is typically linked to gene transcription, histone acetylation is known to exist in regions of the genome that are transcriptionally silent. Histone phosphorylation likewise displays apparent discrepancies where phosphorylation of serine 28 of histone H3 is linked to chromosome condensation (24). Thus, the histone code hypothesis has emerged in an attempt to explain the role of histone post-translational modifications.

Essentially the histone code hypothesis takes into account the variety of modifications that occur on the histone tails and states that these modifications serve as a mark to signal the recruitment of additional effector molecules to the histone tails (22, 23). In support of this hypothesis, numerous proteins have been identified which recognize post-translational modifications on histones. One primary example of this is the heterochromatin protein 1 (HP1), which interacts with methylated lysine 9 of H3 and functions by maintaining silenced DNA (heterochromatin) (27, 28). There is also evidence that one modification may serve as a signal in the deposition or prevention of another modification. Indeed the tri-methylation of lysine 4 on histone H3, a signal recently linked to gene transcription, requires the prior ubiquitination of lysine 123 on histone H2B. Alternatively, phosphorylation of serine 10 on histone H3, a modification linked to histone acetylation and gene activation, inhibits the methylation of lysine 9 on H3, a signal that is recognized by HP1 and is consistent with heterochromatin and gene silencing (29-31).

The histone code is attractive because it provides an explanation for the exceptions seen in the previous model. As such, the presence of acetyl or phosphoryl groups alone is not enough to determine an outcome, but rather how these signals are read that is the determining factor. As a result of the multiple sites available to any modification, it is possible to envisage how a specific modification may result in different signals depending on both the location of the modification, as well as the presence of additional modifications. In short, the numerous sites
available for post-translational modification, the diversity of the post-translational modifications that may be effected, and the method by which these signals are interpreted, all add complexity to the regulatory mechanism of chromatin, a complexity that the histone code hypothesis attempts to resolve (22-24).

1.1.3 Histone Acetylation and its Role in the Histone Code Hypothesis

Histone acetylation is carried out by a group of enzymes referred to as histone acetyltransferases (HATs), acetyl groups are removed by histone deacetylase (HDAC) complexes (32, 33). HAT complexes require acetyl co-enzyme A as a substrate and function by transferring the acetyl group from this substrate to specific lysine residues within both the globular domain as well as the tails of histone proteins (19, 34, 35). Of all the post-translational modifications that occur within the histone tails, histone acetylation is the best studied due to the established link between histone acetylation and gene expression (36).

Since the initial discovery that histone acetylation correlated to gene expression, the identification of two groups of enzymes, HATs and HDACs, that are required to maintain steady state levels of histone acetylation, has fueled interest in this modification (24). Additionally, the conservation of acetylation sites within the histone tails, as well as HATs and HDACs, is suggestive of the importance of this modification (24, 37). Recognition of the non-random nature of histone acetylation, where histones are preferentially acetylated in regions of the genome that are actively undergoing transcription, was a further indication of the importance of histone acetylation in gene transcription (25).

Apart from its role in transcription, histone acetylation has been implicated in nucleosome core particle assembly. Newly synthesized core histones H3 and H4 are acetylated prior to their incorporation into the nucleosome (37). While it is not necessary that both H3 and
H4 be acetylated, the acetylation of either H3 or H4 is essential for proper complex formation (37). Histone acetylation also modulates the folding of the nucleosome, and is important for maintaining an “open” chromatin structure (38, 39). In short, all of the functions associated with histone acetylation suggest a very important role for this modification within the cell.

In the yeast *Saccharomyces cerevisiae* there are at least eight different proteins shown to exhibit HAT activity through either *in vitro* or *in vivo* assays (19, 32, 40). Three of these proteins are found in complexes which have been shown to contain specificity toward acetylation of the histone H3 tail including the *GCN5*-dependent HAT complexes SAGA, SLIK/SALSA and ADA (41-44), the *ELP3*-dependent elongator complex (45) and the *SAS3*-dependent NuA3 complex (46). Primarily, acetylation of histone H4 is carried out by only one HAT, the *ESA1*-dependent NuA4 complex (47). While Sas2p, the catalytic subunit of the SAS complex, has been shown to acetylate H4, its function seems to be that of regulating heterochromactc boundaries by opposing the function of yeast silencing proteins, specifically the Sir2p HDAC (48-52). Unlike all other known HATs in yeast, NuA4 is the only essential HAT where disruption of NuA4 results in cell cycle arrest, indicating the essential function associated with H4 acetylation (47, 53).

Correlations are shown to exist between histone acetylation and regions of the genome that are actively undergoing transcription (54). Thus, acetylation of histones is thought to alter the structure of chromatin through several different mechanisms. First, it is thought that the neutralization of the positively charged lysine residues, associated with addition of acetyl groups results in a weakening of the interaction of DNA with histone proteins (39, 55, 56). Additionally, acetyl groups on histone tails have been shown to recruit chromatin remodeling complexes to the nucleosome (57). One such example of this recruitment is shown with the yeast ATP-dependent chromatin remodeling complex Swi/Snf which interacts with acetylated histones H3 and H4 through its subunit, Swi2 (40). As with Swi2, other chromatin modifying
Table 1.1 Yeast HAT Enzymes and Their Properties (19, 32, 40-46)

<table>
<thead>
<tr>
<th>Yeast HAT Enzymes</th>
<th>Putative Substrates</th>
<th>HAT Containing Complexes</th>
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<tbody>
<tr>
<td>Gcn5p</td>
<td>K9, 18, 23 and 27 of H3</td>
<td>ADA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SLIK/SALSA</td>
</tr>
<tr>
<td>Hat1p</td>
<td>K5 and 12 of H4 – functions prior to nucleosome assembly</td>
<td>Associates with Hat2p</td>
</tr>
<tr>
<td>Hpa2p</td>
<td>H3 and H4 tail domains</td>
<td>Unknown</td>
</tr>
<tr>
<td>Elp3p</td>
<td>H3 and H4 tail domains</td>
<td>Elongator Complex</td>
</tr>
<tr>
<td>Nut1p</td>
<td>General acetylation of histone tails</td>
<td>Mediator Complex</td>
</tr>
<tr>
<td>Spt10p</td>
<td>K56 of H3</td>
<td>Interacts with Spt21p</td>
</tr>
<tr>
<td>Sas2p</td>
<td>K16 of H4</td>
<td>SAS complex</td>
</tr>
<tr>
<td>Sas3p</td>
<td>K14 and 23 of H3</td>
<td>NuA3</td>
</tr>
<tr>
<td>Esa1p</td>
<td>K4 and 7 of H2A and K5, 8, 12 and 16 of H4</td>
<td>NuA4</td>
</tr>
</tbody>
</table>

Complexes have been shown to interact with acetylated histones, and this interaction is dependent upon the presence of a bromodomain, a domain capable of recognizing acetylated lysine residues (58). The recruitment of chromatin modifying complexes through recognition of acetylated lysine is an exemplification of the histone code hypothesis.

Alternatively, transcription factors have been shown to recruit, or target, HATs to promoter regions of genes. Indeed, the molecular basis for the link between histone acetylation and gene activation was made upon the observation that the yeast transcriptional co-activator, Gcn5p, also showed HAT activity (59, 60). Since this initial discovery, a number of transcriptional co-activators have been identified that also possess HAT activity, strengthening the role of histone acetylation in gene activation (32).

The removal of acetyl groups is thought to be consistent with transcriptional silencing (37). As such, the enzymes responsible for removal of acetyl groups, HDACs, are believed to impart a negative influence on transcription (61). HDAC complexes are phylogenetically grouped based on the components found within the complex. In yeast there are three different classes containing a total of ten HDACs (Table 1.1) (61).
Table 1.2 Yeast HDACs and Their Phylogenetic Classes (61)

<table>
<thead>
<tr>
<th>Phylogenetic Class</th>
<th>Yeast HDAC</th>
</tr>
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<tbody>
<tr>
<td>Class I</td>
<td>Rpd3p</td>
</tr>
<tr>
<td></td>
<td>Hos1p</td>
</tr>
<tr>
<td></td>
<td>Hos2p</td>
</tr>
<tr>
<td>Class II</td>
<td>Hos3p</td>
</tr>
<tr>
<td></td>
<td>Hda1p</td>
</tr>
<tr>
<td>Class III</td>
<td>Hst1p</td>
</tr>
<tr>
<td></td>
<td>Hst2p</td>
</tr>
<tr>
<td></td>
<td>Hst3p</td>
</tr>
<tr>
<td></td>
<td>Hst4p</td>
</tr>
<tr>
<td></td>
<td>Sir2p</td>
</tr>
</tbody>
</table>

One yeast HDAC, Sir2p, was originally identified through a genetic screen aimed at identifying mutants that were defective in mating-type silencing (62). Additional characterization recognized Sir2p as an HDAC which predominantly acts upon acetylated lysine 16 of histone H4, a modification that is primarily maintained by the Sas2p HAT (48, 63). This link between HDAC activity and transcriptional silencing provides evidence for the proposed role of histone deacetylation in gene regulation. Another such example is Rpd3p, a class I HDAC which has been implicated in gene repression through its associated H3 and H4 HDAC activity (64). A variety of studies have shown that Rpd3p HDAC activity, through either its targeted recruitment to promoters, or its global histone deacetylase activity, generally correlates to decreased levels of transcription (61, 64, 65).

Regulation of gene expression through acetylation is thought to be dependent on the establishment of an equilibrium between histone acetylation and histone deacetylation, as mediated through non-targeted HAT and HDAC activities respectively. Disruption of this equilibrium is thought to result from the targeted recruitment of either HAT or HDAC complexes to genes, which in turn facilitates either gene activation or repression (66, 67). The targeted recruitment of histone modifying complexes allows for histone modification within
specific gene promoters. The targeting of HATs or HDACs permits the acetylation or deacetylation of discrete regions of the genome depending upon the recruiting factor (68).

Acetylation of specific promoters is effected through the targeting of HATs, through the interaction of HAT complexes, with transcriptional activators (68-70). The yeast co-activator, Gcn5p, is recruited by transcriptional activators such as Gcn4p and Swi5p (69, 70). Gcn5p, is associated with several HAT complexes (41-43), thus through its recruitment, promoters of genes are targeted for acetylation. Similarly, the H4 acetyltransferase, Esa1p, has been shown to be recruited to promoters of ribosomal protein genes through its interaction with the activator Rap1p (71). In an analogous manner, HDACs may also be recruited to promoters through their interaction with suppressors. In yeast, a DNA-bound repressor, Ume6p, has been shown to recruit the Rpd3-Sin3 HDAC complex (66). This targeted recruitment of HATs and HDACs, by activators or repressors respectively, strengthens the proposed function of histone acetylation, namely its role in facilitating gene transcription.

1.1.4 Histone Methylation as a Signal

In a manner analogous to acetylation, histones are also modified by the addition of methyl groups on either lysine or arginine residues, within the amino-terminal tails and within the globular domain of H3 and H4 (28, 72, 73). Unlike histone acetylation, however, histone methylation has been linked to transcriptional repression and activation depending on the specific nature of the modification (27). In higher eukaryotes, there is significant evidence to suggest that methylation is involved in gene silencing (24). Heterochromatin protein 1 (HP1), a protein which maintains heterochromatin, and is therefore thought to be involved in gene silencing, has been shown to recognize methylated lysine 9 of histone H3 (27, 28). There is, however, no such an interaction known in yeast (22). Heterochromactic regions of the genome
are maintained though a collection of proteins including Sir3p and Sir4p and the HDAC, Sir2p, and seem to be dependent upon levels of acetylation rather than methylation (13, 49, 50, 74). Thus, while discrepancies seem to persist in higher organisms, methylation seems to correlate with gene activation in yeast (54, 75).

Although methylation as a modification is anticipated to be an important signal, when compared to other modifications, little is known about its properties. Due to the discrepancies which exist regarding methylation and transcription, it has been difficult to assign a role to this particular modification (28). Studies on this modification have also been made difficult as a result of the lack of information regarding the proteins that are responsible for methylating histones (28). Also, unlike acetylation and phosphorylation, addition of methyl groups does little to alter the positive charge of basic lysine or arginine residues, thus making it difficult in the past to distinguish between modified and unmodified histones (24). This problem, however, has solved in part with the recent advancement of commercially available antibodies to various methylated histone tail residues. Lysine residues may also be mono-, di-, or tri- methylated, adding yet an additional complexity to this type of modification (24, 27, 28).

While less is known regarding the role of methylation as a modification, its importance as a signal regulating histone modification is beginning to emerge. Recent evidence has shown that histone methylation is associated with regions of the genome that are transcriptionally active (76). Additional studies in yeast have indicated that methylation is required for activity of HAT complexes (77). Consistent with the histone code hypothesis, this recent evidence would suggest that methylation of residues within histone H3 may regulate gene expression through mediating levels of histone acetylation.

In yeast, two histone methyltransferases (HMTs), Setlp and Set2p, bearing specificity towards histone H3 residues lysine 4 and 36 respectively, have recently been implicated in transcription (78, 79). Tri-methylation of lysine 4 on histone H3 is synonymous with actively
transcribed genes. Set1p, is a component of the COMPASS complex, and is responsible for effecting the tri-methylation of lysine 4 (80). This tri-methylation of lysine 4 by Set1p appears to be regulated at several levels and is absolutely dependent on H2B ubiquitination by the Rad6p complex (30, 31, 81).

Additionally, Set1p has been shown to associate with the carboxy-terminal domain (CTD) of Rpb1, the largest subunit of RNA polymerase II (RNAPII) and this interaction is dependent upon phosphorylation of the CTD (82). The CTD contains the consensus repeat, Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which is highly conserved in eukaryotic cells. Serine residues within this repeat are extensively phosphorylated in the elongating complex (the complex in which RNAPII is actively transcribing DNA). The phosphorylation of the CTD is concomitant with the switch of RNAPII from the pre-initiation complex to the elongating complex, and is mediated, at least in part, by the kinase activity of TFIIH. Phosphorylation of the CTD is therefore representative of the conversion of RNAPII from a complex involved in promoter recognition, to one that is involved in elongation (83).

The interaction of Set1p with the CTD of RNAPII requires the phosphorylation of serine 5 by Kin28p, a TFIIH associated kinase which is shown to mediate the transition from initiation to elongation. Thus, Set1p preferentially associates with RNAPII at the 5' end of genes (80, 84). Similarly Set2p, has been shown to associate with RNAPII, and like Set1p, this association is dependent on phosphorylation of the CTD. The association of Set2p with RNAPII requires phosphorylation of an alternate site within the CTD, serine 2. The phosphorylation of serine 2 is carried out by the Ctk1p kinase, which functions during the later stages of elongation (85-88). Therefore, lysine 4 methylation is usually confined to the promoter and regions of the gene immediately downstream of the promoter (89), while lysine 36 methylation is usually spread out throughout the coding sequence (75, 80).
Recognition of the methylation signal is primarily carried out by chromodomain containing proteins (57). However, additional domains have recently been identified, such as the Tudor domain, which bears homology to chromodomains, and is shown to interact with arginine in a methyl-dependent manner (90). Chromodomains, while capable of interacting with a variety of substrates, display specificity towards methylated lysine residues (91). Initially linked to gene silencing, due to its presence in the heterochromatin protein HP1, chromodomains have since been shown to exist in a number of histone modifying complexes, including HATs (57). Recently, the chromodomain of Chd1p, a component of SLIK and SAGA HAT complexes, has been shown to interact with methylated lysine 4 of histone H3 (77). Reminiscent of the histone code hypothesis, this proposed link between initiation of transcription and histone acetylation, as mediated by histone methylation, suggests an intriguing mechanism for the targeted recruitment of HATs to genes undergoing transcription.

1.2 The Inhibitor of Growth (ING) Protein Family

Identification of the first human ING protein, ING1, was accomplished by looking at the differential expression of growth inhibitors between normal epithelial cells and a breast cancer cell line (92). Since their initial discovery, numerous splice variants of ING1 as well as other family members have been identified, all of which bear substantial homology in their C-terminal region (93). Contained in the C-terminal regions of all ING proteins is the protein motif known as the plant homeodomain (PHD) finger which is highly conserved throughout ING proteins (93-95). While the functional roles of all human ING proteins is not yet fully understood, those that are known suggest very diverse roles for this protein family.
ING proteins are shown to play a role in tumor suppression as down regulation or loss of ING1 expression is coincident with tumor progression (93, 96). Similarly, abrogate expression of ING3 and ING4 is also seen in several types of tumors (92, 97). Though these proteins have been shown to play a role in tumor suppression, the mechanism whereby tumor suppression is accomplished is not entirely understood. Some clues to their mechanism have come through the identification of interacting partners of ING proteins. A splice variant of ING1, p33\(^{\text{ING1b}}\), has been shown to associate with the transcriptional activator p53, and perhaps this interaction modulates the function of p53 (97). In support of this, studies have shown that in the absence of p33\(^{\text{ING1b}}\), p53 is unable to negatively regulate cell proliferation (96). Additionally, p33\(^{\text{ING1b}}\) over-expression promotes apoptosis in a p53 dependent manner, while reduced expression of p33\(^{\text{ING1b}}\) attenuates the ability of p53 to induce apoptosis (92, 98).

ING proteins have also been shown to be involved with protein complexes that interact with DNA. The ING1 splice variant p33\(^{\text{ING1b}}\) has been shown to associate with PCNA indicating a role for ING proteins in DNA damage repair (99). This interaction appears to be accentuated by DNA damage, and is thought also to play a role in mediating apoptosis through alteration of the structure of PCNA (92, 93). ING proteins also display a role in the regulation of chromatin structure. Several splice variants of ING1, as well as other ING family members, are shown to associate both physically and functionally with HAT and HDAC complexes (92, 93). This interaction of ING proteins with histone modifying complexes suggests a possible mechanism whereby ING proteins suppress tumor formation. Thus, it is possible that the tumor suppressive function of ING proteins is achieved, at least in part, through the regulation of chromatin structure.
1.3 The Three Yeast ING Homologs are Found Exclusively in Histone Modifying Complexes

1.3.1 The NuA3 HAT Complex

NuA3 is a multiprotein complex with a molecular weight of approximately 400 kDa (100). Originally identified based on its ability to acetylate histone H3 in vitro, NuA3 was shown to preferentially acetylate lysine 14 and to a lesser extent lysine 23 within the H3 tail (42, 101). Although NuA3 shows HAT activity toward purified proteins, it is unclear as to whether or not it is capable of acetylating H3 in vivo. The NuA3 complex is comprised of the catalytic subunit, Sas3p, along with at least four other subunits including, Yng1p, Ntol1p, TAF14p and Eaf6p [L. Howe personal communication] (46, 100). While some work has been done in the characterization of Yng1p, the yeast homolog of human ING, little is known about the remaining three subunits of NuA3.

NuA3 purified from strains lacking YNG1 is shown by in vitro HAT assays to have a significant reduction in HAT activity (100). Additionally, in vitro assays performed with NuA3 purified from yng1Δ strains show a decreased interaction with nucleosomes, as compared to wild type NuA3 (100). This diminished ability of NuA3, lacking Yng1p, to interact with nucleosomes suggests a possible role for Yng1p, namely acting as a mediator for NuA3s interaction with the nucleosome. Yng1p, as an ING homolog, also contains the conserved PHD finger domain within its C-terminus (94, 100). Though deletion of YNG1 is not deleterious to the cell, over-expression of YNG1 results in an inhibition of cell growth. Removal of the PHD finger abolishes the toxicity associated with YNG1 over-expression, suggestive of a functional role for this motif (L. Howe, personal communication). The Ntol1p subunit, although not an ING
homolog, does contain a PHD finger domain like Yng1p, which appears to play some structural role within the NuA3 complex (L. Howe personal communication).

Disruption of SAS3 results in only minor phenotypes, notably restoration of silencing to a partially defective *HMR* locus, while deletion of another H3 HAT, *GCN5*, causes a more severe phenotype characterized by slow growth (42, 102). Despite these growth phenotypes, deletion mutants of either SAS3 or GCN5 are still viable. However, a synthetic lethality phenotype is observed if both *GCN5* and SAS3 are deleted in conjunction with one another, as sas3Δgcen5Δ strains are inviable (103). Interestingly, sas3Δ is not synthetically lethal with either ada2Δ or ada3Δ, where loss of either of these genes results in disruption of all known Gcn5p-dependent HAT complexes (103). Thus, the sas3Δgcen5Δ synthetic lethality is not dependent upon Gcn5p-dependent HAT complexes (103). The fact that this synthetic lethality is not due to the loss of Gcn5p-dependent HAT activity suggest that Gcn5p may have a function that is unrelated to histone acetylation.

To date, only genes that encode components of NuA3 have been shown to display similar genetic interactions with respect to *GCN5* and *ADA2*, that is, synthetic lethality with gcen5Δ but not ada2Δ. All other deletion mutants that are known to be synthetically lethal with gcen5Δ, such as swilΔ, are also synthetically lethal with ada2Δ (104). As such, this sas3Δ specific phenotype was used to show that Yng1p is required for *in vivo* HAT activity as yng1Δ gcen5Δ strains are extremely sick (100). Interestingly, Yng1p lacking the PHD finger is still able to rescue the yng1Δ gcen5Δ growth defect (100). These results, along with the over-expression toxicity data indicate that Yng1p may have both PHD dependent and PHD independent functions.
1.3.2 The H4 Specific NuA4 HAT Complex

NuA4 is a large multiprotein complex of approximately 1.3 MDa in size and contains at least 13 subunits (see Table 1.2) (105). NuA4 was originally identified through the use of in vitro HAT assays, based on its preference to acetylate histone H4 (42). The catalytic subunit of NuA4 has been identified as Esa1p (47) and shows in vitro HAT activity towards H4 and to a lesser extent H2A (42, 101). Unlike other HAT complexes found in yeast, NuA4 is essential to the cell as disruption of the NuA4 complex is lethal (106, 107). Consistent with the proposed role of histone acetylation in facilitating gene transcription, it has recently been shown that Esa1p occupies the promoters of all actively transcribed genes in yeast (108). Furthermore, both yeast and human NuA4 complexes are shown to have, in addition to, but not exclusive of, HAT activity. These associated functions with the NuA4 complex are quite varied and include activities such as transcriptional co-activation, DNA damage repair, and cell cycle control, indicating evolutionarily conserved roles for these complexes (109, 110).

Table 1.3 The Subunits of NuA4 and Their Associated Functions (105, 111, 112)

<table>
<thead>
<tr>
<th>Sub-complexes of NuA4</th>
<th>NuA4 Subunits</th>
<th>Associated Function</th>
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<tr>
<td>Piccolo NuA4 complex</td>
<td>Esa1p</td>
<td>HAT activity</td>
</tr>
<tr>
<td></td>
<td>Epllp</td>
<td>Anchors Piccolo NuA4 to the recruitment complex</td>
</tr>
<tr>
<td></td>
<td>Yng2p</td>
<td>Required for efficient HAT activity and G2/M progression</td>
</tr>
<tr>
<td>Transcription, DNA repair recruitment module</td>
<td>Tra1p</td>
<td>Activator interaction module, also found in SAGA and shown to be involved in targeted recruitment</td>
</tr>
<tr>
<td></td>
<td>Act1p</td>
<td>Shared subunits of the chromatin remodeling complex, SWR1. Perhaps involved in counteracting the spread of heterochromatin.</td>
</tr>
<tr>
<td></td>
<td>Arp4p</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Yaf9p</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Eaf2p</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Eaf1p</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Eaf3p</td>
<td>Perhaps regulates global acetylation</td>
</tr>
<tr>
<td></td>
<td>Eaf5p</td>
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<tr>
<td></td>
<td>Eaf6p</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Eaf7p</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
The NuA4 complex consists of a stable core sub-complex known as Piccolo NuA4, which is sufficient for NuA4 HAT activity (113). Formation of the NuA4 complex requires the association of numerous other proteins with the picNuA4 complex imparting a host of other functions associated with NuA4, including targeted acetylation of promoters (105). The picNuA4 complex is comprised of the catalytic subunit Esa1p, Epllp and the yeast ING homolog Yng2p. Though the picNuA4 complex is capable of interacting with nucleosomal substrates in vivo, it is incapable of being recruited by transcriptional activators, and as such, its functional relevance is unclear. Therefore, it has been suggested that this complex is responsible for maintaining global levels of histone acetylation through a non-targeted mechanism (113, 114). Interestingly, as shown through in vitro HAT assays, disruption of YNG2 negatively affects the ability of picNuA4 to acetylate nucleosomes indicating a role of Yng2p in modulating the HAT function of NuA4 (114).

Additional proteins that associate with picNuA4 impart a greater diversity to the function of NuA4. The essential protein, Tra1p, is a homolog of human TRRAP and has been shown to associate with the picNuA4 complex (47, 115). This is significant because TRRAP, a known component of the human NuA4 complex, has been shown to recruit HAT complexes to promoters of genes through its interaction with transcriptional activators (116). Likewise, in yeast, Tra1p has been shown to be involved in a similar mechanism through its interaction with acidic activators (111). The NuA4 complex has also been shown to play a role in transcriptional co-activation, as examples of NuA4 functioning as a co-activator in both yeast and human cells have been described (105). NuA4 is also shown to play a role in DNA damage repair as its catalytic subunit, Esa1p and an additional subunit, Arp1p, are specifically recruited to double strand break sites in vivo (105). Finally, strains which are deficient in NuA4 HAT activity show
a delay in the G2/M transition phase of the cell cycle indicating a role for NuA4 in cell cycle control (53).

Although Yng2p is not required for cell viability, yng2Δ mutants are severely compromised for growth (53). Loss of Yng2p causes a substantial reduction in the ability of the complex to acetylate nucleosomes, as indicated through in vitro HAT assays (107). The human tumor suppressor protein p53, is shown to interact with NuA4 in a Yng2p dependent manner, and this interaction is required for p53 dependent transcriptional activation in yeast (107, 117). In comparison, the human NuA4 complex has associated with it ING3, suggestive of a link between histone acetylation and p53 dependent gene activation (109). Despite the importance Yng2p displays toward the NuA4 complex, little is known as to the mechanism whereby Yng2p regulates HAT activity.

1.3.3 The Rpd3-Sin3 HDAC Complex

The Rpd3-Sin3 HDAC complex contains a number of proteins subunits including the catalytic subunit, Rpd3p, and the yeast ING homolog, Pho23p (118, 119). Rpd3p was originally linked to transcriptional silencing of a variety of genes including the phosphate responsive gene PHO5 under conditions of high levels of inorganic phosphate (120). The role of Rpd3p in histone deacetylation was identified through characterization of the yeast HDAC complex histone deacetylase A (HDA) (65). The catalytic subunit of HDA, Hda1p, was identified through the purification and analysis of yeast HADC complexes (121). Rpd3p was subsequently identified as an HDAC based on the sequence similarity of RPD3 with HDA1 (65). Additionally, deletion of RPD3 has been shown to coincide with a reduction in the levels of both H3 and H4 acetylation indicating these as the target of its deacetylase activity (64). Though
the Rpd3-Sin3 complex is not essential to yeast, disruption of the complex results in various pleiotropic phenotypes including hypersensitivity to cycloheximide, mating defects, and an inability to sporulate as homozygous diploids (119, 122).

Pho23p was identified through a screen which looked at mutants capable of constitutively expressing PHO5, suggestive of a role for Pho23p in the Rpd3-Sin3 complex (123). Biochemical analysis using immunoprecipitation assays revealed that Pho23p is a stable component of the Rpd3-Sin3 complex (119). Loss of Pho23p negatively affects the HDAC activity of the Rpd3-Sin3 complex as in vitro HDAC assays reveal a greater than 50% reduction in the complex’s ability to deacetylate nucleosomal substrates (95). However, as with Yng2p, little is known as to the mechanism of Pho23p within the Rpd3-Sin3 HDAC complex.

1.4 The Role of Yng1p in the NuA3 Complex and its Applications to Other ING homologs

The central focus of this study was to investigate the role of the yeast ING homolog, Yng1p, within the NuA3 complex. Previous work has shown that while Yng1p is required for both the in vitro and in vivo function of NuA3, it is not required to maintain complex integrity. Thus, we proposed that Yng1p functions in mediating the interaction of NuA3 with chromatin which in turn regulates the HAT activity of this complex.

To investigate the possibility that Yng1p interacts with the nucleosome, we developed the chromatin pull-down assay. Through the use of this assay, in conjunction with genetic analysis, we have shown that NuA3 interacts with the nucleosome and that this interaction is dependent upon Yng1p as well as the H3 tail. Using the sas3A phenotype, we have shown that NuA3 functions in vivo as a HAT, and this function is dependent upon the lysine 14 residue
within the H3 tail. Furthermore, through both genetic and biochemical approaches, we were able to show that methylation of two lysine residues within the H3 tail are required for the interaction and function of NuA3.

The two other ING homologs in yeast, Yng2p and Pho23p, are also found in histone modifying complexes (117). Therefore, we speculated that these other ING homologs were performing similar functions within their respective complexes. However, we failed to identify any mediating function as being performed by either Yng2p or Pho23p. This discrepancy is similar to that seen with the over-expression of human ING cDNAs within yeast, where only ING2 is toxic when over-expressed. We thus surmise that while some ING proteins function in a manner similar to what we have described for Yng1p, others function through mechanisms that have yet to be described.
Chapter 2 – Materials and Methods

2.1 Preparation of Yeast Strains and Plasmids

All yeast strains used in this study are listed in table 2.1. Yeast strain manipulation, including all transformations, plating, sporulation and screening were carried out using standard protocols (124). TAP tagging of *HTB1* required additional PCR product for a successful insertion into the genome. To this end 3x100μL reactions were concentrated, and 40μL of the concentrated product was transformed into the cell. PCR purifications as well as gel purifications were performed using the Wizard SV Gel and PCR Clean-up system (Promega Corporation, Madison, WI).

The plasmid p*HHT2*, with the wild type *HHT2-HHF2* locus was created by ligation of the SpeI fragment from the plasmid pDM18 (125) into the SpeI site of the vector pRS414 (126). *phht2Δ3-29* was created by the insertion of annealed phosphorylated oligonucleotides (5’-GATCCACGCAACACTCCACAATGGCCAGACCATCTA-3’ and 5’-CCGGTAGATGGTTGCTTGAGTGTTTGCTT-3’) into the BamHI and AgeI sites of *pHHT2*. Plasmids containing point mutations in *HHT2* were created by site directed mutagenesis using Stratagene’s QuickChange Site-Directed Mutagenesis Kit.

Plasmids used in the *YNG1* over-expression screens were all constructed using the pGAL416 vector (126). The plasmid pY1OE was constructed through the incorporation of the *YNG1* open reading frame (ORF) directly downstream of the *GAL1* promoter. For *YNG1* expression analysis, the pGAL.*YNG1.HA.416* plasmid was constructed by first incorporating the HA tag along with a *CYC* terminator into the pGAL.416 vector, using KpnI and SalI restriction sites downstream of the *GAL1* promoter. The plasmid pY2OE was created by subcloning the
YNG1 ORF into the pGAL.HA.CYC.416 vector (the pGAL.416 expression vector with the 3xHA.CYC terminator cassette inserted adjacent to the GAL1 promoter) downstream of the GAL1 promoter and directly adjacent to the 3xHA.CYC cassette. The insertion was accomplished through the use of the BamHI and SalI restriction sites corresponding to the 5' and 3' ends respectively. Similar to the creation of pY1OE, the plasmids pING1OE – pING5OE were constructed by insertion of ING cDNAs (courtesy of G. Li and J. Cote) into the pGAL.416 vector. All insertions were done downstream of the GAL1 promoter using the restriction sites BamHI and HindIII for ING1, ING3 and ING5, SpeI and BamHI for ING2, and SpeI and HindIII for ING4.

To increase levels of GAL1 induction of YNG1 in set1Δ mutants, the plasmid pY3OE was created. This involved cloning the GAL1 promoter into the high copy vector pRS426. For this the vector pGAL.416 and the plasmids pY1OE and pY2OE were digested with the restriction enzymes Kpn1 and Sac1. The vector pRS426 (126) was similarly digested with Kpn1 and Sac1. The digested vector was treated with calf intestinal phosphatase (CIP) to dephosphorylate the cut ends to reduce ligation of the vector to itself. All digested products were gel purified and ligations were performed overnight at 16°C. These ligations produced the vector pGAL.426 and the plasmids pGAL.YNG1.426 (pY3OE) and pGAL.YNG1HA.426 (pY4OE).

pYNG2 consists of the YNG2 ORF along with 5' and 3' flanking sequences containing an endogenous Kpn1 site (~1,500 bp upstream) and an engineered Sac1 site (~1,000 bp downstream) respectively. Digestion of the amplified product containing the YNG2 ORF was performed by ligation of PCR product into the pGEM-T vector (Promega Corporation, Madison, WI) followed by a double digestion of the ligated vector and gel purification of digested products prior to ligation into the vector pRS416.

For the insertion of the N-terminally FLAG tagged HTB1 ORF we used the plasmid pFLAG-HTB1. This was plasmid was obtained through using the vector FLAG-pET11d which
contained the FLAG epitope tagged *HTB1* with an in-frame fusion of the FLAG epitope onto the N-terminus of *HTB1* (127). The FLAG-*HTB1* gene was subcloned into pRS415 containing the *LEU2* biosynthetic marker resulting in the pFLAG-HTB1 plasmid (126). Digestion with the restriction enzyme BstxI created a single cut site within the *LEU2* ORF. The digested product was then transformed into yeast strains YDM001 and YDM002 using standard procedures, and transformants were selected for on synthetic drop-out media lacking leucine.
<table>
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<th>Strain</th>
<th>Description</th>
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<td>FY602</td>
<td>matα his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63</td>
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<td>750</td>
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<td>matα his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 NTO1TAP::TRP1 sas3::HISMX6 LEU2::SAS3HA</td>
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Table 2.2 Plasmids Used in This Study

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2.2 PCR Techniques

All gene tagging and deletions were accomplished using high efficiency transformation of PCR product obtained using Taq polymerase enzyme. A 100 μL reaction was the typical reaction volume for PCR product to be used in either an insertion or deletion. Screening for insertions or deletions was done using PCR with genomic DNA as a template. All reactions using Taq polymerase enzyme for amplification were carried out using the standard elongation protocol (92°C for 30 s. with 30 cycles of 92°C for 45 s., 55°C for 45 s. and 72°C for 1 min).

The subcloning of YNG2 as well as all ING cDNAs was carried out using the PCR enzyme Pfu Turbo from Stratagene. Primers were designed to anneal within (and adjacent to the ends of) the ORFs, and included the necessary restriction sites. All amplifications were done in 50 μL volumes. Set-ups for Pfu Turbo reactions involved 1 μl of Pfu Turbo enzyme, 5 μL of Pfu Turbo 5x buffer, 4 μL of 2.5 mg/mL dNTPs and 0.25 μL of each primer (initial concentration of 100 mM). Concentrations for templates varied depending upon the source. A total of 100ng of template was added to each reaction for templates derived from genomic DNA, while only 50ng of template was added to the reactions for templates derived from plasmid DNA. All Pfu Turbo amplifications followed the same basic program (95°C for 30 s. followed by 15 cycles of 95°C for 30 s., 55°C for 1 min and 68°C at 1 min for each kb amplified).

2.3 Preparation of Whole Cell Extracts for Chromatin Pull-down Assays

Inoculations from freezer stocks were performed in 5 mL of yeast extract-peptone-dextrose (YPD) and allowed to grow overnight at 30°C with shaking. Whole cell extracts were prepared by diluting overnight cultures in 50 mL of YPD to an optical density at 600nm (OD_{600}) of 0.5 and growing cultures at 30°C to an OD_{600} of 2.0. Cultures were harvested by
centrifugation (3 min. at 4,000 rpm) at 4°C. Cells were washed in 25-mL of distilled water and subsequently resuspended in 500 μL of IPP 150 buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% NP40 with 1 mM PMSF and 2 μg/mL pepstatin A). An equivalent volume of glass beads were added to the cell suspensions and samples were vortexed for 3 min using a Vortex Genie (Scientific Industries, Bohemia, NY). Cell lysates were clarified by centrifugation (5 min at 14,000 rpm) at 4°C.

2.4 Chromatin Pull-down Assays

Bradford assays were performed on all whole cell extracts to normalize for bulk protein content. IgG sepharose 6 Fast Flow resin (Amersham Biosciences, Piscataway, NJ) was washed 4x with cold IPP 150 buffer and aliquoted in 20 μL volumes. To each 20 μL aliquot, 400 μL of normalized whole cell extracts were added. Samples were then rotated at 4°C for 2 h. Resin was washed 3x with 20 volumes of cold IPP 150 buffer prior to boiling in SDS sample buffer. Purified proteins were then analyzed using western blot analysis with anti-HA antibodies (Roche).

2.5 Preparation of Whole Cell Extracts for Calmodulin Pull-downs

Inoculations from freezer stocks were performed in 5 mL of yeast extract-peptone-dextrose (YPD) and allowed to grow overnight at 30°C with shaking. Whole cell extracts were prepared by diluting overnight cultures into 4x1 L of YPD to an appropriate optical density at 600nm (OD₆₀₀) allowing cultures to grow overnight at 30°C with shaking to an OD₆₀₀ of 2.0. Cultures were harvested by centrifugation (10 min at 4,000 rpm) at 4°C. Each 1 L portion was
washed in 100 mL of distilled water. Cells from each 1 L culture were combined and subsequently resuspended in 60 mL of calmodulin binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM CaCl$_2$, and 0.1% NP40 with 1 mM PMSF and 2 μg/mL pepstatin A). The cell suspensions were added to an 80 mL bead-beater chamber (BSP bead-beater, Biospec Products, Bartlesville, OK) along with ~20 mL of glass beads. Cells were broken open by 10 cycles of bead-beating (15 s. bead-beating followed by 1 min rest). Lysates were clarified by centrifugation (10,000 rpm for 30 min) resulting in the whole cell extract.

2.6 Calmodulin Pull-down Purifications

Calmodulin affinity resin (Stratagene, Cedar Creek, TX) was washed 4x with cold calmodulin binding buffer and aliquoted in 200 μL volumes. To each 200 μL aliquot, 60-mL whole cell extracts were added. Samples were then rotated at 4°C overnight. Resin was washed 3x with 20 volumes of cold calmodulin binding buffer. Purified proteins were then eluted off the calmodulin resin by washing resin with calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-Cl pH8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM EGTA, and 0.1% NP40). Three washes were performed using 200 μL of elution buffer. The first wash involved rotating resin with elution buffer for 30 min at 4°C. Subsequent washes were performed in a stepwise fashion with no incubation period. Western blot analysis, with anti-HA antibodies (Roche), was used to determine which elution contained the maximal amount of purified protein.

2.7 Preparation of Whole Cell Extracts for YNG1 Expression Analysis
All plasmids used for YNG1 expression analysis, along with their corresponding vectors (as a control) were transformed into yeast strains that were to be tested for resistance to YNG1 over-expression. Transformants were grown up in 50 mL of synthetic drop-out media lacking uracil, with galactose as the carbon source. All strains were harvested at an OD\textsubscript{600} of 1.0. Whole cell extracts were prepared in 300 µL of 5x SDS-PAGE loading buffer and 10 µL of each whole cell extract was added to 10 µL of 2.5x SDS loading buffer and boiled for 5 min at 100°C. Expression analysis was performed by western blot analysis (on 10 µL of prepared extracts) using anti-HA antibodies (Roche).

2.8 The H3 Tail Pull-Down (\textit{in vitro} analysis)

The pH3GST plasmid (13) was transformed into BL21 (DE3) cells and expression of the H3-GST construct was induced by growing transformed cells in 500 mL of LB + with 1.0mM of IPTG for 3 h. at 37°C. Cells were harvested by centrifugation (4000 rpm for 10 min) and washed with 25 mL of 1x phosphate buffered saline (PBS). Cells were resuspended in 1 mL of 1xPBS to which an equivalent volume of glass beads were added and cells were broken open by vortexing for 3 min using a Vortex Genie (Scientific Industries, Bohemia, NY). 25 µL of 20% Triton-X-100 was added to each lysate and samples were rotated for 20 min at 4°C.

Purification of the H3 tail constructs was accomplished using glutathione sepharose resin (Amersham Biosiences, Piscataway, NJ). Lysates were clarified by centrifugation (14,000 rpm for 5 min) and added to 200 µL of glutathione resin that had been washed 3x with 1xPBS. The lysates were rotated with the glutathione resin for 2 h. at 4°C. The resin was then washed 3x with cold 1xPBS.
The 200 µL of resin that was used in the pull-down was divided into two 100 µL portions. Using glutathione elution buffer (50mM Tris-Cl pH 8.0, 10mM reduced glutathione) purified H3-GST constructs were eluted off the glutathione resin, for one of the divided portions, and subsequently used for HAT assays. The H3-GST constructs of the remaining portion were left bound to the glutathione resin as an immobilized substrate for its use with the \textit{in vitro} pull-down assays.

\textit{In vitro} pull-downs were performed by diluting 20 µL of purified NuA3 into 180 µL of calmodulin elution buffer. To this dilution 7.5 µL of glutathione resin (containing the immobilized H3-GST construct as described above) was added and allowed to rotate at 4°C for 2 h. Resin was washed 3x with 20 equivalents of cold calmodulin elution buffer. 30 mL of 2.5x SDS loading buffer was then added to the resin which was then boiled for 5 min at 100°C. Precipitation of NuA3 was then detected using western blot analysis with anti-HA antibodies (Roche).

2.9 Histone H3 Acetylation Analysis

H3 acetylation western blots were performed on \textit{setl}Δ and \textit{set2}Δ mutants in both wild type and \textit{gcn5}Δ mutant backgrounds. Strains were grown and harvested as described previously. Whole cell extracts were prepared in 400 µL of IPP 150 with PMSF and pepstatin. Whole cell extracts were normalized for bulk protein content using a Bradford assay prior to addition of SDS sample buffer. Western blots were performed using anti-acetyl-H3 antibodies (Biotech, Lake Placid, NY).
2.10 *In vitro* HAT Assays

HAT assays were performed following the standard protocol. For each individual HAT assay was performed by adding, 4 μL of 5xHAT buffer (250 mM Tris-Cl pH 8.0, 25% glycerol, 0.5 mM EDTA, 250 mM KCl, 5 mM DTT and 50 mM butyrate), 1 μL of substrate (histones or nucleosomes), 2 μL of purified HAT, and 2μL of $^3$H acetyl coenzyme A (MP Biomedicals, Irvine, CA), brought to a final volume of 20 μL with distilled H$_2$O. Samples were incubated at 30°C for 30 min. The total volume was then spotted onto a P81 filter (cut in half) and allowed to dry for 10 min. Spotted samples were washed 3x ~80 mL HAT wash buffer (from 10x stock – 32.5g NaHCO$_3$, 12g Na$_2$CO$_3$ in 1 L of distilled H$_2$O) for 5 min. each. Filters were then washed briefly in acetone and allowed to dry for 20 min. Amounts of $^3$H acetyl-coA present in each sample were determined by placing filters in scintillation vials with 5 mL of scintillation cocktail (Fisher Chemical, Fair Lawn, NJ) counting radioactive units with the use of a scintillation counter (Beckman Industries).

2.11 Plasmid Shuffle

Each plasmid containing either the wild type *HHT2* gene or some mutant version thereof was transformed into the parent strain FY2162 (125) or one of its derivatives. Selection for transformants was carried out on synthetic drop-out media lacking tryptophan. A total of six ten-fold serial dilutions were performed for each clone in water. Each of these dilutions were spotted on synthetic complete (SC) media (control) and 5'-FOA plates (0.1% of 5'-flouroorotic acid) (to select for loss of the plasmid *pHHT2/URA3*, containing the wild type *HHT2-HHF2* locus) and grown at 30°C for 3 days.
2.12 YNG1 Over-expression Screen

The yeast strain, YPH1662 [P. Hieter] was transformed with the plasmid, pGAL.YNG1.416. This query strain was then robot pinned onto media lacking uracil. The query strain was mated to each of the strains contained within the yeast deletion set by pinning each deletion mutant on top of the previously pinned query strain and grown for one day at 25°C. The resulting MATα/α zygotes were pinned onto synthetic drop-out media lacking lysine and uracil and grown for two days at 25°C. A second round of diploid selection was carried out by repinning and growing for an additional day at 25°C. Diploids were pinned onto sporulation media (2% agar, 1% potassium acetate, 0.1% yeast extracts, 0.05% glucose, supplemented with histidine, and leucine) and incubated for nine days at 25°C. To select for the growth of MATα spore progeny, spores were plated onto synthetic drop-out media lacking histidine and arginine and containing canavanine and incubated for two days at 25°C. The MATα cells were repinned onto synthetic drop-out media lacking histidine, arginine and uracil with canavanine for a second round of selection and incubated at 25°C for 1 day. Next, to select for spores that contained the deletion mutation from the parent strain, MATα haploids were pinned onto synthetic drop-out media lacking histidine, arginine and uracil with canavanine and G418 sulfate and allowed to grow at 25°C for one day. Finally, to select for mutants that were resistant to the over-expression of YNG1, MATα spores were repinned onto synthetic drop-out media lacking histidine, arginine and uracil with canavanine and G418 sulfate and galactose (as the sole carbon source) to induce expression of YNG1. These strains were then incubated for one day at 25°C. Resistance to YNG1 over-expression was assessed on a growth/no growth basis. ¹

¹ All work done on the YNG1 over-expression screen involving the yeast deletion set was performed by Dr. Kristin Baetz. The yeast deletion set and robot pinning equipment were compliments of Dr. Philip Hieter.
Chapter 3 – Results

3.1 NuA3 Interactions with the Nucleosome

3.1.1 Yng1p is Required for \textit{in vitro} Function of NuA3

The H3 specific histone acetyltransferase (HAT) complex, NuA3, is a multiprotein complex containing at least four subunits. One recently identified subunit, Yng1p (a yeast ING homolog), has been shown to play a role in NuA3 function (100). Both biochemical and genetic studies performed on Yng1p have indicated that it is required for the \textit{in vitro} and \textit{in vivo} HAT activity of NuA3 (100). We therefore wanted to use these published results to assess the capability of a new purification scheme. Thus, the initial question we wished to addressed focused on whether or not we could reproduce the biochemical data showing that Yng1p is required for the \textit{in vitro} HAT activity of NuA3. The previously described protocol used for purifying NuA3 for \textit{in vitro} HAT assays involved affinity purification of NuA3, through the incorporation of a FLAG epitope on the C-terminus of the NuA3 subunit TAF14 (100).

Here we proposed to purify NuA3 from both wild type and \textit{yng1\Delta} strains using an affinity purification technique employing the tandem affinity purification (TAP) epitope. The TAP epitope was inserted at the C-terminus of the Ntolp subunit of NuA3 as opposed to a FLAG epitope insert as described previously (100). Additionally, a 3xHA tag was inserted at the C-terminus of Sas3p, the catalytic subunit of NuA3. Affinity purification and subsequent elution of purified NuA3 was done in accordance with the outlined protocol for calmodulin pull-down purification (128). To ensure that roughly equal amounts of NuA3 were purified from either wild type or \textit{yng1\Delta} strains western blot analysis was performed on the purified samples using anti-HA antibodies to normalize levels of NuA3 with respect to Sas3p. The result of this
normalization showed that more NuA3 was isolated from the yng1Δ strain as compared to wild type (Fig. 3.1A). The HAT activity of each sample was investigated through the use of HAT assays. HAT activity of each purified complex (using 1:1 dilution of NuA3 purified from yng1Δ strains) was determined by assaying the amount of titrated acetyl-CoA that was transferred onto recombinant histones purified from bacteria. In agreement with previously published data, loss of the Yng1p subunit results in a substantial reduction in the HAT activity of NuA3 in vitro (Fig. 3.1B) (100).

3.1.2 The Chromatin Pull-down Assay

Having reproduced the results that Yng1p is required for the in vitro HAT activity of NuA3, we next wished to explore the possible functional roles of Yng1p. While the evidence that Yng1p is required for NuA3 function was quite conclusive, the role of Yng1p within the NuA3 complex still remained unclear. An additional study had shown that Yng1p is not required for the integrity of the NuA3 complex, where strains lacking YNG1 still contain an otherwise intact NuA3 (100). One obvious possibility for the role of Yng1p function is mediating the interaction of NuA3 with the nucleosome. Indeed preliminary work, done in vitro, has indicated that Yng1p appears to play such a role within the cell (100). With these considerations in mind, we were interested in investigating the hypothesis that Yng1p mediates the interaction of NuA3 with the nucleosome. While an obvious method to test this hypothesis would be the use of a chromatin immunoprecipitation (ChIP) assay, there are no reports, to date, of Sas3p being “ChIPed” anywhere. Therefore, due to the inability to ChIP Sas3p to any region of the genome, it became necessary to develop an alternative approach to study the proposed interaction of Yng1p with the nucleosome.
Figure 3.1 Ynglp and its role in the NuA3 complex.

(A) More NuA3 is recovered from yng1Δ strains. NuA3 purification was performed through C-terminal insertion of the TAP tag into NTO1 in both wild type and yng1Δ strains expressing Sas3HA3. Purified samples were normalized through western blot analysis using immunodetection for HA. (B) Yng1p is required for NuA3 in vitro HAT activity. HAT assays were performed on purified recombinant histone substrates using NuA3 purified from wild type and yng1Δ (1:1 dilution used) strains containing C-terminal NTO1TAP. Wild type strain containing no TAP insertion was used as a negative control. Experiments were done in triplicate where results represent the mean and error bars indicate the standard deviation between all three trials.
It was reported that the H2B subunit of the histone octamer could be epitope tagged without causing any deleterious effects to the cell (127). Therefore, we proposed a type of co-immunoprecipitation (co-IP) experiment in which H2B would be epitope tagged in conjunction with an additional (and unique) epitope tagging of Sas3p of the NuA3 complex. For the original design we constructed a strain that contained a FLAG tag on the N-terminus of HTB1 (gene encoding for H2B) through use of the digested pFLAG-HTB1 plasmid (see Materials and Methods). Subsequent transformation and homologous recombination of the digested product resulted in the expression of the N-terminally tagged FLAG-H2B construct. These transformations were done into the previously described strains with the genotypes SAS3HA, NTO1TAP and SAS3HA, NTO1TAP, yng1Δ (YDM001 and YDM002 respectively). Therefore, although Nto1p contained a C-terminal TAP tag in both strains it was not required for any experimental procedures. Pull-downs were then performed using anti-FLAG antibodies immobilized on sepharose. Although this approach showed potential, there were still some difficulties concerning the consistency of the results. Possibly due to the interaction of anti-FLAG resin with the TAP tag present on the Nto1 subunit, immunoprecipitation was detected in the negative control (Sas3HA3, Nto1pTAP).

To address the problem encountered with the FLAG tag insertion, new strains were developed in which the C-terminus of Sas3p once again contained the HA epitope insertion, while the C-terminus of H2B was TAP tagged (insertion of the TAP tag in HTB1). Pull-downs were then performed (following the chromatin pull-down protocol) on the strains containing Sas3HA3 (negative control) and Sas3HA3, H2BTAP followed by anti-HA western blot analysis. As evidenced by the anti-HA western blot, co-purification of Sas3p was detected in the H2BTAP tagged strain but not in the untagged negative control (refer to Fig. 3.2A). The initial data obtained from this experiment indicated that NuA3 could indeed be co-purified with the nucleosome.
Although no observable phenotypes were noted with the inclusion of the TAP tag onto the C-terminus of H2B, it remained possible that, although *HTB1TAP* was being expressed, its gene product was not being incorporated into the nucleosome. Due the presence of two genes responsible for encoding H2B (*HTB1* and *HTB2*) it was suggested that the gene product corresponding to the untagged H2B product, *HTB2*, was the only H2B product being incorporated into nucleosomes (129). Therefore, concern was raised regarding the potential exclusion of H2BTAP from the nucleosome. To address this concern, an additional anti-acetyl H3 western blot was performed on the affinity purified extracts. The concept behind performing this western resided in the fashion with which the histone octamer assembles to form the nucleosome core particle. In the step-wise fashion that nucleosomes are assembled, the (H3-H4)$_2$ tetramer interacts with newly synthesized DNA onto which the two H2A-H2B dimers associate (6). It therefore follows that if H3 could be detected in the purified extracts the other core histones, H2A, H2B, and H4, would also likely be present. As shown in figure 3.2, acetylated H3 is detected in precipitates containing the TAP tag (Fig. 3.2A). Thus, the presence of H3 would suggest that H2BTAP is associating with other histone proteins resulting in the formation of histone octamers and ultimately nucleosomes.

### 3.1.3 The NuA3 – Nucleosome Interaction is Dependent on Yng1p

The development of the chromatin pull-down assay promised a method whereby the interaction NuA3 with the nucleosome could be studied. The chromatin pull-down assay was therefore employed to address the previously proposed question which addressed whether or not Yng1p was required to mediate NuA3 interaction with the nucleosome. To this end, strains were designed to assess comparative differences between the NuA3 – nucleosome interaction seen in both wild type and *yng1Δ* strains through the use of chromatin pull-down assays.
Construction of the strains took place in a manner similar to that previously described (containing Sas3pHA, H2BTAP insertions). However, due to the requirement of this experiment for strains lacking Yng1p, insertions were done in both wild type and yng1Δ strains. Pull-downs were performed on SAS3HA, yng1Δ (negative control) and SAS3HA, yng1Δ, HTB1TAP along with the wild type epitope tagged strains as before, following the chromatin pull-down protocol. Co-precipitation of NuA3 with H2BTAP was once again detected with anti-HA western blot analysis. As shown in figure 3.2B, less NuA3 co-precipitated with H2B in the strain lacking Yng1p as compared to wild type. Furthermore, by way of control, an anti-acetyl H3 western was performed which indicated that approximately equal amounts of H3 were being pulled down in both wild type and yng1Δ strains (Figure 3.2B). Although western blot analysis revealed only a modest reduction in NuA3 co-precipitation upon loss of Yng1p, this result is highly reproducible providing preliminary evidence that Yng1p facilitates NuA3 interaction with the nucleosome. These results are in accordance with previously published genetic and biochemical data suggesting that the requirement of Yng1p for the HAT function of NuA3 may be a result of Yng1p mediating the NuA3 – nucleosome interaction (100).

3.2 The H3 Tail is Required for NuA3 Function

Development of the chromatin pull-down assay proved to be a valuable technique for investigating Yng1p role in the cell. Results obtained from this study, in accordance with previous published work, indicated that NuA3 interacts with the nucleosome and this interaction is dependent upon Yng1p (100). While the chromatin pull-down assay was used to show that NuA3 interacted with the nucleosome, it was not clear what region of the nucleosome was required for this interaction. Possible regions of interaction for NuA3 with the nucleosome included nucleosomal DNA, as well as the core histone proteins. Therefore, we wished to
Figure 3.2 NuA3 interacts with chromatin and this interaction requires Yng1p.

(A and B) Chromatin pull-down experiments were performed on the indicated strains expressing Sas3HA3. Co-precipitation of NuA3 with H2BTAP was detected through anti-HA western blot analysis. Samples were normalized using anti-acetylated H3 western blot analysis.
examine possible regions of interaction with respect to the nucleosome. Initially we began our investigation using an *in vitro* technique, however due to technical problems with the assay it was necessary to explore the use of an alternative method. Although the chromatin pull-down assay was designed to study Yng1p, we realized that its use as a biochemical tool was not limited to the study of Yng1p alone. Thus, based on the success of the chromatin pull-down assay, in identifying Yng1p as a possible interacting partner with the nucleosome, as well as its adaptability, we devised an alternate application for this assay, namely its use in mapping the site of interaction of NuA3 with the nucleosome.

### 3.2.1 *In vitro* NuA3 Pull-down Using Recombinant H3 tails

With the evidence pointing toward Yng1p mediating the interaction of NuA3 with the nucleosome, we wished to ascertain what part of the nucleosome NuA3 (and perhaps Yng1p) was interacting with. HAT complexes have been shown to acetylate histones in a manner that is not random, but rather preferentially acetylate certain regions of the genome (69). Indeed, correlations have been drawn between regions of the genome that are hyper acetylated and regions that are actively undergoing gene transcription (54, 130). Some clues as to how HATs preferentially acetylated promoters of actively transcribed genes have come from the identification of HAT recruitment by certain transcriptional activators (111). While targeted recruitment of HATs suggests a mechanism whereby histones are acetylated in a specific manner, other mechanisms of HAT recruitment have recently been suggested. Reminiscent of the histone code hypothesis, the acetylation status of histones has been shown to have a synergistic relationship with other post-translational modifications of residues within histone tails (23, 26). Indeed, one recent report has shown that methylation of lysine 4 of histone H3 facilitates the HAT activity of other H3 HAT complexes (77). These reports suggest that HAT complexes may, at least in part, associate with the histone tails themselves.
Although previous data provided little evidence as to where on the nucleosome NuA3 would interact, it was reasoned that due to NuA3 being a histone H3 specific HAT, a likely site of interaction on the nucleosome would be the H3 tail itself (46, 103). Furthermore, we wished to obtain more information regarding the proposed interaction of Yng1p with the nucleosome. To explore this proposed functional role of Yng1p further, we wanted to address the possibility that Yng1p is required for NuA3 – histone interactions. To this end, an *in vitro* pull-down assay was developed, which was designed to assess the interaction of recombinant histone H3 tails with purified NuA3.

Recombinant histone H3 tails were constructed by inserting the nucleotide sequence corresponding to amino acid residues 1 through 46 of the H3 tail into the pGEX2T expression vector containing the *LacZ* promoter (13). Induction and subsequent purification of the H3-GST construct was performed in accordance with the outline protocol resulting in two 100μL portions of glutathione resin containing the bound H3-GST construct. The H3-GST construct was eluted off of one of the 100μL portions and this eluted H3-GST construct was used as the histone substrate for *in vitro* HAT assays.

As a control, we performed HAT assays, in conjunction with *in vitro* pull-down assays, on the purified H3-GST construct to provide additional evidence that NuA3 interacted with the H3-GST construct. These HAT assays were performed using NuA3 purified from both wild type and *yng1Δ* strains (as described previously using the calmodulin pull-down protocol) where both the GST (negative control) and H3-GST purified constructs were used as histone substrates. Results showed, in agreement with previous data, that loss of Yng1p does negatively affect the HAT activity of NuA3 on the H3 tail (Figure 3.3A).

Bound GST (negative control) and H3-GST tail constructs were then used to pull-down NuA3 purified from either wild type or *yng1Δ* strains. The interaction of NuA3 with the
recombinant H3 tail constructs were assayed through western blot analysis. The preliminary results we obtained from this assay indicated that these constructs could indeed pull-down purified NuA3 (Figure 3.3B). However, this technique showed problems especially in the area of reproducibility of results. As we had shown previously with the use of the chromatin pull-down assay, loss of Yng1p had a negative effect on NuA3 – nucleosome interaction. Therefore, although we observed an interaction of NuA3 with the H3-GST construct we were unable to reproduce the data seen in our chromatin pull-down experiment. That is, loss of Yng1p did not have any negative effects on the ability of NuA3 to bind the H3-GST construct.

3.2.2 Loss of the H3 Tail is Disruptive to the NuA3 – Nucleosome Interaction

Due to the problems that surrounded the in vitro pull-down protocol, we needed to develop an alternative method for assaying the interaction of NuA3 with the H3 tail. One success of the in vitro pull-down assays was that the preliminary results obtained seemed to suggest that the H3 tail does interact with NuA3. To assess this interaction further we decided to modify our chromatin pull-down assay and investigate the in vivo interaction of NuA3 with the H3 tail. To test this possibility, a mutant strain was constructed in which amino acid residues 3-29 of the H3 tail were deleted. Deletions were done on pHHT2 resulting in the plasmid phht2Δ3-29 which was subsequently transformed into the strain FY2162 (125) (in which all genomic copies of H3 and H4 genes were deleted) in accordance with the plasmid shuffle protocol. As a control, the wild type plasmid pHHT2 was also transformed into FY2162. It should be noted that while we refer to the plasmids pHHT2 and phht2Δ3-29 as expressing wild type or truncated versions of HHT2 respectively, they also contain a wild type copy of HHF2 (the gene that encodes for histone H4) allowing for expression of both H3 and H4 genes.
Figure 3.3 NuA3 Interacts with the H3 Tail in vitro.

(A) NuA3 acetylates recombinant H3 tails, and requires Yng1p for this HAT activity. HAT assays were performed on NuA3 purified from either wild type or yng1Δ strains containing a C-terminal TAP epitope insertion within the Nto1p subunit of NuA3 (untagged NuA3 used as a control). Purified recombinant H3 tail constructs (with amino acids 1-46 of the H3 tail) containing a C-terminal GST tag were used as histone substrates for NuA3 HAT activity, with GST alone as a control. (B) NuA3 interacts with recombinant H3 tails. NuA3 in vitro pull-downs were performed by incubating purified NuA3 (calmodulin pull-down protocol) with the H3-GST construct which was immobilized on glutathione resin. Interactions were assessed using western blot analysis with anti-HA antibodies.
Chromatin pull-downs were then performed in each of these strains through epitope tagging of both Sas3p and H2B (C-terminal HA and TAP tags respectively). Co-precipitation of NuA3 with H2B was detected by anti-HA western blot analysis, which revealed that loss of the H3 tail results in a substantial reduction in the interaction of NuA3 with the nucleosome (Figure 3.4A). To ensure that the reduction of NuA3 interaction seen in the HA western was not due to less nucleosomes being pulled down in the \textit{hht2}A3-29 mutant, as compared to wild type, a control western was performed using anti-acetyl H4 antibodies. The anti-acetyl-H4 western revealed that essentially equal amounts of H4 were present in both the wild type and \textit{hht2}A3-29 mutant strain pull-downs suggesting that loss of Sas3HA signal was due to a reduction in the interaction of NuA3 with the nucleosome (Figure 3.4A).

3.2.3 Genetic Analysis of NuA3 Interaction With the H3 Tail – The \textit{sas3}\Delta Phenotype

The requirement for an alternative method in studying the interaction of NuA3 with the nucleosome led to the development of a genetic assay employing the use of a \textit{sas3}\Delta specific phenotype. As described previously, deletion of \textit{SAS3} in conjunction with \textit{GCN5} results in a synthetic lethality (103). Furthermore, this synthetic lethality is not dependent upon \textit{GCN5}-dependent HAT activity as loss of either \textit{ADA2} or \textit{ADA3} in conjunction with \textit{SAS3} is not lethal. Additionally, this \textit{sas3}\Delta phenotype seems to be confined to components of the \textit{SAS3}-dependent acetylation pathway. This is seen for mutants of components within the NuA3 complex which result in loss of NuA3 HAT activity such as \textit{yng1}\Delta and \textit{ntol1}\Delta. When either of these mutations is combined with the \textit{gcn5}\Delta mutation, severe growth defects are observed. The double mutant \textit{gcn5}\Delta\textit{yng1}\Delta exhibits severe synthetic growth defects while \textit{gcn5}\Delta\textit{ntol1}\Delta mutants are synthetically lethal (100). However, as seen with the \textit{sas3}\Delta mutant either \textit{yng1}\Delta or \textit{ntol1}\Delta
mutations in combination with an \( \text{ada2}\Delta \) mutation results in no observable growth defects (L. Howe personal communication) (100).

Thus, as described previously, the properties of this \( \text{sas3}\Delta \) specific phenotype provides a useful tool in the identification and study of components within the Sas3p dependent acetylation pathway (103). Therefore, to explore genetically the possibility that H3 is required for NuA3 – nucleosome interaction, \( \text{GCN5} \) and \( \text{ADA2} \) were deleted in strains which carried deletions in both \( \text{HHT1} \) and \( \text{HHT2} \) loci and \( \text{HHT2} \) was expressed from a \( \text{URA3} \) based plasmid. \( \text{TRP1} \) based plasmids expressing \( \text{HHT2} \) and \( \text{hht2}\Delta 3-29 \) were transformed into these deletion mutants. The resulting deletion mutants were then plated on 5'-FOA to select for the \( \text{URA3} \) based plasmid loss.

We anticipated that if the H3 tail was required for NuA3 interaction, deletion of the H3 tail, in conjunction with \( \text{gcn5}\Delta \) would result in a synthetic lethality. However, due to the nature of this particular \( \text{sas3}\Delta \) phenotype, we would also expect that deletion of the H3 tail in \( \text{ada2}\Delta \) mutants would not be lethal. When subjected to negative selection for the \( \text{URA3} \) based plasmid, no growth was observed for \( \text{gcn5}\Delta \text{hht2}\Delta 3-29 \), while \( \text{ada2}\Delta \text{hht2}\Delta 3-29 \) strains were still viable (Fig. 3.4B). This synthetic lethality demonstrates that the H3 tail displays a \( \text{sas3}\Delta \) specific phenotype, supporting the hypothesis that NuA3 depends on the H3 tail for its interaction with the nucleosome.

### 3.2.4 NuA3 Function is Dependent on Lysine 14

The Sas3p phenotype exhibited by the \( \text{gcn5}\Delta \text{hht2}\Delta 3-29 \) synthetic lethality indicated that NuA3 requires interaction with chromatin for function (100). However, it is possible that although NuA3 is recruited to nucleosomes, its acetylation target resides somewhere other than histone proteins. Alternatively, it is possible that NuA3 is interacting with some part of the
Figure 3.4 The H3 tail is required for NuA3 function.

(A) Chromatin pull-down assays were performed on the indicated Sas3HA3 expressing strains. Samples were normalized using anti-acetyl H4 western blot analysis. (B) Yeast strains YLH224 (hht1-hhf1Δ hht2-hhf2Δ), YLH289 (hht1-hhf1Δ hht2-hhf2Δ gcn5Δ), and YLH290 (hht1-hhf1Δ hht2-hhf2Δ ada2Δ) containing the indicated plasmids were plated on either synthetic complete medium (control) or synthetic complete medium with 5′-FOA and incubated at 30°C for 3 days.
nucleosome other than histone H3. For NuA3 to function there must be two events which occur: first NuA3 must bind to its substrate (perhaps a histone) and second it must acetylate the histone. Deletion of the H3 tail resulted in the disruption of the putative acetylation target of NuA3, the H3 tail, as well as the proposed site of NuA3 interaction, the H3 tail itself. To address these possibilities, we wanted to look at whether or not loss of the putative acetylation target of NuA3 within the H3 tail exhibited a sas3A phenotype. It has been previously reported that NuA3 acetylates lysine 14 (and to a lesser extent lysine 23) of H3 (103). Therefore, to test for the existence of a sas3A phenotype we once again employed the \textit{gcn5A ada2A} genetic interactions with sas3A mutants to test the importance of lysine 14 for the function of NuA3.

Point mutations were carried out using site-directed mutagenesis on the plasmid pHHT2. The resulting mutant plasmid \textit{phht2K14R} was transformed into either \textit{gcn5A} or \textit{ada2A} mutant strains which also contained deletions of both \textit{HHT1} and \textit{HHT2} loci with \textit{HHT2} being expressed from a \textit{URA3} based plasmid. Strains were then plated on 5' FOA to select for cells which have loss of wild type \textit{HHT2}. Selection for the \textit{TRP1} based \textit{phht2K14R} plasmid showed severe growth defects in the \textit{gcn5A} mutant strain (Fig. 3.5A). Consistent with the sas3A phenotype, this mutation was tolerated in the \textit{ada2A} mutant strain which showed no discernable growth defects as compared to wild type (Fig. 3.5A) (103).

Although the sas3A phenotype is exhibited by the lysine 14 mutants, it is possible that the resulting synthetic lethality is due to loss of interaction of NuA3 with the nucleosome. Thus, to examine whether or not lysine 14 is required for NuA3 interaction, we performed a chromatin pull-down assay on the lysine 14 mutant. Sas3p was tagged with 3xHA epitope on its C-terminus in the strain expressing \textit{phht2K14R}, and NuA3 was co-precipitated with the nucleosome by TAP tagging the C-terminus of H2B. Figure 3.5B shows that the ability of NuA3 to bind chromatin is not impaired in lysine 14 mutants. Therefore, through use the H3 K14R
A.

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B.

![Image](image7.png)

**Figure 3.5 NuA3 function is dependent on lysine 14.**

(A) Yeast strains YLH224 (hht1-hhf1Δ hht2-hhf2Δ), YLH289 (hht1-hhf1Δ hht2-hhf2Δ gcn5Δ), and YLH290 (hht1-hhf1Δ hht2-hhf2Δ ada2Δ) containing the indicated plasmids were plated on either synthetic complete medium (control) or synthetic complete medium with 5′-FOA and incubated at 30°C for 3 days. (B) Chromatin pull-down assays were performed from the indicated Sas3HA3 expressing strains and the resulting samples subjected to western blot with immunodetection for HA. Samples were normalized by immunoblotting for TAP tagged Htb1p.
mutant we were able to differentiate between the two steps necessary for NuA3 HAT function. Mutation of lysine 14 to arginine removed the putative acetylation target of NuA3 while leaving the hypothesized interaction site of NuA3, the H3 tail, in tact. These results suggest that while lysine 14 is necessary for the function of NuA3, it functions at a step downstream of nucleosome binding. Furthermore, these results complement the H3 tail truncation mutant data in suggesting that the H3 tail is required for NuA3 binding.

3.2.5 The YNG1 Over-expression Phenotype

If over-expressed, the gene encoding the Yng1p subunit of NuA3, YNG1, results in inhibition of cell growth. Based on the data previously obtained regarding the role of Yng1p in mediating NuA3 interaction, it was assumed that the toxicity associated with YNG1 over-expression is the result of its interaction with the nucleosome, and more specifically, the H3 tail. The current model used to explain this toxicity suggests that free Yng1p (Yng1p that is not incorporated into NuA3) binds to the H3 tail. This binding of free Yng1p is favored as a result of a disruption in the equilibrium established between normal levels of Yng1p and its substrate, the H3 tail. The net result of this disequilibrium is an overloading of free Yng1p on the H3 tail, effectively blocking access of NuA3 (and possibly other modifying enzymes) to the H3 tail, and consequently interfering with the normal functions associated with the histone tail. Therefore, it was reasoned that if NuA3 interacts with the H3 tail, and does so through its subunit, Yng1p, loss of the H3 tail should restore growth to a strain in which YNG1 is over-expressed.

To investigate the possibility that Yng1p interacts with the H3 tail, strains containing deletions in HHT1 and HHT2 loci with a TRP1 plasmid expressing either HHT2 or hht2Δ3-29 were transformed with the expression vector pY1OE. As a control, the vector, pGAL.416 was transformed into both wild type and hht2Δ3-29 mutant strains to ensure that the vector did not
interfere with cell growth and to control for the growth rate in the *hht2Δ3-29* mutant strain. The viability of transformed strains was tested through dilution plating assays on both synthetic drop-out plates lacking uracil (URA) (negative control with dextrose as sole carbon source) and URA with galactose. The toxicity associated with *YNG1* over-expression seen in wild type strains was shown to be alleviated in the mutant strain *hht2Δ3-29* (Fig. 3.6A). The resistance of *hht2Δ3-29* to *YNG1* over-expression is suggestive of an interaction between Yng1p and the H3 tail, providing additional support for an interaction between NuA3 and the H3 tail.

It is possible that the resistance seen in *hht2Δ3-29* mutants is due to a reduction in *YNG1* expression from the *GAL1* promoter. To address this concern we wanted to examine the levels of Yng1p within the strains containing the pY1OE vector. To investigate the levels of Yng1p between strains we needed to develop a method that addressed several problem areas. First, we did not have available to us any antibodies that would recognize Yng1p directly; therefore we would need to incorporate an epitope tag within Yng1p. A second issue that required attention was the associated toxicity of *YNG1* over-expression. The toxicity of *YNG1* over-expression in wild type cells would make it impossible to assess the level of Yng1p within the mutant strain relative to that seen in wild type.

To perform this assay a 3xHA epitope tagged *YNG1* gene was created with an in-frame fusion of the 3xHA epitope onto the C-terminus of *YNG1* (see Materials and Methods). Rather fortuitously, incorporation of the 3xHA epitope at the C-terminus of Yng1p addressed both issues simultaneously as addition of this epitope at the C-terminus of Yng1p alleviated its associated toxicity. The resulting plasmid, pY20E, was transformed, into both wild type and *hht2Δ3-29* mutant strains. The vector, pGAL.HA.CYC.416, was also transformed into mutant and wild type strains as a negative control. This was done to ensure that the vector was not adversely affecting the cell, and to show that the signal observed in the western blot represented
the expression of HA tagged Yng1p. Whole cell extracts of the transformed strains was then performed according to protocol. YNG1 expression in both wild type and hht2Δ3-29 mutant strains was then examined by western blot analysis of whole cell extracts, using anti-HA antibodies. As shown in the western blot (Fig. 3.6B), levels of Yng1p were consistent in both the wild type and hht2Δ3-29 mutant strains indicating that the resistance to YNG1 over-expression in the hht2Δ3-29 mutant was not due to reduced levels of Yng1p within the cell.

3.3 Understanding the Interaction of NuA3 with the Nucleosome – An Exemplification of the Histone Code Hypothesis

Through the use of both genetic and biochemical techniques we have shown that NuA3 interacts with the nucleosome. Additionally, our data suggests that the H3 tail is required for this interaction of NuA3 with the nucleosome. While we felt that we had sufficient evidence to say that NuA3 interacts with the H3 tail, we were unsure as to the mechanism regulating the binding of NuA3 to the H3 tail. As other groups have shown in the past, the binding of factors that catalyze modifications to the histone tails are regulated by the histone code (22-24). Histone acetylation has been shown to be dependent upon prior histone modifications by other factors. Recent studies have indicated that the binding of the HAT, SAGA, to the H3 tail is facilitated by methylation or, as shown within some promoters, phosphorylation of the H3 tail (26, 77, 131). These findings indicate that the regulation of HATs, at least to some degree, is accomplished by the histone code. With these findings in mind, we next wanted to address whether or not NuA3 was regulated through a mechanism involving the histone code, and if so, how.
Figure 3.6 Loss of the H3 tail rescues growth in strains over-expressing YNG1.

(A) The yeast strains YDG010 (pHHT2) and YDG009 (pHHT2Δ3-29) were transformed with the indicated plasmids and dilution plated on synthetic drop-out media lacking uracil with either dextrose (negative) or galactose as the carbon source. (B) GAL1 induction is not compromised in mutants lacking the H3 tail. YNG1 expression analysis was performed by transforming the plasmid pY2OE, with the vector alone as a control, into YDM209 and YDM210. Levels of Yng1HA3 were monitored through western blot analysis using immunodetection for HA. Prior to blocking, blots were ponceau stained to ensure equal loading of samples.
As stated previously, YNG1 is toxic when over-expressed, providing what we refer to as the YNG1 over-expression phenotype. Using the knowledge gained from previous experiments, regarding Yng1p, we felt that resistance to YNG1 over-expression, within a mutant, would result because the associated mutation caused a disruption of the NuA3 – nucleosome interaction, as mediated by Yng1p. Therefore, to begin our investigations we made use of this Yng1p associated phenotype by performing a screen aimed at identifying mutants that were resistant to YNG1 over-expression. Once we were able to isolate mutants, we then followed up the characterization of these mutants through biochemical analysis. Loss of an interaction between NuA3 and the nucleosome, in all of the mutants, was assessed with the use of the chromatin pull-down assay. Thus, the main goal of this investigation was to identify any mutants that disrupted the NuA3 – nucleosome interaction. As such, it was anticipated that the information obtained from mutants, showing resistance to YNG1 over-expression, would indicate factors that were involved in regulating the interaction of NuA3 with the nucleosome.

3.3.1 The YNG1 Over-expression Screen

Prior to the development of the chromatin pull-down experiment, attempts were made to establish a genetic method to study the Yng1p interaction with the nucleosome. The requirement of this genetic approach was a readily identifiable Yng1p associated phenotype that had applications for use in a genetic screen. Due to the severity of the YNG1 over-expression phenotype, this Yng1p associated phenotype was chosen as the bases for the genetic screen. Thus, development of the genetic screen was dependent upon the assumption that Yng1p mediates NuA3 interaction with the nucleosome.
The information that was hoped to be gained from this screen included identification of any other factors involved in mediating NuA3 interaction with the nucleosome (via Yng1p), such as enzymes that modify the histone tails, as well as the possible identification of the region of the nucleosome which interacts with NuA3. Thus, the goal was to isolate mutants that were resistant to YNG1 over-expression, and through subsequent characterization of these mutants, draw conclusions regarding the method whereby NuA3 interacts with the nucleosome.

3.3.2 Development of the YNG1 Over-expression Screen – Spontaneous Mutagenesis

Originally the YNG1 over-expression screen was designed using spontaneous mutagenesis of haploid strains of each mating type. Strains of both mating types were transformed with plasmids containing YNG1 under the control of the GAL1 promoter. These strains were then plated onto media that contained galactose as its only carbon source. The rational was that through spontaneous mutagenesis, strains would develop a resistance to YNG1 over-expression when subjected to the conditions stated above. A collection of mutant strains from each mating type were isolated and subsequently mated against the opposite mating type. From the matings, attempts were made to arrange these mutants into complementation groups. Based on their arrangement within complementation groups, these mutants could then be characterized through the use of a genomic library.

The results that we obtained from this spontaneous mutagenesis approach were difficult to interpret. While we were able to obtain mutants that were resistant to YNG1 over-expression, difficulty arose when attempts were made to arrange these mutants into complementation groups. Processing these mutants resulted in the conclusion that the isolated mutants were actually plasmid mutants. It appeared that mutations in the plasmid containing the YNG1 gene under to control of the GAL1 promoter was giving rise to the resistance that we saw in our
mutant collection. Therefore, it became necessary to develop a new approach to screen for mutants resistant to YNG1 over-expression.

### 3.3.3 YNG1 Over-expression in the Yeast Deletion Set

As an alternative approach to spontaneous mutagenesis, a genetic screen was designed in which YNG1 was over-expressed throughout the yeast deletion set. Use of the deletion set for this genetic screen held the potential to allow recognition of entire genes whose products were responsible for mediating the interaction of Yng1p with the nucleosome. Identification of these genes would take place by examining which deletion mutants show resistance to YNG1 over-expression, and then rationalizing these results by taking into account their respective role within the cell. Based on the putative role of Yng1p in the cell, if a deletion mutant was shown to be resistant to YNG1 over-expression, the gene product of the deleted gene would be assumed to be involved in mediating Yng1p interaction with the nucleosome. Typical gene targets that we anticipated to be identified using this screen included genes encoding for either, proteins that modified histones, or proteins that were known to interact with histones/nucleosomes in some manner.

The over-expression of YNG1 in the yeast deletion set consisted of the transformation of the \textit{URA3} based expression vector pY1OE into a strain with mating type \( \alpha \). The transformed mat \( \alpha \) strain was then mated to each strain contained in the deletion set. These mated strains were subsequently sporulated followed by a selection process designed to isolate haploid strains containing both the gene deletion, as well as the plasmid pY1OE (refer to materials and methods for a detailed description of experimental design). Mutant strains were screened for resistance to \textit{YNG1} over-expression by plating the isolated haploid strains on \textquotesingle UR\textquotesingle (as a control) and \textquotesingle URA\textquotesingle with galactose media. Resistance to \textit{YNG1} over-expression was scored on a growth/no growth
basis with resistance being characterized as growth on URA galactose media. Initial results from this screen showed that deletion mutants, \textit{lge1Δ, duslΔ} and the unnamed ORF \textit{ylr177Δ} were all resistant to \textit{YNG1} over-expression (Fig. 3.7).

The resistance seen in the \textit{lge1Δ} mutant proved interesting due to the functional role associated with Lge1p. Lge1p is a component of the Rad6 complex which has been shown to ubiquitinate lysine 123 of histone H2B (81). Although this ubiquitination does not directly target the H3 tail, it does appear to serve as a signal for the di- and tri-methylation of lysine 4 of histone H3 by the Set1p containing COMPASS complex (78). Indeed, it has been reported that, while mono-methylation of lysine 4 of histone H3 by Set1p is not dependent upon the Rad6 complex, di- and tri-methylation of this residue, by Set1p, does require the prior ubiquitination of H2B by the Rad6p complex. (30, 31, 81, 132). Moreover, in terms of NuA3 function, this methylation of H3 by Set1p is suggestive of a possible signal to facilitate the interaction of NuA3 with the nucleosome. It should be noted that the reason the \textit{setlA} mutant was not identified as a possible candidate in the deletion mutant screen was the result of the absence of a \textit{setlA} mutant in the yeast deletion set.

To test the possibility that Set1p is required for NuA3 interaction, we examined the effect of \textit{YNG1} over-expression in a \textit{setlA} mutant. In addition to \textit{SET1}, \textit{YNG1} over-expression was also examined in the mutants \textit{lge1Δ} and \textit{rad6Δ}, mutants for upstream components of the Set1p methylation pathway, as well as another methyltransferase, Set2p (\textit{set2Δ} mutant). Set2p is a histone methyltransferase which has been shown to methylate specifically lysine 36 of the H3 tail (79). These experiments were carried out by transforming each mutant with the \textit{URA3} based pY10E plasmid (with the vector alone as a control) followed by dilution plating assays on 'URA and 'URA with galactose. As a negative control, all strains were transformed with vector alone to ensure that the vector was not adversely affecting the cell. Dilution plating assays
showed that, compared to wild type, the set1Δ mutant along with ile1Δ and rad6Δ mutants showed resistance to YNG1 over-expression, while the deletion mutant of the other H3 methyltransferase, SET2, did not exhibit any resistance (Fig. 3.7). The resistance associated with deletion mutants of the Setlp methylation pathway suggested the possibility that methylation of lysine 4 of histone H3 is required for the interaction of Ynglp.

3.3.4 GAL1 Induction in Deletion Mutants

Setlp mediated methylation of the H3 tail has been correlated to regions of the genome that are actively undergoing gene transcription (54). Furthermore, loss of SET1 has also been shown to adversely effect the transcription of as many as 80% of the genes in S. cerevisiae (133). In addition, other studies have shown that Setlp methylation is required for efficient expression of a number of GAL genes in yeast (134). Thus it is possible that the resistance seen in set1Δ mutants is due to a reduction in YNG1 expression from the GAL1 promoter. To address this concern we wanted to examine the levels of Ynglp within the strains containing the pYlOE vector. Due to the same constrains as before, namely no Ynglp antibody and the associated toxicity in wild type strains, we once again made use of the 3xHA tagged YNG1 construct, pY2OE. This plasmid, along with the vector alone (as a negative control) was transformed into all of the deletion mutant, as well as wild type, strains. Transformed strains were grown to an OD600 of 1.0 and whole cell extracts were prepared as outlined (refer to Materials and Methods). YNG1 expression was assessed through western blot analysis of whole cell extracts using anti-HA antibodies. As shown in the western, levels of Ynglp were reduced in the set1Δ mutant while all other mutants showed roughly similar levels of YNG1 expression as that seen in wild type (Fig. 3.8).
**Figure 3.7 Yng1p interaction is dependent upon the Set1p methylation pathway.**

Yeast strains YLH101, YLH208, YLH220, YLH201, YLH206, YLH210, YDM003 and YLH211 were transformed with the indicated plasmids and plated on synthetic drop-out media lacking uracil with either dextrose (negative control) or galactose as the sole carbon source.
Figure 3.8 Disruption of SET1 negatively affects GAL1 induction.

YLH101 was transformed with either the vector pGAL.HA.CYC.416 (negative control) or pY20E. Yeast strains YLH206, YLH208 and YLH220 were transformed with pY20E. Strains were grown in 50mL of URA galactose. YNG1 expression was assessed by monitoring levels of Yng1HA3 through anti-HA western blot analysis on whole cell extracts. Blots were ponceau stained prior to blocking to ensure that equal levels of bulk protein were loaded.
As a result of the levels of \textit{YNGL} expression being reduced within the \textit{setlA} mutant, it was necessary to bring the levels of expression for \textit{YNGL} up to the same as that seen for wild type. To this end, we subcloned \textit{YNGL} along with the \textit{GAL1} promoter into the high copy vector pRS426 resulting in the plasmid, pY3OE. Also, in the same manner as that described previously, we subcloned \textit{YNGL} with the HA.CYC cassette into pRS426, referred to as pY4OE. Both of the resulting plasmids and their corresponding vectors were transformed into each of the mutant strains tested.

Toxicity of \textit{YNGL} over-expression was once again examined by through dilution plating assays. Strains transformed with either pY3OE or vector alone (as a negative control) were plated on both 'URA and 'URA with galactose. As before, all strains that contained deletions of genes within the Setlp methylation pathway were resistant to \textit{YNGL} over-expression, while the wild type strain did not exhibit this resistance (Fig. 3.9A).

Strains transformed with the plasmid pY4OE (and the vector alone as a control) were grown to an OD$_{600}$ of 1.0 and whole cell extracts were prepared as described previously (refer to Materials and Methods). Western blot analysis revealed that the level of \textit{YNGL} expression, seen in the \textit{setlA} mutant, was roughly the same as that seen in wild type (Fig. 3.9B). Thus, it was reasoned that loss of Setlp as well as other components within the Setlp methylation pathway confer a resistance to \textit{YNGL} over-expression. This resistance then argues for an interaction between Ynglp and methylated lysine 4 of H3.
A.

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B.

Figure 3.9 The set1Δ mutant is resistant to elevated levels of YNG1 expression.

(A) Mutants of the Set1p methylation pathway are resistant to over-expression of YNG1 from the high copy vector pRS426. Yeast strains YLH101, YLH208, YLH206 and YLH220 were transformed with pY3OE and the vector pGAL.426 as a control. Serial dilutions of transformed strains were carried out on synthetic drop-out media lacking uracil with either dextrose (negative control) or galactose as the sole carbon source. (B) The high copy vector, pRS426, increases expression of YNG1 in the set1Δ mutant. YLH101 was transformed with either the vector pGAL.HA.CYC.426 (negative control) or pY40E. Yeast strains YLH206, YLH208 and YLH220 were transformed with pY40E. YNG1 expression was assessed by monitoring levels of Yng1HA3 through anti-HA western blot analysis on whole cell extracts. Blots were ponceau stained prior to blocking to ensure that equal levels of bulk protein were loaded.
3.3.5 YNG1 Over-expression in Histone H3 Tail Mutants

Using both chromatin pull-down assays and synthetic growth defect analysis (looking for the presence of the sas3Δ phenotype) we have provided a substantial amount of evidence to suggest that NuA3 interacts with the tail of histone H3. Previous work done in this study also indicated that Yng1p was required for NuA3 interaction with chromatin. Thus, we wanted to use the YNG1 associated phenotype to investigate the effect of substitution mutants had on Yng1p interaction. The purpose of this experiment was two-fold: first, it allowed us to assess directly any alterations in Yng1p interaction with the nucleosome and second it provided an additional genetic approach to investigate the necessity (with respect to NuA3 function) of specific residues within the H3 tail.

Based on the data that we obtained from the YNG1 over-expression screen it seemed as though NuA3 binding, as mediated by Yng1p, to the nucleosome was dependent upon the Set1p methylation pathway. However, we wanted additional evidence to indicate that it was the methylation signal, and not some other role of aspect of Set1p, was influencing the NuA3 – nucleosome interaction. Therefore, an approach that would allow the direct assessment of the effect of methylation on Yng1p binding was required. The approach that was developed involved the creation of histone H3 tail mutants. These mutants contained substitution mutations at lysine residues 4 and 36, the targets of Set1p and Set2p methylation respectively. A substitution mutant of lysine 14, the target of NuA3 acetylation, was also created as a control for this assay.

As indicated by previous data, it was anticipated that mutation of lysine 4 would impart resistance to YNG1 over-expression. The mutation of lysine residue 36 was not expected to
show resistance as loss of Set2p did not impart YNG1 over-expression resistance to the strain. Similarly mutation of lysine 14, the putative target of NuA3 acetylation and as such an upstream factor of NuA3 binding, was not expected to impart resistance to YNG1 over-expression.

All histone H3 mutants were created by transforming plasmids, according to the plasmid shuffle protocol, which contained lysine to arginine substitutions (created using site directed mutagenesis) at lysine residues 4, 14, and 36 within the H3 tail. All mutants were transformed with pY10E as well as the vector alone (as a control). The viability of all transformed strains in the presence of YNG1 over-expression was then assessed through dilution plating analysis on URA (as a control) and URA with galactose.

The mutant strain containing the substitution at lysine 14 was not resistant to the over-expression of YNG1 (Fig. 3.10A). This result, as mentioned previously, was anticipated because while lysine 14 is required for NuA3 function, it does not play a role in mediating NuA3 interaction with the nucleosome, as evidenced through the chromatin pull-down assay. The lysine 4 substitution mutant was resistant to YNG1 over-expression, a result that is in agreement with the previous data obtained from the YNG1 over-expression screen. Mutation of the lysine 36 residue, the methylation target of Set2p, did not impart resistance to a strain in which YNG1 was over-expressed (Fig 3.10 A). This result was also in agreement with our previous over-expression assays in which loss of the methyltransferase, Set2p, does not impart resistance to YNG1 over-expression.

To ensure that the resistance to YNG1 over-expression, seen in the lysine 4 mutant, was not due to reduced levels of YNG1 expression, we comparatively examined the levels of Ynglp between wild type and the lysine 4 mutants. As described previously, strains were transformed with the plasmid pY20E and vector alone (as a control). Transformed strains were grown to an OD$_{600}$ of 1.0 and whole cell extracts were prepared as outlined (refer to Materials and Methods).
YNG1 expression was assessed through western blot analysis of whole cell extracts using anti-HA antibodies. Western blot analysis of whole cell extracts showed that the levels of Yng1p were essentially equally between wild type and the lysine 4 mutant strains (Fig. 3.1OB). These results seem to indicate that methylation of lysine 4 by Set1p facilitated Yng1p mediated NuA3 binding to the H3 tail. In contrast, the susceptibility to YNG1 over-expression, seen in both the set2Δ and lysine 36 substitution mutants, suggested that Yng1p does not mediate the interaction of NuA3 with the H3 tail via the lysine 36 residue.

3.3.6 Additional Evidence for the Interaction of NuA3 with the H3 Tail

As shown through both genetic and biochemical approaches NuA3 appeared to interact with the nucleosome and quite conclusive evidence was gathered to suggest that the region of the nucleosome responsible for this interaction was the H3 tail. Furthermore, additional evidence seemed to indicate that Yng1p was required to mediate NuA3 interaction with the H3 tail. The next step was then to investigate what factors influenced the binding of Yng1p, and NuA3, with the H3 tail. The clue used to direct the initial investigation came from the genetic screen in which we over-expressed YNG1. From this screen we identified the Set1p methylation pathway as being required for Yng1p interaction. Having obtained this information, we wanted to extend our investigation by looking at the interaction of NuA3 with the nucleosome in mutants that contained deletions in genes encoding for factors necessary for, the Set1p methylation pathway. Therefore, the purpose of this approach was to obtain further information regarding the binding of NuA3 to the nucleosome, and the factors that influenced this interaction.
Figure 3.10 Loss of Set1p methylation site, lysine 4 of H3, alleviates \textit{YNG1} associated toxicity.

(A) Yng1p interacts with lysine 4 of histone H3. Yeast strains YLH224, YDM175, YDM176 YDM203 and YDM204 were transformed with the indicated plasmids. Serial dilutions of transformed strains were done on synthetic drop-out media lacking uracil and containing either dextrose (negative control) or galactose as the sole carbon source. (B) Loss of the Set1p methylation site, lysine 4 of H3, does not affect transcription from a \textit{GAL1} promoter. Yeast strains YLH224 and YDM175 were transformed with the indicated plasmids. \textit{YNG1} expression levels were monitored through western blot analysis of whole cell extracts probing for Yng1HA3 with anti-HA antibodies. Prior to blocking, blots were ponceau stained to ensure equal loading.
The use of the chromatin pull-down assay in showing that NuA3 interacts with the H3 tail proved the adaptability of this assay. As such, we wanted to apply this approach to investigate other factors that may influence the binding of NuA3 to the H3 tail, specifically factors within the Setlp methylation pathway. Additionally, we wished to investigate whether other histone modifications influenced the interaction of NuA3 with the nucleosome. Thus, we proposed to perform chromatin pull-downs in deletion mutants of histone modifying enzymes and evaluate the NuA3 – nucleosome interaction in these mutants as compared to wild type. The use of the chromatin pull-down assay promised an approach that allowed us to assess biochemically the factors that influence the interaction of NuA3 with the nucleosome.

3.3.6 Histone Modifiers Set1p and Set2p are Required for NuA3 Interaction with Chromatin

The results we have obtained thus far indicated that NuA3 binds and subsequently acetylates nucleosomes in vivo. It has been previously reported that post-translational modification of histones serves to regulate the interaction of HAT complexes. For example the HAT complex SAGA has been reported to preferentially modify histones that are methylated at lysine 4 and phosphorylated at serine 10 (26, 77). To investigate the effects of various histone post-translational modifications on the NuA3 – nucleosome interaction we attempted to incorporate the chromatin pull-down assay into a series of deletion mutants. These deletion mutants included set1Δ, rad6Δ and lge1Δ mutants; the gene products of which are shown to be necessary for the Setlp methylation pathway (30, 31, 81, 135). Additional mutants that were tested included deletion mutants of SNF1 and GCN5. The gene product of SNF1, Snf1p, has been shown to phosphorylate serine 10 of H3 (131) while Gcn5p is H3 specific HAT shown to acetylate lysine 9, 18, 23 and 27 with differing affinities depending upon the complex in which
it is found (103, 136, 137). The mutants \textit{dus1A} and \textit{ylr177A} were also included in the pull-down experiment due to the resistance they showed in the \textit{YNG1} over-expression screen. The resistance that these mutants showed to \textit{YNG1} over-expression may indicate a function for their gene products in mediating the binding of NuA3 with the nucleosome. Finally, as a result of the fact that \textit{set2A} mutants were not resistant to \textit{YNG1} over-expression, we included the \textit{set2A} mutant in the pull-down assay as a negative control.

The experimental set-up was performed in the same manner as outlined previously; a 3xHA tag is inserted at the C-terminus of Sas3p while a C-terminal insertion of the TAP tag is effected in H2B. Chromatin pull-downs were performed in accordance to the chromatin pull-down protocol. Co-precipitation of NuA3 with the nucleosome in each of the mutant pull-downs was detected using anti-HA western blot analysis. Preliminary evidence gathered from these pull-downs revealed that the \textit{set2A} mutant was deficient in precipitating NuA3 with the nucleosome. This result was somewhat unexpected, as \textit{set2A} mutants were not resistant to \textit{YNG1} over-expression.

To investigate the role of Set2p in NuA3 interaction further, an additional mutant strain was created in which both \textit{SET1} and \textit{SET2} genes were deleted. Pull-downs were once again performed on all mutants including the double \textit{set1A set2A} mutant. While the \textit{set1A} mutant showed, at best, only a minor reduction in the co-immunoprecipitation of NuA3 with H2B, both the \textit{set2A} mutant and the double mutant \textit{set1A set2A} showed a substantial reduction in the precipitation of NuA3 with H2B, as compared to wild type (Fig. 3.11A). With the exception of \textit{Igel1A (rad6A} showing only a very modest reduction), all other mutants tested showed no effect whatsoever on the precipitation of NuA3 with H2B (Fig. 3.11B). These results agree with the genetic data previously obtained, namely that mutants of upstream components of the Set1p methylation pathway negatively effect Yng1p interaction.
The pull-down data shown in figure 3.11, in conjunction with the results obtained from the \textit{YNG1} over-expression experiment, support the hypothesis that Set1p methylation enhances the interaction of NuA3 through its subunit, Yng1p. However, a new twist to this developing model arose from the apparent role Set2p methylation plays in mediating NuA3 interaction. As indicated by the \textit{YNG1} over-expression screen, Set2p methylation does not seem to be recognized by Yng1p, as loss of Set2p or mutation of its target site (lysine 36 of H3) does not abolish the toxicity associated with \textit{YNG1} over-expression. While methylation mediated by Set2p may not be recognized by Yng1p, it is possible that some other component of NuA3 (or an additional mediating factor) may be involved. Thus, while the \textit{YNG1} over-expression data failed to indicate the importance of Set2p methylation with respect to NuA3, the results from our chromatin pull-down assay indicated quite strongly that Set2p is involved in mediating NuA3 interaction with the nucleosome.

3.3.7 \textbf{Set1p and Set2p are Required to Maintain Steady-State Levels of Acetylation}

Using both genetic and biochemical approaches we have shown that NuA3 interaction with the nucleosome is dependent upon \textit{SET1} and \textit{SET2}. This dependence of NuA3 on methyltransferase activity suggested the possibility that methylation of H3 by both Set1p and Set2p mediates NuA3 dependent histone acetylation. Thus, we wished to assess whether loss of \textit{SET1} and \textit{SET2} affected steady-state levels of histone acetylation \textit{in vivo}.

To this end an assay was designed to shown the relative levels of bulk histone acetylation in both wild type and mutants through western blot analysis. For this assay strains containing deletions in either \textit{SET1}, \textit{SET2} or both \textit{SET1} and \textit{SET2} were analyzed by preparing whole cell extracts and normalizing these extracts according to bulk protein content through the
Figure 3.11 Histone H3 modifiers, Set1p and Set2p, are required for NuA3 interaction with chromatin.

(A and B) Chromatin pull-down assays were performed from the indicated Sas3HA3 expressing strains and the resulting samples subjected to western blot with immunodetection for HA. Samples were normalized by immunoblotting for TAP tagged Htb1p.
use of a Bradford assay. Assessment of the levels of H3 acetylation were carried out using anti-acetyl H3 western blot analysis on the normalized whole cell extracts.

Although other antibodies are available which recognize specific acetylated residues within H3, they are optimized for use in ChIP. Our lab has tried several of these ChIP optimized antibodies for use in western blot analysis, however; with no success. As such, to analyze lysine 14 acetylation levels we only had available to us the anti-acetyl H3 antibody, which recognized acetylated lysine residues 9 and 14. An added complexity to the design of this experiment arose from a previous study showing that the anti-acetyl lysine 9,14 H3 antibody primarily recognizes the acetylated lysine 9 epitope, due to the fact that there is either more acetylated lysine 9 in the cell, or alternatively, acetylated lysine 9 is a better epitope (138). Another recent study indicated that the lysine 4 methylation of histone H3 enhances GCN5-dependent H3 acetylation by the SAGA and SLIK/SALSA HAT complexes (77). Consequently, it was necessary to perform this experiment in mutant strains created within a gcn5A background to rid the cells of lysine 9 acetylation, as well as to remove any other effects that loss of H3 methylation may have on the Gcn5p acetylation pathway.

As shown through western blot analysis, there is no noticeable difference in H3 acetylation levels in strains lacking SET1 and SET2 when compared to wild type (Fig. 3.12). Consistent with the effect seen in strains lacking SAS3, loss of SET1 and SET2 alone does not appear to have a significant impact on H3 acetylation. Hence, to refine our investigation to look specifically at the role of SET1 and SET2 on NuA3 mediated acetylation we performed this experiment in strains derived from a gcn5A mutant background. Western blot analysis revealed that loss of SET2 in conjunction with gcn5A resulted in a loss of H3 acetylation. Interestingly, a more dramatic decrease in H3 acetylation was observed in the set1Δ set2Δ double mutant (in the gcn5A background), suggesting that both SET1 and SET2 mediate acetylation of the H3 tail in
Figure 3.12 The Set1p and Set2p methyltransferases mediate the steady state levels of histone acetylation of bulk histones.

Normalized amounts (~100 µg) of whole cell extract from the indicated strains were subjected to western blotting and immunodetection for acetylated histone H3. As a control, mutants created in a GCN5 wild type background were included.
vivo. These results in conjunction with the chromatin pull-down data, showing a decrease in the interaction of NuA3 in both set1Δ and set2Δ mutants, indicate that Set1p and Set2p methylation (on lysine residues 4 and 36 of the H3 tail respectively) are required for the function of NuA3.

### 3.3.8 Histone H3 Residues lysine 4 and 36 are Required for NuA3 Function

Based on the data presented above, it appears that methylation of lysine 4 and 36 of histone H3 are required for NuA3 interaction with chromatin, thus mediating NuA3 function. However, despite the functional dependence of NuA3 on SET1 and SET2, it is possible that Set1p and Set2p are methylating some other non-histone substrate, and it is this methylation that is responsible for mediating the NuA3 – nucleosome interaction. To investigate this issue in more detail we created three histone H3 substitution mutants using site-directed mutagenesis. These mutants were created through substitution of lysine to arginine at lysine residues 4, 36 and both 4 and 36 within the H3 tail. All mutations were carried out on a TRP1 based plasmid expressing HHT2. To ensure that all isolated plasmids contained the desired mutations, all plasmids were subjected to sequencing prior to their transformation into yeast strains. Mutant plasmids were then transformed individually into a strain containing deletions of HHT1 and HHT2 loci with a URA3 based plasmid expressing HHT2. Strains were then plated onto 5' FOA to rid the strains of wild type HHT2 leaving only the TRP1 based plasmid expressing mutated HHT2 within the cell. Chromatin pull-downs were performed in each of the three resulting mutant strains by TAP tagging the C-terminus of H2B. To detect the co-immunoprecipitation of NuA3 with the nucleosome, a 3xHA tag was inserted into the C-terminus of Sas3p. Co-precipitation was then detected through anti-HA western blot analysis, which showed that mutation of all mutants causes a significant reduction in the interaction of NuA3 with chromatin as compared to wild type (Fig. 3.13A).
While the H3 residues lysine 4 and 36 are required for NuA3 to interact with chromatin, it was not clear as to whether or not these residues are required for NuA3 function. Therefore, to address this question we employed a genetic technique that once again took advantage of the sas3Δ phenotype. It was reasoned that if either of these lysine residues were required for NuA3 function, mutation of either (or both) in conjunction with deletion of GCN5 should result in either a synthetic lethality or synthetic growth defect. With this question in mind, each of the three mutants was introduced into strains lacking either GCN5 or ADA2, as previously outlined. All strains exhibited no growth defects when grown at 30°C, however; mutation of either lysine 4 or 36 created a cold sensitivity in conjunction with gcnaΔ as evidenced by growth defects in strains when incubated at 20°C.

Although wild type yeast strains are capable of growing within a range of temperatures, the normal incubation temperature for culturing yeast is 30°C, a temperature which results in the most efficient growth (124). One observable phenotype of mutations occurring in yeast is the inability of yeast strains to grow at certain temperatures. These mutant strains are referred to as temperature sensitive mutants. Temperature sensitive mutants may be either heat sensitive (inability to grow at elevated temperatures) or cold sensitive (inability to grow at reduced temperatures) based on their sensitivity to temperatures relative to the optimal incubation temperature, 30°C. The cold sensitivity that occurred with these H3 tail mutants within a gcnaΔ background argued for a loss of ability of NuA3 to interact with the nucleosome. In terms of our model, decreased temperatures would impair an already compromised interaction of NuA3 with the nucleosome, in the absence of Setlp or Set2p methylation, while elevated temperatures would compensate to promote an interaction. Characteristic of the sas3Δ specific phenotype, these growth defects were not observed in ada2Δ mutant strains, as wild type growth was seen at 20°C (Fig. 3.13B). Furthermore, substitution of both lysine 4 and 36 to arginine in
conjunction with *gcn5Δ* showed the most severe growth defects when grown at 20°C. These phenotypes correlated strongly with previous biochemical data including; the effect of *set1Δ* and *set2Δ* mutations on bulk histone acetylation, as well as dependence of NuA3, as indicated through chromatin pull-down assays, on *SET1* and *SET2* methylation for its interaction with the nucleosome.

3.3.9 Establishing a Role for Yng1p in the Interaction of NuA3 With the Nucleosome

Through both biochemical and genetic approaches, we have provided evidence that the yeast ING homolog, Yng1p, is involved in mediating the interaction of NuA3 with the nucleosome. As evidenced by the *YNG1* over-expression data, the interaction of Yng1p with the H3 tail appeared to be dependent upon the methylation of lysine 4 by the histone methyltransferase Setlp. While the *YNG1* over-expression data did not indicated any dependence of Yng1p binding on Set2p methylation, additional biochemical and genetic data did implicate Set2p methylation in facilitating NuA3 binding.

In an attempt to explain this apparent discrepancy, it was reasoned that while Yng1p depended upon lysine 4 methylation, it was possible that an additional subunit of NuA3 was also involved in interacting with the nucleosome. The dependence of this proposed additional subunit, on Set2p mediated methylation of lysine 36, would therefore explain the loss of NuA3 interaction in mutants lacking the lysine 36 methylation signal. This discrepancy, along with the absence of any characterized domains within Yng1p that are known to recognize methylated lysine residues, prompted us to explore the possibility that an additional subunit of NuA3 was involved in interacting with the nucleosome.
Figure 3.13 Histone H3 residues lysine 4 and 36 are required for NuA3 function.

(A) Chromatin pull-down assays were performed from the indicated Sas3HA3 expressing strains and the resulting samples subjected to western blot with immunodetection for HA. Samples were normalized by immunoblotting for TAP tagged Hblp. (B) Yeast strains YLH224 (hhtl-hhf1Δ hht2-hhf2Δ), YLH289 (hhtl-hhf1Δ hht2-hhf2Δ gcn5Δ), and YLH290 (hhtl-hhf1Δ hht2-hhf2Δ ada2Δ) containing the indicated plasmids were plated onto yeast extract, peptone, dextrose media and incubated at 30°C for 2 days and 20°C for 3 days.
Due to the absence of chromodomains within any of the known subunits of NuA3, attempts were made to rationalize the apparent ability of NuA3 to recognize, and interact with, methylated lysine residues within the H3 tail. With the PHD finger being present in two of the known subunits of NuA3, Yng1p and Nto1p (100), it was thus proposed that this motif was responsible for recognizing methylated lysine residues. This hypothesis was attractive as it was suggestive of an alternative subunit of NuA3, Nto1p, which may be capable of recognizing lysine 36 within the H3 tail.

To investigate the possibility that Nto1p was responsible for mediating NuA3 interaction with the nucleosome, an experiment was designed in which the viability of cells was tested in the presence of NTO1 over-expression. The rational behind this experiment resided in the proposed function of Nto1p. If Nto1p was mediating the interaction of NuA3 with the nucleosome, over-expression of NTO1, like over-expression of YNG1, should result in inhibition of cell growth. Furthermore, removal of its proposed site of interaction (lysine 36 of H3) should alleviate the toxicity associated with NTO1 over-expression.

To this end the NTO1 ORF was subcloned into the pGAL.416 expression vector. The resulting plasmid, pGAL.NTO1 was transformed into a wild type strain as well as the lysine 36 substitution mutant. The viability of transformed strains was assessed through dilution plating on both URA and URA with galactose. Unfortunately there was no growth defects observed with NTO1 over-expression in either wild type or mutant strains (Fig. 3.14A). This result, although negative, does not rule out the possibility that Nto1p is playing a role in mediating NuA3 interaction with the nucleosome. While the NTO1 ORF was successfully cloned into the pGAL.416 vector, it is possible that NTO1 is not expressed. Due to the size of the gene product (~86 kDa), or improper folding, the cell may target Nto1p for degradation after protein synthesis. Finally, NTO1 may be expressed, and Nto1p may be stable, however, Nto1p may only
be able to interact with the nucleosome when it is incorporated into the NuA3 complex and not when it is a single entity.

The proposed role for the PHD finger of Yng1p in facilitating a nucleosomal interaction is not unprecedented. Previous studies have implicated the PHD fingers of mammalian histone modifying complexes as being responsible for mediating the interaction of their respective complex with nucleosomes (139, 140). Therefore, to investigate further the proposed role of the Yng1p PHD finger in its interaction with the nucleosome, we wanted to address whether or not loss of the PHD finger affected the interaction of NuA3 with the nucleosome. To this end a mutant strain containing a deletion in \textit{YNG1} corresponding to the nucleotide sequence encoding for the PHD finger was created, \textit{yng1ΔPHD}. Chromatin pull-down assays were then performed on \textit{yng1A} and \textit{yng1ΔPHD} mutant strains as well as wild type, as a negative control, through epitope tagging of the C-terminus of H2B with the TAP tag as well as the C-terminus of Sas3p with a 3xHA tag. Co-precipitation of NuA3 with the nucleosome was detected through western blot analysis using anti-HA antibodies. As shown previously, loss of Yng1p results in a reduction of co-precipitation of NuA3 with the nucleosome as compared to wild type. However, in the \textit{yng1ΔPHD} mutant there was no observable difference in the levels of NuA3 immunoprecipitated with the nucleosome relative to the wild type strain (Fig. 3.14B). These results argue against a role for the Yng1p PHD finger in mediating the NuA3 – nucleosome interaction, and suggest that another region of Yng1p is responsible for this interaction. Thus, while we have identified Yng1p as a candidate for mediating the interaction of NuA3 with the nucleosome, through the recognition of methylated lysine 4 of histone H3, additional work is required to explain the role of lysine 36 methylation in terms of NuA3 – nucleosome interaction.
Figure 3.14 Properties associated with NuA3 subunits that contain the PHD finger.

(A) Over-expression of NTO1, unlike YNG1, is not toxic in yeast. Yeast strain YLH101 was transformed with the indicated plasmids. Serial dilutions were done on synthetic drop-out media lacking uracil with either dextrose (negative control) or galactose as the sole carbon source. (B) The PHD finger of Yng1p is not required for Yng1p interaction with the nucleosome. Chromatin pull-down assays were performed on the indicated strains containing Sas3HA3 insertions. Co-precipitation of NuA3 was detected through anti-HA western blot analysis.
3.4 Applications of Yng1p Proposed Role as a Mediator of NuA3 – Nucleosome Interaction

Prior to the commencement of this study, little was known of Yng1p, the yeast homolog of the human tumor suppressor protein ING1. Previous work has shown that strains containing simultaneous deletions in both GCN5 and YNG1 are inviable, indicating the necessity of Yng1p in the function of NuA3 (100). Here we have shown through chromatin pull-down experiments that loss of Yng1p is confluent with a decreased interaction of NuA3 with chromatin. Additionally, over-expression of YNG1, while toxic in wild type cells is tolerated in strains lacking the H3 tail. Furthermore, loss of the histone methyltransferase, Setlp, or its target residue, lysine 4 of histone H3, also imparts a resistance to a strain in which YNG1 is over-expressed. Taken together, these results suggest a role for Yng1p in mediating the interaction of its associated complex, NuA3, with methylated lysine 4 of H3. Having developed a functional model for Yng1p, we then wanted to take this information and apply it to the two other ING homologs known to exist in yeast, Yng2p and Pho23p (94). Additionally, we wanted to explore the possibility that human tumor suppressor proteins function in a manner analogous to that seen in Yng1p. Thus, the goals of these experiments were to investigate the functional roles of Yng2p and Pho23p with respect to their associated complexes, and to extend our knowledge of Yng1p to the study of human ING proteins.

3.4.1 Yng2p is Not Required for NuA4 – Nucleosome Interaction

NuA4 is a 13 subunit complex having an approximate molecular weight of 1.3 MDa. Although some groups report H2A as a substrate, the main target of NuA4 HAT activity is the histone H4 tail (105), (141). The ING homolog Yng2p is found exclusively in NuA4/picNuA4
as determined via HPLC and tandem mass spectrometry. Loss of Yng2p causes a greater than 50% reduction in NuA4 HAT activity on nucleosomal substrates in vitro and is coupled with a reduction in NuA4 complex size (107). Additionally, loss of Yng2p results in a decrease in complex substrate specificity in vitro indicative of its importance for the function of NuA4. Also, deletion of YNG2 results in strains displaying severe growth defects (113). Therefore, the requirement of Yng2p for NuA4 function as well as the severity of the associated phenotype of a yng2Δ mutant strain led us to postulate that Yng2p may therefore be playing a role in mediating the NuA4–nucleosome interaction in vivo.

We were interested in designing an experiment to investigate whether or not loss of YNG2 affected the interaction of NuA4 with the nucleosome in vivo. Thus, to test the possibility that Yng2p is mediating the interaction of NuA4 with chromatin we performed chromatin pull-down assays on both wild type and yng2A strains. In both strains the nucleosome was precipitated by epitope tagging the C-terminus of H2B with the TAP tag while co-precipitation of NuA4 was tested through the incorporation of a 3xHA tag on the C-terminus of Epllp, an integral subunit of NuA4. Co-precipitation followed by western blot analysis using anti-HA antibodies revealed that loss of Yng2p had some effect on the ability of NuA4 to interact with the nucleosome (Fig. 3.15A). As a control an additional anti-IgG western was run, which recognized the TAP tag of H2B, to ensure equal loading of immunoprecipitates (Fig. 3.15A).

Though it appeared that Yng2p had some effect on NuA4 interaction it was felt that this explanation alone was not sufficient, due to the severity of yng2Δ phenotypes. Thus, it was possible that Yng2p was playing a structural role within NuA4. To investigate this proposed function further, we inserted the TAP epitope on the C-terminus of Eaf6p, a subunit of NuA4 whose functional significance remains unknown, in conjunction with a 3xHA tag insertion at the C-terminus of Epllp in both wild type and yng2Δ strains. NuA4 was then purified in accordance
with the chromatin pull-down protocol, and the complex integrity was examined through anti-HA western blot analysis. While Epllp was shown to co-precipitate with Eaf6p in the wild type strain, no such interaction was shown to exist in strains lacking Yng2p (Fig. 3.15B). Upon initial inspection this result seemed to indicate that loss of Yng2p is concomitant with a disruption of the NuA4 complex. However, in vitro data suggested that NuA4 is still functionally active in the absence of Yng2p. This is shown where NuA4 purified from mutant strains is still capable of acetylating H4 substrates, albeit to a much reduced extent (107). To rationalize this discrepancy, we offered the possibility that although the NuA4 complex remains essentially intact, deletion of YNG2 results in loss of one or more peripheral subunits. Thus, it is possible that loss of Yng2p is accompanied by loss of the ostensibly non-essential subunit, Eaf6p.

To clarify the issue concerning the fate of Eaf6p we performed another chromatin pull-down in both wild type and yng2Δ strains using a TAP tag insertion into the C-terminus of Epllp, while Eaf6p contained a C-terminal 3xHA insertion. Co-precipitation was detected through anti-HA western blot analysis and, as anticipated, loss of the Eaf6pHA signal was consistent with the loss of Yng2p (Fig 3.16). As a control, we examined the effect that re-incorporation of Yng2p had on the stability of Eaf6p by transforming the plasmid pYNG2 into the yng2Δ mutant. As shown in figure 3.16, expression of YNG2 within the cell is consistent with the incorporation of the Eaf6p subunit into the NuA4 complex. While the evidence is very weak to suggest that Yng2p is required for the interaction of NuA4 with the nucleosome, in vitro evidence suggests that Yng2p is necessary for efficient HAT activity by NuA4. Although further work is required to elucidate its role, the dependence exhibited by Eaf6p on Yng2p for its incorporation into the NuA4 complex may provide a clue as to the functional role Yng2p is playing within the NuA4 complex.
Figure 3.15 The role of Yng2p within the NuA4 complex.

(A) NuA4 interacts with chromatin and loss of Yng2p does not affect this interaction. Chromatin pull-down assays were performed on the indicated strains containing Epl1HA3 insertions. Co-precipitation of NuA4 with H2B was detected through anti-HA western blot analysis. Samples were normalized through anti-IgG western blot analysis. (B) Disruption of YNG2 is consistent with loss of Eaf6p from the NuA4 complex. Co-precipitation experiments were performed on the indicated strains containing Epl1HA3 insertions. Co-precipitations were detected using anti-HA western blot analysis.
3.4.2 Loss of Pho23p Does Not Affect the Interaction of Rpd3 With Chromatin

Unlike Yng1p and Yng2p which are components of HAT complexes, Pho23p is found in the histone deacetylase (HDAC) complex, Rpd3-Sin3. Pho23p is a critical component of the Rpd3-Sin3 complex where loss of Pho23p results in a greater than 50% reduction in HDAC activity on nucleosomes in vitro (119). Thus, due to the important role which Pho23p appears to play in Rpd3-Sin3 function, we hypothesized that its functional role, like Yng1p, may be mediating the interaction of its respective complex with chromatin.

Initially we wished to address whether Pho23p was involved in maintaining the structural integrity of the Rpd3-Sin3 complex. Previous studies showed that, while the in vitro HDAC activity of the Rpd3-Sin3 complex, purified from strains lacking Pho23p, was reduced relative to wild type, the complex still contained HDAC activity (95). Thus, while unlikely, it was possible that the reduction in HDAC activity was due to a disruption of the complex. To examine the stability of the Rpd3-Sin3 complex in the absence of Pho23p, co-precipitation experiments were performed on both wild type (negative control) and pho23A strains. The TAP epitope was inserted onto the C-terminus of the integral subunit Sds3p, additionally a 3xHA tag was inserted onto the C-terminus of another integral protein, Rpd3p. Co-precipitations were performed according to protocol using IgG resin. Complex stability was assessed using anti-HA western blot analysis, which looked at the co-precipitation of Rpd3pHA3 with Sds3pTAP. As shown in figure 3.17A, loss of Pho23p did not affect complex stability, as approximately equal levels of Rpd3pHA3 are present in both wild type and pho23A strains. Therefore, the role that Pho23p plays with respect to the Rpd3-Sin3 complex function appears to reside somewhere other than the stability of the complex.
Figure 3.16 Yng2p is required for incorporation of Eaf6p into the NuA4 complex.

Wild type YNG2 was re incorporated into yng2Δ strains by transforming YDM102 with pYNG2 (along with the empty vector as a control). Chromatin pull-down experiments were performed on the indicated strains containing Eaf6HA3 insertions. Co-precipitation of NuA4 was detected using anti-HA western blot analysis.
To test the proposed role for Pho23p in mediating the Rpd3-Sin3 – nucleosome interaction, chromatin pull-down assays were performed on wild type and \textit{pho23A} strains. As with other chromatin pull-downs, the C-terminus of H2B was TAP tagged and immunoprecipitation of the Rpd3-Sin3 complex was detected by insertion of a 3xHA tag on the C-terminus of the integral subunit Rpd3p. Anti-HA western blot analysis revealed that loss of Pho23p showed no negative effect on the ability of Rpd3-Sin3 to bind chromatin as compared to wild type (Fig. 3.17B). To rule out the possibility that less nucleosomes were being pulled down in the wild type strain, a control western was performed using anti-acetyl H3 antibodies (Fig. 3.17B). While \textit{in vitro} evidence indicates that Pho23p is important for the function of the Rpd3-Sin3 complex, Pho23p is not required for the structural integrity of the complex nor does it appear to have an affect on the ability of this complex to interact with chromatin. Therefore, as with Yng2p with respect to NuA4, further study is warranted to determine the role of Pho23p within the Rpd3 complex.

3.4.3 The Human Tumor Suppressor Protein ING2 is the Functional Equivalent of the Yeast ING Homolog, Yng1p

Existence of human tumor suppressor homologs in the yeast \textit{Saccharomyces cerevisiae} provides an effective approach to establish the fundamental principles regarding the function of tumor suppressor proteins. In this study we have provided convincing evidence that the yeast ING homolog, Yng1p, functions through mediating the interaction of its associated complex NuA3 with the nucleosome. Due to the prevalence of human ING proteins in complexes that are known to associate with chromatin, it is interesting to think that perhaps some, if not all, human ING proteins function in a manner analogous to Yng1p (92). It was therefore reasoned that if
Figure 3.17 The function of Pho23p within the Rpd3-Sin3 complex.

(A) Pho23p is not required for the integrity of the Rpd3-Sin3 complex. Co-precipitation experiments were performed on the indicated strains containing Rpd3HA3 insertions. Co-precipitation was detected through anti-HA western blot analysis. Samples were normalized through immunodetection of Sds3TAP. (B) The interaction of Rpd3-Sin3 with chromatin is not dependent upon Pho23p. Chromatin pull-down assays were performed on the indicated strains containing Rpd3HA3 insertions. Co-precipitation of Rpd3-Sin3 with H2B was detected through anti-HA western blot analysis. Samples were normalized through anti-acetyl H3 immunodetection.
any ING homolog shared a function similar to Yng1p, over-expression of that ING within yeast should recapitulate the phenotype associated with \textit{YNG1} over-expression, namely inhibition of cell growth.

As an initial experiment to test if such was the case, we examined the associated phenotypes for the over-expression of all 5 human INGs in the yeast \textit{Saccharomyces cerevisiae}. All 5 ING cDNAs were subcloned into the pGAL.416 expression vector and subsequently transformed into a wild type yeast strain, along with the vector alone as a negative control. Resistance to the over-expression of all INGs was then assessed using a dilution plating assay on both 'URA (as a control) and 'URA with galactose. Severe growth defects were observed for strains in which the human tumor suppressor, ING2, was over-expressed (Fig. 3.18A). These preliminary results suggest that the human tumor suppressor protein, ING2, functions in a manner similar to that seen in Yng1p.

To provide additional support for the proposed function of ING2, we looked at the over-expression of ING2 in mutant strains that were shown to be resistant to the over-expression of \textit{YNG1}. These strains included the histone modifier mutants, set1A and set1A set2A, with set2A as a negative control, in addition to the H3 tail mutants hht2A3-29 and the substitution mutant hht2K4R. Over-expression of ING2 in all mutants with the exception of the negative control, set2A, showed growth similar to that seen in strains containing the vector alone, this indicated a rescue of growth, as compared to wild type which shows essentially no growth at all (Fig. 3.18B). The ability of these mutants to rescue growth in the presence of ING2 over-expression provided further evidence that ING2 functions through a similar pathway as does Yng1p. While the results presented in this study are very preliminary, it is promising to think that the information that we have obtained regarding the role of Yng1p may be applied to understanding the roles of tumor suppressor proteins in higher organisms.
Figure 3.18 The functional roles of human ING proteins.

(A) Over-expression of the human ING protein ING2 is toxic in yeast. The strain YLH101 was transformed with the plasmids indicated. Dilution plating was performed on synthetic drop-out media lacking uracil with either dextrose (negative control) or galactose as the sole carbon source. (B) Mutants that lack the H3 tail or are deficient in lysine 4 methylation are resistant to ING2 over-expression. Yeast strains YLH220, YDM003, YDM181, YLH101, YDM209, YDM210, YDM175 and YDM209 were transformed with the indicated plasmids. Dilution plating was performed on synthetic drop-out media lacking uracil with either dextrose (negative control) or galactose.
Chapter 4 – Discussion

At the commencement of this study we had one central goal in mind – the characterization of Yng1p function within the NuA3 complex. As our research progressed the focus of the study split into two main aspects of investigation. With an interest in factors that affect NuA3 dependent acetylation, we began investigating the effect of other histone post-translational modifications on NuA3 mediated histone acetylation. The result of subsequent investigation lead to the development of a model which suggests that histone methylation acts as a signal to mediate the interaction of NuA3 with the H3 tail. The second aspect of our study concerned itself with the original problem, namely the role of Yng1p within the NuA3 complex. Though the direction of each line of investigation is unique, the techniques used and information gathered complement one another, and are not mutually exclusive. Thus, the information presented, while discussed separately, should be thought of as such.

4.1 The Role of the NuA3 Complex in Histone H3 Acetylation

Numerous histone modifying complexes have been isolated and characterized, giving rise to groups of modifying complexes differing in both substrate specificity as well as the type of modifications they affect. These posttranslational modifications serve as the basis of the histone code hypothesis which implicates these modifications as signals or mediators for other modifying complexes to interact with the chromatin and ultimately regulate gene expression (22). Therefore, significant interest is being placed on the development and use of methods to identify novel protein – nucleosome interactions. Here we have described a dual focus method, employing both a biochemical (chromatin pull-down assay) as well as a genetic aspect, to
characterize the interaction of the H3 specific HAT NuA3 with the nucleosome. With the use of this approach, we have shown that a subunit of NuA3, Ynglp, interacts with the nucleosome via the H3 tail, while methylation of specific lysine residues within H3 tail facilitates NuA3 interaction.

4.1.1 NuA3 Acetylates Histone H3 in vivo

The histone H3 specific HAT, NuA3, was originally identified based on its ability to acetylate histones in vitro (46). Initial results indicated that like the GCN5-dependent HAT complexes, the NuA3 complex acetylated lysine 14 of H3. However, NuA3 showed a preference for in vitro acetylation of lysine 14 and to a lesser extent lysine 23, unlike Gcn5p which was shown to also acetylate lysine residues 9 and 18 in addition to 14 (103). While the Gcn5p containing HAT complex SAGA shows some acetyltransferase activity toward lysine 23, no in vitro acetyltransferase activity is shown toward this residue with ADA, another GCN5-dependent HAT complex (137). Thus, while it showed similarities with other H3 specific HAT complexes, NuA3 appeared to have a unique functional role.

Disruption of SAS3 in conjunction with GCN5 results in a synthetic lethality. This synthetic lethality is rescued by the reincorporation of either SAS3 or GCN5 into the cell. Interestingly, disruption of SAS3 in conjunction with either ADA2 or ADA3 is not synthetically lethal, whereas loss of either Ada2p or Ada3p disrupts all Gen5p containing HAT complexes (103). As such, the synthetic lethality incurred upon loss of both GCN5 and SAS3 may not be the result of loss of H3 HAT activity. An added complexity results, however; by additional evidence which indicates that the sas3Δ gen5Δ synthetic lethality is due to loss of acetyltransferase activity (103). Taken together, these results suggest that either or both Sas3p and Gen5p may perform another role in addition to histone acetylation. One such possible
function is the acetylation of other non-histone substrates. While no other substrates have yet to be identified, Gcn5p has been shown to exhibit *in vitro* acetyltransferase activity toward Sinlp, suggestive of a capability to acetylate non-histone substrates (103).

In this study we have addressed the possibility that the primary target of NuA3 acetylation resides somewhere other than histone H3. Through the use of chromatin pull-down assays we have shown that Sas3p is capable of interacting with the nucleosome *in vivo*. In addition, the interaction of Sas3p with the nucleosome is dependent upon the H3 tail, suggestive of a region of interaction for NuA3 with the nucleosome.

The synthetic lethality associated with the deletion of *SAS3* in a *gcn5Δ* mutant background is unique to proteins involved in the NuA3 acetylation pathway and as such we refer to this genetic property as a *sas3Δ* specific phenotype. Thus, this particular *sas3Δ* specific phenotype provides a useful genetic tool in elucidating components of the NuA3 acetylation pathway. Through examining the viability of mutants that were created in *gcn5Δ* mutant backgrounds it is possible to evaluate the role proteins are responsible for playing in the NuA3 acetylation pathway. We have shown that deletion of the H3 tail in a *gcn5Δ* mutant background results in a synthetic lethality, showing a dependence of NuA3 on the H3 tail for function. This is consistent with the requirement of the H3 tail for NuA3 interaction with the nucleosome. Furthermore, a substitution mutation of lysine 14 to arginine 14 (K14R) of histone H3 also recapitulated the *sas3Δ* specific phenotype. These results indicate that loss of lysine 14 acetylation is creates a synthetic growth defect in a *gcn5Δ* background suggesting that lysine 14 is required by NuA3 for its function. While it is still possible that NuA3 is acetylating a target other than histones, these results strongly suggest that NuA3 functions as a HAT *in vivo*.

The synthetic lethality associated with histone H3 Δ3-29 mutant and the synthetic growth defect seen in the K14R mutant, when in a *gcn5Δ* background, has been reported
previously (138). Here we have shown that while lysine 14 is required for function, it is not required for the interaction of NuA3 with the nucleosome. This result suggests that lysine 14 plays a role in NuA3 function downstream of its interaction with the nucleosome. Furthermore, previous work has indicated that the histone H3 lysine 9 to arginine 9 substitution mutant is not synthetically lethal with \textit{gcn5A}. This result is in agreement with the Gcn5p dependent acetylation pathway which implicates lysine 9 as a target of Gcn5p associated complexes. Indeed, others have shown that Gcn5p preferentially acetylates lysine residues 9, 18, 23 and 27 but not lysine 14 of the H3 tail \textit{in vivo} (136). The preferential acetylation, seen \textit{in vivo}, of lysine residues other than lysine 14 by Gcn5p, in conjunction with the \textit{gcn5A} K14R synthetic lethality data, suggests strongly that lysine 14 is being acetylated by a HAT other than Gcn5p. With the K14R mutant exhibiting a \textit{sas3A} phenotype, we suggest that NuA3 is the HAT responsible for acetylating lysine 14.

As shown through \textit{in vitro} HAT assays on nucleosomal histones, lysine 14 of histone H3 is readily acetylated by either Gcn5p or NuA3 (101, 103). However, as subsequent experiments by others have indicated, Gcn5p does not appear to play a role in lysine 14 acetylation \textit{in vivo} (136, 137). Paralleling this anomaly, the elongator complex of yeast shows a preference for acetylating lysine 14 \textit{in vitro}, however; additional studies performed \textit{in vivo} indicate that other lysine residues within the H3 tail are the preferred acetylation targets of the complex (142, 143). Therefore, it appears that while various HAT complexes are capable of acetylating the lysine 14 residue \textit{in vitro}, there are additional factors of the surrounding environment within the cell which control access to this particular residue \textit{in vivo}.

In an attempt to explain the preferential acetylation of lysine 14 by the NuA3 complex several possibilities have been suggested. One explanation that we have put forth is that acetylation of lysine 14 by NuA3 occurs before either Gcn5p or the elongator complex has an
opportunity to access this residue for subsequent acetylation. The NuA3 complex has been shown to interact with the yeast facilitates chromatin transcription (FACT) complex. Shown to have a role in DNA replication, the FACT complex may recruit NuA3 shortly after DNA replication occurs (46). This recruitment by the FACT complex may allow for the preferential acetylation of lysine 14 by NuA3 prior to other HATs targeting chromatin. An alternative explanation we provide suggests that lysine 14 is situated in the nucleosome in such a way that HATs other than NuA3 have difficulty in accessing, and acetylating this residue in vivo.

4.1.2 Methylation as a Signal for NuA3 Acetylation

A great deal of work has been done showing a positive correlation between histone acetylation and regions of the genome which are undergoing active transcription (37, 40, 54, 108, 136). In agreement with this, studies have shown that complexes effecting this acetylation are targeted to specific regions of the genome through various mechanisms (108). The HAT complexes SAGA and NuA4 have been shown to associate with transcriptional activators and are thus directed towards the promoter regions of genes (111, 144-148). The elongator complex is known to associate with the elongating form of RNA polymerase II and is therefore assumed to acylate nucleosomes in regions that are undergoing transcription (45). Prior to the work done in this study, no mechanism, as such, had been described for NuA3. Although it had been shown to interact with FACT, a complex which plays a role in modulating chromatin structure, evidence existed that placed the FACT complex in a great abundance over the NuA3 complex (46). Due to the disparity in the relative abundance of these complexes, it is assumed that the cell must possess an alternative mechanism to target the NuA3 complex to specific regions within the genome.
One possible explanation that we have put forth regarding the recruitment of NuA3 to the nucleosome involves the signaling of NuA3 through prior methylation of histones. In support of this hypothesis we find that loss of SET1 and SET2 results in a reduction in steady state levels of acetylation on bulk histones. Using western blot analysis we have shown that simultaneous disruption of the histone methyltransferases SET1 and SET2 results in a reduction of H3 acetylation. In addition to disruption of histone acetylation, loss of SET1 and SET2 result in a decreased interaction of NuA3 with the nucleosome. Further investigation also revealed that mutation of either lysine 4 or 36 (targets of Setlp and Set2p methylation respectively) also negatively affected the NuA3 - nucleosome interaction. These results, when taken together, argue for a role of methylation in NuA3 recruitment to the nucleosome.

The recruitment of both Setlp and Set2p to the nucleosome is facilitated by the association with RNAPII (76). This interaction of histone methyltransferases with the RNAPII would suggest that histone acetylation, if it is indeed mediated by methylation, is linked to transcription. Numerous studies have been undertaken which have looked at the existence of any correlations between histone H3 acetylation and gene transcription. One study employed ChIP analysis, using a highly specific antibody directed toward the acetylated form of lysine 14 of histone H3, to study genome wide acetylation levels of ORFs (130). Positive correlations were shown to exist between lysine 14 acetylation and coding regions of the genome. Interestingly, lysine 23 of histone H3, which has been shown to be a target of NuA3 acetylation *in vitro*, also showed a positive correlation between acetylation and transcriptional activity. While a positive correlation was shown to exist between acetylated lysine residues 14 and 23 in coding regions, the opposite situation was shown to persist in intragenic regions. Furthermore, both histone methylation and acetylation have been correlated to transcriptionally active regions of the genome. Another study used ChIP analysis to investigate the genome wide methylation and acetylation patterns of histone H3 in yeast. Results from this study indicated that methylation of
lysine 4 and acetylation of H3 both correlated with regions that were transcriptionally active (149).

The proposed role of methylation in mediating histone acetylation is not unprecedented. 

SET1 and SET2 have been shown to mediate NuA4 interaction with certain promoters, and thus facilitating acetylation of lysine 8 of the histone H4 tail (75). Associated with DNA damage repair, serine 1 phosphorylation of the H4 tail results is concomitant with decreased levels of H4 acetylation. However, methylation of arginine 3 of H4 has been shown to increase acetylation in H4 tails containing the serine 1 phosphorylation signal (150). Methylation of arginine 3 of H4 in mammalian cells, a modification that is effected by a protein arginine methyltransferase (PRMT1), also displays a positive correlation with H4 acetylation (151). Further evidence that methylation plays a role in mediating histone acetylation was provided through a study investigating H3 HAT complexes. Here it was shown that lysine 4 methylation enhanced acetylation of histone H3 by SAGA and SLIK/SALSA complexes (77). While we failed to observe any substantial reduction in H3 acetylation levels in our set1A mutant, as shown through western blot analysis, one explanation could be that the bulk of acetylation was performed by a Gcn5p containing HAT complex other than SAGA or SLIK/SALSA complexes.

Mediation of the HAT complexes NuA4, SAGA and SLIK/SALSA by methylation is not entirely surprising when one considers that all these complexes contain subunits containing chromodomains. Although chromodomains have been implicated in binding methylated lysine residues (91), no known subunits of NuA3 contain a canonical chromodomain. It is therefore possible that an additional domain contained within a subunit of NuA3 is responsible its interaction with methylated lysine residues. While there exists few examples of protein domains other than the chromodomain that are capable of associating with methylated lysine residues, recent studies have identified several such motifs. The WD40 motif of the protein WDR5, an H3 lysine 4 methyltransferase found in vertebrates, has been shown to recognize methylated lysine
4 residues of H3 (152). Similarly, the Tudor domain, shown to have homology to chromodomains has been suggested to play a role in interacting with chromatin (153). The identification of novel protein domains capable of recognizing methylated lysine residues suggests the possibility that an additional domain (or domains) contained within a subunit of NuA3 is responsible its interaction with methylated lysine residues.

In an attempt to explain the dependence of the NuA3 – nucleosome interaction on Setlp and Set2p histone methyltransferase, we proposed that the PHD finger of Yng1p, a motif of which little is known, recognizes lysine residues in a methyl dependent manner. However, due to our in vivo chromatin pull-down data, which showed loss of the PHD finger having little effect on the ability of NuA3 to bind nucleosomes, this did not appear to be the case. An alternative hypothesis is that histone methylation is required for an additional modification, and it is this secondary modification that is recognized by Yng1p. An example of such is the dependence of the yeast Isw1p ATPase on SET1. While SET1 is required for its function, Isw1p is unable to bind methylated peptides in vitro (154).

4.2 The Proposed Function of Yeast ING Homologs and Their Applications to Human ING Proteins

4.2.1 Yng1p and its Role in the NuA3 Complex

Since their initial discovery, ING proteins have been implicated in numerous key regulatory events within the cell. Initial work done on a splice variant of ING1 p33ING1 showed that the function of ING1 was dependent upon the tumor suppressor protein p53. Thus, ING1 activity was linked to transcriptional regulation (96). Subsequent research has revealed that several splice variants of ING1 have been shown to associate with HAT and HDAC complexes
There is however some discrepancy regarding the role ING proteins are playing in these complexes as some groups report that placement into either HAT or HDAC complexes is dependent upon the particular isoform (92). In contrast to this, others have reported that the same splice variant, p33\textsuperscript{ING1}, is found in both HAT as well as HDAC complexes. Despite their seemingly opposing functions, association of ING proteins with histone modifying complexes suggests that ING proteins function, at least in part, by regulating histone acetylation.

In a situation similar its human homolog, ING1, the yeast ING protein, Yng1p, is shown to associate with a HAT complex, NuA3 (100). Through \textit{in vitro} HAT assays we have shown that loss of Yng1p results in abolishment of the associated HAT activity of NuA3. While interruption of \textit{YNG1} is consistent with loss of NuA3 HAT activity, it has been shown that this effect is not due to the disruption of the NuA3 complex (100). With Yng1p shown to be essential for NuA3 function and implications for ING proteins in interacting with chromatin we hypothesized that Yng1p functioned by mediating NuA3 interaction with the nucleosome. In support of this, we have shown that loss of \textit{YNG1} is accompanied by a reduction in NuA3 interaction with chromatin, as shown through chromatin pull-down assays.

Though loss of Yng1p does not completely abolish NuA3 interaction with the nucleosome, it is possible that the destabilization consistent with the disruption of \textit{YNG1} is sufficient to abolish activity. One possible explanation for the ability of NuA3 to interact with the nucleosome in the absence of Yng1p is that another subunit of NuA3 is also responsible for mediating this interaction. Despite the ability of this other subunit to mediate an interaction, it is not sufficient to support the function of NuA3. Furthermore, as shown previously, \textit{YNG1} displays the \textit{sas3A} specific phenotype as \textit{yng1A gcn5A} mutants are extremely sick (103). Taken together, these results suggest that Yng1p is required by NuA3 to mediate its interaction with the nucleosome.
4.2.2 YNG1 Over-expression as a Genetic Tool

The over-expression of YNG1 in wild type yeast cells results in an inhibition of cell growth. Consistent with this, studies have shown that apoptosis is induced in cells where the splice variant of ING1, p33\textsuperscript{ING1b}, is over-expressed (92). Though the mechanism of YNG1 toxicity is not understood, it appears to be dependent upon the highly conserved PHD finger domain found in the C-terminus of Yng1p as loss of the PHD finger alleviates the associated toxicity of YNG1 over-expression (L. Howe personal communication).

While we are unsure as to the mechanism whereby Yng1p interacts with the H3 tail we have proposed that the YNG1 over-expression toxicity is due to the interaction of Yng1p with the H3 tail. Furthermore, we hypothesize that the interaction of free Yng1p with the H3 tail, in conditions where YNG1 is over-expressed, disrupts the interaction of other factors with the H3 tail, and perhaps the nucleosome itself. These disruptions, which are potentiated by the association of free Yng1p with the H3 tail, result in the inhibition of cellular growth. This may be a reflection of Yng1p blocking access to the nucleosome, of other factors necessary for chromatin maintenance. Alternatively, binding of free Yng1p in conditions of YNG1 over-expression may result in locking chromatin into a constitutively active, or inactive, state. Although we offer little proof of the actual mechanism which we propose, the elements of our model appear to hold true for the experiments that we have performed employing this phenotype.

Using the YNG1 over-expression phenotype we have identified that the histone modification, effected by Set1p, is necessary for NuA3 – nucleosome interaction. We thus proposed that Yng1p interacts with the H3 tail through the recognition of methylated lysine 4. This is supported by data showing that either loss of SET1 or mutation of its target residue, lysine 4, imparts to the cell a resistance towards YNG1 over-expression. Further work
employing chromatin pull-down assays identified an additional modification, methylation of lysine 36 by Set2p, as being required for NuA3 interaction. Though Set2p mediated methylation of lysine 36 is necessary for NuA3 – nucleosome interaction, set2Δ mutants are not resistant to YNG1 over-expression. Consistent with this, mutation of lysine 36 does not repress the toxic effects associated with YNG1 over-expression. Thus, it is possible that while Yng1p is recognizing methylated lysine 4 an additional subunit of NuA3 is responsible for interacting with methylated lysine 36. This possibility is in agreement with chromatin pull-down experiments showing that loss of Yng1p does not completely abolish NuA3 interaction with chromatin.

4.2.3 The Yng1p PHD Finger

Though it remained unclear how Yng1p interacts with the H3 tail we proposed that the PHD finger of Yng1p is responsible for the NuA3 – nucleosome interaction. The PHD finger, as found in the Yng1p subunit of NuA3, is a zinc-finger like motif that occurs in a variety of proteins and is thought to play a role in mediating transcription (155). A recent study has shown that loss of PHD finger domains of putative mammalian HAT complexes is concurrent with loss of HAT activity (156). In further support of this proposed role, it has recently been reported that the PHD finger of the human transcriptional cofactor p300 is required for nucleosomal binding (139). Additionally, the PHD finger of the human chromatin remodeling complex NoRC, in conjunction with an associated bromodomain, has been shown to be required for interaction of the complex with acetylated histone H4 (140). Thus, it seemed an attractive possibility that the PHD finger of Yng1p, and perhaps an additional subunit of NuA3, Ntolp, was functioning by recognizing methylated lysine residues within the H3 tail.
Despite the intriguing possibility that the PHD finger is responsible for interacting with the nucleosome, there is considerable evidence to suggest that such is not the case. It has been shown that strains lacking the PHD finger of Yng1p are able to rescue the gcn5A yng1A synthetic lethality, indicating that the PHD finger is not essential for Yng1p function (100). Furthermore, loss of the PHD finger does not appear to have any affect on the ability of NuA3 to interact with the nucleosome. Evidence suggesting that loss of the PHD finger eliminates the toxicity of \textit{YNG1} over-expression should also be taken with caution, as we are unsure that \textit{YNG1D}PHD is actually being expressed. Preliminary work also suggests that Nto1p is not involved in an interaction with the nucleosome, as over-expression of \textit{NTO1} is not toxic to the cell. However, as with the \textit{YNG1D}PHD over-expression data, this result should be taken with caution as we were unsure whether or not \textit{NTO1} was being expressed.

The two other yeast ING homologs, Yng2p and Pho23p, are found in the NuA4 and Rpd3-Sin3 complexes respectively. As seen with Yng1p, loss of either of these proteins from their respective complexes is disruptive to the associated enzymatic activity of the complex (107, 117). However, unlike Yng1p, over-expression of either \textit{YNG2} or \textit{PHO23} is not toxic to the cell (L. Howe personal communication). These results suggest that these ING homologs do not function by interacting with chromatin. Supporting this, we have shown through chromatin pull-down assays that loss of either of these proteins does not significantly disrupt the interaction of their associated complex with the nucleosome. Interestingly, the PHD fingers of Yng2p and Pho23p display significant homology with the PHD finger of Yng1p, but the function of this motif is, as of yet, unknown. Therefore, the disparity of function seen between yeast ING proteins, despite the similarity of their PHD fingers, suggests that the PHD finger is performing a role other than facilitating an interaction with the nucleosome. Thus, it seems likely that Yng1p interacts with the nucleosome through its N-terminus.
4.2.4 Application of Yng1p Function to Human ING Proteins

Work done on human ING proteins and their yeast homologs has revealed a number of similarities in these proteins which exist between these organisms. Various splice variants of ING1 have been shown to interact with the transcriptional regulator p53 (96). Subsequent studies done in yeast have shown that Yng2p is also capable of interacting with p53 both in vitro and in vivo and this interaction promotes acetylation of histone H4 (107, 117). Furthermore, the human homolog of NuA4, hNuA4, is shown to have associated with it several ING proteins, including p33ING1b and ING3 (105, 109). A number of human ING proteins have been shown to associate with histone modifying complexes similar to the situation seen for yeast ING proteins. As is the case with YNG1 over-expression, some, if not all, human ING proteins are toxic to the cell when over-expressed (92).

While there is a great deal of similarity is shown to exist between yeast and human ING proteins, little is known about the functional role that ING proteins are playing within the cell, especially in their interactions with histone modifying complexes. Having shown that Yng1p interacts with the nucleosome, we were anxious to extend this line of research to include human ING proteins. Thus, we proposed that if any human ING protein possessed a function similar to that shown for Yng1p, over-expression of this gene in yeast should be toxic considering the high conservation of histones and histone post-translational modifications. Based on this hypothesis, ING2 appears to share functional homology to Yng1p as over-expression of ING2 shows results identical to that seen for YNG1 over-expression. This difference in behavior between ING proteins reflects the situation seen in yeast, where only Yng1p appears to mediate the interaction of its associated complex with chromatin, while the functional roles of Yng2p and Pho23p have yet to be determined. Therefore, it is possible that ING2 functions, at least in part, by interacting with the nucleosome, leaving other ING proteins to operate through different mechanisms.
4.3 Concluding Statement

The study of chromatin remodeling and histone modifications has really begun to emerge within the last several years. Identification of the yeast transcriptional activator Gcn5p as a HAT aided in the understanding of the role that these protein complexes play in yeast transcription. Development of the histone code has helped to refine the proposed mechanisms whereby histone post-translational modification regulates transcription. The persistence of ING proteins in HAT complexes from yeast to humans indicates a conserved function for these proteins in histone modification. As an explanation for their role as tumor suppressors in human tissue cells, it is interesting to suppose that the function of ING family members is linked to histone modification. In support of this possibility, we have shown that the yeast ING homolog, Ynglp, mediates the interaction of the NuA3 complex with nucleosomes. The conserved functions between yeast and human ING proteins in both structure, and putative interacting partners, leads us to suggest that one mechanism whereby ING proteins function is through their interaction with the nucleosome. Although work done in this study is fundamental, and direct applications to human ING proteins would be presumptuous, it does provide a base for the additional work necessary for the clarification of ING proteins, and their associated function.
Figure 4.1 The NuA3 acetylation pathway – a proposed model.

The H3 tail is methylated at lysine residues 4 and 36 by the histone methyltransferases Setlp and Set2p respectively. This methylation serves as a mark to facilitate the interaction of NuA3 with the H3 tail. The interaction of NuA3 with methylated lysine 4 is mediated by Yng1p while interactions with methylated lysine 36 appears to be mediated by another unknown subunit of NuA3, or an additional unidentified factor. The interaction with methylated lysine residues permits the association of the histone acetyltransferase complex NuA3, with its target residues lysine 14 and 23, which it subsequently acetylates.
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