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FUNCTIONAL CHARACTERIZATION OF THE SAS (SOMETHING ABOUT SILENCING) COMPLEX IN BUDDING YEAST SACCHAROMYCES CEREVISIAE

A Thesis in

Biochemistry, Microbiology, and Molecular Biology

by

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ABSTRACT

The yeast SAS2 (Something About Silencing 2) gene encodes a member of MYST (hMOZ, yYbf2/Sas3, ySas2, and dTIP60) family of histone acetyltransferases (HATs) and is involved in transcriptional silencing at all silent loci (HML, HMR, telomeres, and rDNA) in Saccharomyces cerevisiae. Here we show that the native SAS complex is a trimeric protein complex composed solely of Sas2, Sas4, and Sas5. The SAS complex is capable of acetylating core histones and nucleosomes through its catalytic subunit Sas2. The conserved MYST domain in Sas2 is essential for both the silencing function and histone acetyltransferase activity of the SAS complex. More importantly, SAS exclusively acetylates lysine 16 of histone H4, and this specific lysine preference corresponds to the role of SAS complex in antagonizing the spreading of Sir proteins at chromosome ends.

In addition to the SAS complex, histone variant H2A.Z also plays an important role in anti-silencing function at subtelomeric regions. We demonstrate that SAS2 and HTZ1 synergistically regulate a subset of genes near telomeres for transcription activation. In the absence of SAS2, H4 lysine 16 acetylation and H2A.Z are both reduced at subtelomeric regions. Furthermore, mutation at H4 lysine 16 causes the same H2A.Z loss. When recruited, SAS enhances the H4 lysine 16 acetylation and the subsequent H2A.Z enrichment. Our data suggest that acetylation of H4 lysine 16 by SAS is required for incorporation of H2A.Z near telomeres. As a result, the presence of H4 lysine 16 acetylation and H2A.Z thus synergistically prevents the ectopic propagation of heterochromatin.
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Chapter 1

Introduction

1.1 Hierarchy in Chromatin Organization - Chromatin, Nucleosomes, and Histones

In the nucleus of eukaryotic cells, the genomic DNA is highly folded and compacted via associations with histones and other nonhistone proteins to form the dynamic polymer known as chromatin. The basic repeating unit of chromatin is the nucleosome core particle, which contributes to the first level of higher-order organization of chromosomal DNA. The nucleosome core particle, linker DNA, and histone H1 make up the complete nucleosome. Regular nucleosomal arrays form a 10-nm fiber of chromatin that folds into a 30-nm fiber upon the incorporation of the linker histone H1. While the exact arrangement of nucleosomes within the chromatin fiber is poorly understood, experimental data as well as modeling support a helical packing model (Widom 1989; Woodcock et al. 1993; Beard and Schlick 2001). Each nucleosome core particle is comprised of a histone octamer that is wrapped with 146 bp of DNA to form a tight left-handed superhelix structure, which leads to the approximate sixfold-length compaction of the DNA (Luger et al. 1997). Two copies of H3 and H4 form the central tetramer, which interacts with two H2A-H2B heterodimers through a specific interface between H2B and H4. Besides the relatively unstructured N- and C-terminal tails, all core histones share a common central structural element called the histone fold, which is essential for nucleosome structure. These histone fold domains are required for
the formation of H2A-H2B heterodimers, H3-H4 heterodimers, (H3/H4)_2 tetramer through H3-H3 interaction, and the subsequent histone octamer through H2B-H4 interaction (Arents et al. 1991). In addition, they account for the direct interactions between DNA and the histone octamer. The crystal structure of the nucleosome reveals a main globular body within the structure, while the more flexible and charged histone N-terminal tails are predominantly external to the nucleosome, revealing sites for post-translational modifications (Luger et al. 1997).

The synthesis of all core histones is tightly regulated and occurs exclusively during S-phase (Osley et al. 1986; Freeman et al. 1992). The histone proteins are then deposited by histone chaperones to newly replicated DNA to form the highly stable nucleosomes. Nucleosomes then form higher-order chromatin fibers, which are in turn further condensed during mitosis to from individual chromosomes (Krude and Keller 2001). As a result, the compact architecture of chromatin naturally presents significant repression effects on all cellular processes that use the underlying DNA as a template. In order for transcription, DNA repair, replication, and recombination to take place, the DNA-histone interactions within nucleosomes must be loosened or remodeled to allow access for regulatory protein complexes (Ehrenhofer-Murray 2004).

1.2 Transcriptional Silencing in *Saccharomyces cerevisiae*

The compact nature of chromatin structure partitions chromosomes into distinct functional regions. While euchromatin often refers to regions of DNA that are actively transcribed, the heterochromatic regions consist of highly condensed chromatin and are
directly linked to transcriptional silencing. Unlike gene-specific repression that is mediated by transcriptional repressors, “silencing” is a form of position-dependent and gene-independent transcriptional repression in which chromatin structure influences gene expression over large regions of the genome (Rusche et al. 2003). Transcriptional silencing involves the establishment of a repressive chromatin structure, followed by propagation and maintenance of this silent chromatin. Once established, the silenced state of chromatin is heritable in most if not all species. In yeast, it persists through mitotic and meiotic cell divisions, and the specific chromatin structure is replicated during the process of chromosome duplication (Rusche et al. 2003). The epigenetic silencing phenomena is observed in various organisms including X chromosome inactivation in mammalian cells for dosage compensation and position effect variegation in Drosophila centromeric and telomeric regions (Heard et al. 1997; Hennig 1999). In yeast Saccharomyces cerevisiae, transcriptional silencing occurs at a number of genomic locations, including the HML and HMR mating-type loci, telomeres, and the tandem rDNA array (Huang 2002; Rusche et al. 2003).

1.2.1 Transcriptional Silencing at the HML and HMR Mating-Type Loci

The mating type of haploid yeast, either a or α cells, is determined by expression of the gene cassette from mating-type locus MAT. The genetic information in the MAT locus is donated from either HML for α-specific genes, or from HMR for a-specific genes, and is interconvertable. The switch between a and α cells is catalyzed by the HO
endonuclease, which cleaves the \textit{MAT} locus then transfers a copy of either the \textit{HML} or \textit{HMR} cassette to the \textit{MAT} locus for repair (Haber 1998; Rusche et al. 2003).

To assure mating ability, transcription at \textit{HML} and \textit{HMR} loci must be silenced in haploid yeast. The \textit{HM} loci are flanked by \textit{cis}-acting elements, known as \textit{E} and \textit{I} silencers that are the initiation sites for transcription silencing (Abraham et al. 1984; Feldman et al. 1984). Perhaps the best-characterized silencer is the \textit{HMR-E} silencer, which contains binding sites for several silencing initiating factors, including ORC (Origin Replication Complex), Rap1 (Repressor and activator protein 1), and Abf1 (ARS-binding factor 1) (Sussel and Shore 1991; Foss et al. 1993; Loo et al. 1995; Rusche et al. 2003). It has been confirmed that mutations at these binding sites, as well as genes encoding for these binding factors, cause severe silencing defects at \textit{HMR} locus (Kimmerly et al. 1988). The presence of these three factors in combination leads to the optimal accumulation of Sir proteins (Silent information regulator 1, 2, 3, and 4), which are required for silencing (Moazed et al. 1997). Although none of these Sir proteins are essential for viability, Sir2, Sir3, and Sir4 greatly impact mating-type loci silencing, as well as telomeric silencing. Deletion of \textit{SIR1} causes mild silencing defects at the \textit{HM} loci, however, after binding to the ORC, Sir1 can recruit Sir4 to silencers, which links the function of Sir1 to silencing establishment (Pillus and Rine 1989; Triolo and Sternglanz 1996). Sir3 can be recruited through interactions with either Abf1, Rap1, or Sir4. In addition to being recruited by Sir1 or Rap1, Sir4 also can bring Sir2 to silencers as a Sir2-Sir4 complex (Moretti et al. 1994; Buck and Shore 1995; Cockell et al. 1995; Moazed et al. 1997; Ghidelli et al. 2001). Interestingly, Sir2 is a NAD$^+$-dependent histone deacetylase (HDAC) and its HDAC activity appears to significantly contribute to the
process of heterochromatin spreading (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). Once bound to chromatin, Sir proteins (Sir2, Sir3, and Sir4) can spread over nucleosomes by a unique mechanism in which the sequential histone deacetylation along the chromosome by Sir2 provides a high affinity binding site for Sir3 and Sir4 (which also brings Sir2) (Hecht et al. 1995; Hecht et al. 1996; Carmen et al. 2002), thus leading to the efficient propagation of Sir proteins over several kilobase pairs of DNA.

1.2.2 Transcriptional Silencing at Telomeres

Similar to the position effect variegation found in *Drosophila*, any gene that is placed near a yeast telomere is transcriptionally repressed, a phenomenon known as the telomere position effect (TPE) in yeast (Gottschling et al. 1990). The establishment of transcriptional silencing at telomeres is very similar to that seen at the *HM* loci. Abf1, the ORC complex, and Sir1 are not involved in the process of telomere silencing (Pillus and Rine 1989; Aparicio et al. 1991). Instead, Ku70 and Rap1 are responsible for recruiting Sir proteins to the nucleosome-free telomeric DNA at the ends of chromosomes to initiate telomeric heterochromatin formation (Tsukamoto et al. 1997; Martin et al. 1999; Mishra and Shore 1999). The spreading of Sir proteins away from the telomeres and toward the euchromatic region is dependent on the HDAC activity of Sir2 and the interactions of Sir3 and Sir4 with hypoacetylated histone tails (Rusche et al. 2003). Telomeres are tethered to the nuclear periphery through the interaction between Ku proteins (Ku70 and Ku80) and the myosin-like nuclear pore complex proteins, Mlp1 and Mlp2 (Galy et al. 2000). The perinuclear localization of telomeres appears to be important for silencing
since the absence of any of the tethering components disrupts the telomeric association of Sir proteins, which leads to silencing defects. The tethering of telomeres to nuclear periphery itself, however, is not sufficient to cause silencing (Gotta et al. 1996; Boulton and Jackson 1998; Laroche et al. 1998; Tham et al. 2001).

1.2.3 Transcriptional Silencing at the Tandem rDNA Array

The yeast rDNA array contains 100-200 tandem repeats of 5S and 35S rRNA genes that are highly transcribed by Pol III and Pol I respectively (Petes and Botstein 1977). The insertion of any pol II transcribed gene into the rDNA array results in the transcriptional repression phenomenon known as rDNA silencing (Bryk et al. 1997; Smith and Boeke 1997). Unlike silencing at HM loci and telomeres, Sir2 is the only Sir protein involved in rDNA silencing. Sir2 forms a complex, RENT (REgulator of Nucleolar silencing and Telophase exit), with Cdc14 (Cell division cycle 14) and Net1 (Nucleolar silencing establishing factor and telophase regulator 1), which recruits Sir2 to the rDNA array (Shou et al. 1999; Straight et al. 1999). At present, the mechanism of rDNA silencing is not well understood.

1.2.4 Silenced Chromatin and Transcriptional Repression

The establishment and heritance of silenced chromatin structure has been extensively studied. Nevertheless, the exact mechanism by which the silenced chromatin affects transcription activity is not clear. One possible mechanism proposed for
transcriptional repression involves the physical presence of Sir proteins on chromatin. The binding of Sir proteins to nucleosomes could stabilize the chromatin structure and make it less accessible to the conformational change that is necessary for transcription to take place (Kimura and Cook 2001; Ahmad and Henikoff 2002; Kireeva et al. 2002). Alternatively, the HDAC activity of Sir2 might be the key for transcriptional repression. The high correlation between histone deacetylation and transcriptional repression has long been established (Workman and Kingston 1998), and will be discussed later in this chapter.

1.2.5 Boundaries for Silenced Chromatin

The propagation of heterochromatin, although efficient, is restricted by specific barriers. Two major histone acetylation-related models have been proposed to explain how the spreading of the silenced chromatin is halted. The first model suggests that a cis-acting DNA element serves as a silencing barrier by recruiting chromatin modifiers to change the underlying chromatin structure (Donze et al. 1999). The best example comes from the study on a t-RNA\textsuperscript{THR} gene near the HMR-I silencer. It has been demonstrated that this tRNA gene is capable of efficiently blocking the spreading of silenced chromatin. Interestingly, the barrier activity of the t-RNA\textsuperscript{THR} gene depends on several histone modifiers and chromatin remodelers (Donze and Kamakaka 2001; Oki et al. 2004). It has recently been shown that the histone H3 specific SAGA (Spt Ada Gcn5 Acetyltransferase) complex is directly associated with the barrier t-RNA\textsuperscript{THR} gene (Oki and Kamakaka 2005).
The second model suggests that the heterochromatin-euchromatin boundary is
determined by the competition of two functionally opposite chromatin modifiers. Sir2 has
been shown to specifically deacetylate histone H4 at lysine 16 \textit{in vitro} (Imai et al. 2000;
Tanny and Moazed 2001). At telomeres, the propagation of Sir proteins is antagonized by
the SAS (Something About Silencing) complex, presumably by its histone acetylation
activity (Kimura et al. 2002; Suka et al. 2002). Since the SAS complex is also involved in
silencing at other loci, a similar anti-silencing mechanism might apply to \textit{HM} loci and
rDNA array.

1.3 Chromatin-Modifying Mechanisms

The accessibility of DNA is dynamically regulated through several distinct, but
not mutually exclusive, mechanisms to accomplish the destabilization of DNA-histone
interactions or the removal of all or part of the histone octamer. Over the last ten years,
numerous multiprotein complexes have been identified that contribute to these
mechanisms by remodeling chromatin, covalently modifying histones, and exchanging
histones with non-major histone variants (Berger 2002; Henikoff et al. 2004; Cairns
2005).

1.3.1 Chromatin Remodeling

Chromatin remodeling complexes use energy derived from ATP hydrolysis to
perturb DNA-histone contacts. These ATP-dependent chromatin remodelers are capable
of either unwrapping the nucleosomal DNA, sliding the nucleosome along the DNA, or transferring the histone octamer to another DNA molecule to generate nucleosome-free DNA regions (Tsukiyama 2002; Langst and Becker 2004). To date, all the chromatin remodeling complexes characterized contain a highly conserved ATPase subunit that belongs to SWI2/SNF2 (SWItching deficient /Sucrose NonFermenting) superfamily (Becker and Horz 2002; Tsukiyama 2002; Eberharter and Becker 2004). Based on their sequence features outside the ATPase domain, proteins in this family with known remodeling activity can be further divided into four subfamilies: SWI2/SNF2 (Sudarsanam and Winston 2000), CHD (Chromo-Helicase-DNA binding) (Brehm et al. 2000; Wang and Zhang 2001), ISWI (Imitation SWItch) (Langst and Becker 2001), and INO80 (INOsitol requiring) (Shen et al. 2000). The distinguishing feature of the SWI2/SNF2-type ATPase is the bromodomain, which binds to acetylated lysines on histone tails (Horn and Peterson 2001; Martens and Winston 2003). Likewise, members of the CHD subfamily are characterized by the chromodomains (chromatin organization modifier), which bind to methylated lysines on histone tails (Delmas et al. 1993; Kelley et al. 1999). One or more SANT (Swi3, Ada2, N-CoR, TFIIB-B) domains are present in ISWI remodelers (Elfring et al. 1994; Grune et al. 2003). As for the INO80 subfamily, one interesting property shared in this family is that the ATPase domain contains an insert that splits the conserved region into two segments (Eberharter and Becker 2004). These specific protein domains might be required for the interaction between ATP-dependent chromatin remodelers and other transcriptional regulatory complexes such as histone chaperones and histone modifying enzymes (Hassan et al. 2001; Hassan et al. 2002; Narlikar et al. 2002).
The first discovered and by far the most studied chromatin remodeling complex is the yeast SWI/SNF complex. It was identified through two independent genetic screens for mutants affecting either mating type switching or sucrose fermentation (Workman and Kingston 1998; Sudarsanam and Winston 2000). Genetic and biochemical characterizations have revealed its composition and provided strong evidence for chromatin remodeling activity in an ATP-dependent manner (Workman and Kingston 1998; Vignali et al. 2000a). Systematic genome-wide analyses have implicated the role of SWI/SNF in both gene activation and repression in yeast (Holstege et al. 1998; Sudarsanam et al. 2000; Wang 2003). It has been shown that SWI/SNF can be recruited to specific promoters through direct interaction with sequence-specific transcriptional activators (Prochasson et al. 2003), however, whether SWI/SNF is directly involved in transcription repression is unclear. Nevertheless, the identification of SWI/SNF homologous in human and *Drosophila* do indicate the functional importance of SWI/SNF in evolution (Kwon et al. 1994; Dingwall et al. 1995; Wang et al. 1996).

1.3.2 Post-translational Histone Modifications

Histone proteins can be covalently modified by various multiprotein complexes thus providing another way to alter the chromatin structure. The post-translational modifications on histones include acetylation and ubiquitylation of lysine residues, phosphorylation of serine and threonine residues, and, methylation of lysine and arginine residues (Berger 2002; Eberharter and Becker 2002; Kouzarides 2002; Lachner and Jenuwein 2002). These modifications can occur on the flexible N-terminal tail, as well as
on the exposed residues in the globular domain of histones (Santos-Rosa and Caldas 2005) (Figure 1-1). In addition, the combination of modifications on single histones, single nucleosomes, and nucleosomal domains could establish a “histone code” that specifies unique downstream functions (Strahl and Allis 2000; Turner 2000). Since the main focus in this thesis is a putative histone acetyltransferase complex, histone acetylation and its corresponding enzymes will be discussed later in further detail.

Figure 1-1: Post-translational modification sites on histones. Shown here are the acetylation (Purple), methylation (Blue), phosphorylation (Yellow), and ubiquitylation (Green) sites on histones that have been previously confirmed. (Adapted from Santos-Rosa and Caldas, 2005)
A long-standing model suggests that histone modifications may directly regulate chromatin structure by interfering with histone-histone or histone-DNA interactions. This model is supported by several independent observations (Ausio et al. 1989; Fletcher and Hansen 1995; Tse and Hansen 1997; Hansen et al. 1998; Wolfe and Hayes 1999). In these studies, however, the histones were premodified in an undefined pattern, which makes data interpretation complicated. Nevertheless, the hypothesis is further verified by recent studies using recombinant histones to examine chromatin structure. Dorigo et al. implicated the histone H4 N-terminal tail, especially amino acids 14-19, in maintenance of proper chromatin folding (Dorigo et al. 2003). A more recent study from Shogren-Knaak et al. further demonstrated that the acetylation of H4 lysine 16 is the central switch for controlling higher-order chromatin structure (Shogren-Knaak et al. 2006). Based on the crystal structure of the nucleosome, histone H4 tail contacts the H2A/H2B dimer of the neighboring nucleosome (Luger et al. 1997). Shogren-Knaak’s work confirms that the acetylation of H4 lysine 16 destabilizes this internucleosomal interaction and impairs chromatin fiber compaction.

Covalent histone marks can also indirectly regulate chromatin structure by recruiting a variety of chromatin-related effectors, such as transcription factors, histone modification enzymes, and chromatin remodelers. This concept is reflected in the “histone code” hypothesis, which states that a sequence or combination of specific modifications on histone tails acts as a signal to other proteins and produces distinct biological effects (Strahl and Allis 2000; Jenuwein and Allis 2001). One critical component in this model is the histone modification recognition protein which specifically binds to a certain type of modification through a unique interacting domain,
such as the bromo- (acetylated lysine residue) and chromo- (methylated lysine residue) domain (Matangkasombut and Buratowski 2003; Brehm et al. 2004). These bromodomain or chromodomain containing proteins exert their functions by initiating downstream biological responses that include alteration of chromatin state, DNA repair, and transcription activation and/or repression (de la Cruz et al. 2005).

Interestingly, the same type of modification on different residues could result in opposite effects. For example, methylation at histone H3 lysine 4 correlates with transcription activation, while H3 K9 methylation is tightly linked to silenced heterochromatin and transcription repression (Kouzarides 2002). Moreover, the degree of modification on a particular residue can effect the transcription signaling to a different level. It is clearly demonstrated in vitro that transcription is further repressed when H3 lysine 9 is converted from di-methyl to tri-methyl state (Wang et al. 2003).

1.3.3 Histone Variant Incorporation

The replacement of canonical histones with their counterpart variant appears to influence chromatin structure and dynamics for specific functions (Kamakaka and Biggins 2005). The expression of the major histones is tightly regulated and primarily restricted to S phase of the cell cycle (Osley et al. 1986; Freeman et al. 1992). They are then assembled and deposited into DNA immediately after replication to resume the chromatin state. Unlike the canonical histones, the synthesis and deposition of histone variants occurs throughout the cell cycle in a DNA replication-independent manner, which has considerable impact on cell cycle regulation, chromosome segregation, DNA
repair, genomic stability, heterochromatin barrier formation, and transcription (both activation and repression) (Kamakaka and Biggins 2005; Dhillon et al. 2006). Histones are extremely conserved throughout evolution. Many variants of histone H2A and histone H3 have been identified from different species (Table 1-1). Recent works have also identified two testis-specific H2B variants from human, but their respective functions are not yet understood (Zalensky et al. 2002; Churikov et al. 2004). As for histone H4, it is the most invariable one among all histone proteins and there is no known variant discovered from any species.

Table 1-1: **Nomenclature of histone H2A and H3 variants.**
(adapted from Kamakaka and Biggins, 2005)

<table>
<thead>
<tr>
<th>Histone Variant</th>
<th>Mouse</th>
<th>Human</th>
<th>Drosophila</th>
<th>Tetrahymena</th>
<th>S. cerevisiae</th>
<th>S. pombe</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3-3</td>
<td>h3β</td>
<td>H3.3</td>
<td>Hs3.3</td>
<td>hv2</td>
<td>H3</td>
<td>hht3</td>
</tr>
<tr>
<td>CENP-A</td>
<td>Cenpa</td>
<td>CENPA</td>
<td>Cid</td>
<td>TetCENPA?</td>
<td>Cse4</td>
<td>cnp1</td>
</tr>
<tr>
<td>H2A.Z</td>
<td>H2afz</td>
<td>H2A.Z</td>
<td>H2AvD</td>
<td>hv1</td>
<td>H2A.Z</td>
<td>phl1</td>
</tr>
<tr>
<td></td>
<td>H2afv</td>
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</tr>
<tr>
<td>H2A.X</td>
<td>H2afx</td>
<td>H2A.X</td>
<td>H2AvD</td>
<td>H2AX</td>
<td>H2A</td>
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<td>MacroH2A</td>
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1.3.3.1 Histone H3 Variants

Two H3 variants, H3.3 and CENP-A (CENtromere Protein A), have been intensively studied and their distinct functions have been characterized. The distribution of histone H3.3 is specific to transcriptionally active regions of the chromosome, suggesting that the replacement of H3 with variant H3.3 actually marks active genes for future rounds of transcription initiation (Ahmad and Henikoff 2002; Schwartz and Ahmad 2005; Wirbelauer et al. 2005). It should be noted that, while the deposition of canonical H3 by CAF-I (Chromatin Assembly Factor-I) is replication-dependent, histone variant H3.3 utilizes the HIRA (Histone Regulatory homolog A) complex for its incorporation, which is independent of DNA replication (Ray-Gallet et al. 2002; Loyola and Almouzni 2004; Tagami et al. 2004). Interestingly, the canonical yeast H3 is more homologous to histone variant H3.3 from other species (Ahmad and Henikoff 2002), which is in agreement with the finding that the majority of yeast genome is in a transcriptionally active or competent state (Lohr and Hereford 1979).

The essential histone variant CENP-A is a centromere-specific histone, which has great impact on kinetochoore assembly. It has been suggested that centromeric CENP-A functions as a binding site for kinetochoore proteins during the process of centromere construction. Nonetheless, the machinery for loading CENP-A into centromeres has not yet been identified (Sullivan et al. 1994; Smith 2002; Sarma and Reinberg 2005).
1.3.3.2 Histone H2A Variants

Among all core histones, H2A has by far the largest group of variants that includes H2A.X, H2A.Z, MacroH2A, and H2A-Bar-body-deficient (H2A-Bbd). The most distinguishable difference of these variants to the major H2A is the C-terminal tail, which contains a phosphorylation site in some cases (Jin et al. 2005). MacroH2A and H2A-Bbd are only found in mammals including human and mouse, and have specific chromosomal distributions. The localization of MacroH2A is predominantly on the highly condensed and inactive X chromosome, while H2A-Bbd is specifically found at sites of transcription and in active X-chromosome and autosomes (Costanzi and Pehrson 1998; Pehrson and Fuji 1998; Chadwick and Willard 2001; Malik and Henikoff 2003).

H2A.X variants from different species share a conserved serine residue at the C-terminal tail. Although H2A.X is randomly distributed throughout the genome, it is specifically phosphorylated at this C-terminal serine residue at sites flanking DNA double strand breaks in response to DNA damage (Redon et al. 2002). The yeast homolog of H2A.X is actually the canonical histone H2A (Malik and Henikoff 2003). It has been reported that, in yeast, the phosphorylated H2A induced by DNA double strand breaks (DSBs) can recruit chromatin remodeler INO80 and histone acetyltransferase NuA4 (Nucleosome Acetyltransferase of histone H4)(Bird et al. 2002; Choy and Kron 2002; Downs et al. 2004; van Attikum and Gasser 2005). Evidence showing the direct effects of these complexes on the variant-related DNA repair mechanism is still missing. The role of phosphorylated H2A variant in the DNA damage pathway is better explained in Drosophila. The histone variant of H2A in Drosophila is H2AvD, which has sequence
characteristics similar to both H2A.Z and H2A.X (C-terminal tail) (Redon et al. 2002). Upon DNA damage, the phosphorylated H2AvD enriched near DSBs is acetylated and then replaced with unmodified H2AvD \textit{in vitro} by dTip60 (Tat interacting protein of 60 kDa), a histone acetyltransferase complex (Madigan et al. 2002; Kusch et al. 2004).

\subsection*{1.3.3.3 Histone Variant H2A.Z and the SWR1 Complex}

The histone variant H2A.Z is the most studied H2A variant in terms of function. This highly conserved histone variant constitutes about 5-10 \% of the total H2A histone pool and can be found in a variety of species (Raisner and Madhani 2006). Notably, although not essential in yeast, the absence of H2A.Z causes lethality in higher eukaryotes such as mouse, \textit{Drosophila}, and \textit{Tetrahymena} (Liu et al. 1996; Clarkson et al. 1999; Faast et al. 2001).

Recent works from three independent groups all confirmed the existence of H2A.Z in a specialized \textit{Saccharomyces cerevisiae} chromatin remodeling complex called SWR1 (Sick With Rat8 ts) (Kobor et al. 2004; Krogan et al. 2004; Mizuguchi et al. 2004). Wu’s laboratory used an \textit{in vitro} biochemical method involving recombinant nucleosomal arrays to successfully demonstrate that SWR1 can specifically catalyze the replacement of histone H2A/H2B dimers with variant H2A.Z/H2B dimers in an ATP-dependent manner (Mizuguchi et al. 2004). The catalytic motor in this 13-subunits SWR1 complex is an SWI/SNF type ATPase Swr1 protein, which is essential for H2A.Z deposition (Kobor et al. 2004; Krogan et al. 2004; Mizuguchi et al. 2004). In addition, the Swc2 (Swr1 complex) subunit makes direct contact with H2A.Z and is also required for
transfer of H2A.Z to chromatin (Wu et al. 2005). The SWR1 complex also has a bromodomain-containing Bdf1 (Bromodomain factor 1) protein. Because Bdf1 preferentially binds to acetylated histone H3 and H4 through its tandem bromodomains (Ladurner et al. 2003; Matangkasombut and Buratowski 2003), it is tempting to postulate that it is involved in the recruitment of the SWR1 complex to acetylated nucleosomes.

Interestingly, SWR1 shares several subunits with the NuA4 HAT complex, and both complexes have common regulatory targets for transcription (Kobor et al. 2004; Krogan et al. 2004; Mizuguchi et al. 2004). The close relationship between SWR1 and NuA4 initially raises the possibility that the acetylation by NuA4 can facilitate the targeting of SWR1 to chromatin through binding of Bdf1 to acetylated histones. Recent studies, however, have implicated NuA4 in functions downstream of SWR1 by showing that it acetylates H2A.Z only after its deposition into chromatin (Babiarz et al. 2006; Keogh et al. 2006; Millar et al. 2006).

The biophysical influence of H2A.Z in nucleosome stability and chromatin structure is still ambiguous. Crystal structure of the H2A.Z-containing nucleosomes from *Xenopus* has revealed slight differences compared to the canonical nucleosomes. The presence of H2A.Z changes the surface charge and results in a subtle destabilization of the H2A.Z-H3 interaction within the histone octamer (Suto et al. 2000). It has been suggested that these changes actually alter protein-nucleosome and internucleosomal interactions, as well as higher order chromatin folding (Fan et al. 2004). However, the *in vitro* biochemical characterization of H2A.Z nucleosomes concludes different physical properties depend on the assays performed or the sources of nucleosomes. For example, the *Xenopus* H2A.Z nucleosomes appeared to be more stable based on fluorescence
resonance energy transfer analysis, while opposite results were obtained in yeast, where H2A.Z seemed to dissociate from nucleosomes more easily (Park et al. 2004; Zhang et al. 2005).

Despite the lack of agreement between data from structure and biological functional studies, the histone variant H2A.Z, once incorporated into chromatin, does have significant impact on various cellular processes. Histone H2A.Z contributes to maintenance of genomic integrity as H2A.Z mutants exhibit defects in chromosome segregation, DNA repair, and genome stability (Carr et al. 1994; Madigan et al. 2002; Krogan et al. 2004; Rangasamy et al. 2004). A recent study also suggested a role for H2A.Z in regulation of cell cycle progression (Dhillon et al. 2006). The role of H2A.Z in transcription has been linked to both activation and repression (Santisteban et al. 2000). It is involved in silenced heterochromatin organization in both Drosophila and mammalian cells (van Daal et al. 1988; Leach et al. 2000; Rangasamy et al. 2003; Rangasamy et al. 2004). Although widely distributed throughout the entire yeast genome, the histone variant H2A.Z is non-randomly enriched at the promoters of inactive genes to poise genes for transcriptional activation (Guillemette et al. 2005; Li et al. 2005; Zhang et al. 2005).

Perhaps the most intriguing function of H2A.Z in yeast involves its role in establishing a gene silencing barrier. At the silenced HMR mating locus, H2A.Z is important in the regulation of gene silencing by functioning parallel with barrier elements to prevent the ectopic spreading of heterochromatin (Meneghini et al. 2003). A similar anti-silencing function of H2A.Z was also observed at yeast telomeres. It was initially found by genome-wide microarray analysis that genes dependent on H2A.Z for
transcriptional activation tend to cluster near telomeres. Consistent with this observation, subsequent studies showed that the silencing regulator Sir proteins spread toward euchromatic regions in the absence of H2A.Z (Meneghini et al. 2003; Kobor et al. 2004). Furthermore, this heterochromatic propagation was also noted in deletion of any one of the SWR1 subunit, Swr1, Bdf1, or Yaf9 (Yeast AF-9) (Krogan et al. 2003; Ladurner et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Zhang et al. 2004). Taken together, the anti-silencing function of histone variant H2A.Z appears to play a key role in the restriction of silenced chromatin domains, although the question of the origin of the anti-silencing mechanism remains to be addressed.

1.4 Histone Acetylation

The best-characterized mechanism in post-translational modification is histone acetylation. It was initially linked to transcription activation, but its diverse functions in cell cycle progression, DNA repair, or gene silencing have been subsequently demonstrated (Allfrey et al. 1964; Brownell et al. 1996; Steger et al. 1998; Carrozza et al. 2003). This particular modification is carried out by a class of enzymes known as histone acetyltransferases (HATs), which covalently transfer an acetyl group from acetyl-CoA to the ε-amino group of specific lysine residues on histone N-terminal tails. The reaction is reversible and the acetyl group can be removed by histone deacetylases (HDACs) (Roth et al. 2001; Kurdistani and Grunstein 2003).

Two mechanisms have been proposed to explain how acetylation influences chromatin structure and transcription activity. The acetylation on histones neutralizes the
positive charge of the lysine resides, which leads to the disruption of DNA-histone, histone-histone, internucleosomal interactions and chromatin decondensation (Workman and Kingston 1998). In addition, this epigenetic acetylation marker provides the recognition site for recruiting other chromatin- or transcription-related effectors through the bromodomain-containing proteins, which often significantly affects transcription outcome (Strahl and Allis 2000; Jenuwein and Allis 2001).

Histone acetyltransferases found in the cytoplasm are grouped as type B HATs which only acetylate newly synthesized histones (Ruiz-Carrillo et al. 1975; Allis et al. 1985). On the other hand, nuclear HATs, or type A HATs, are able to acetylate histones in the nucleosome context and are considered to be coupled to transcription (Brownell and Allis 1996). Type A HATs can be further categorized into five subfamilies: (1) Gcn5-related N-acetyltransferase or GNATs (2) MYST HATs (3) p300/CBP HATs (4) basal transcription factor HATs (5) nuclear receptor cofactor HATs (Carrozza et al. 2003). Considerable effort has been directed toward understanding the components, the respective function of each subunit, and the transcription regulation mechanism of these HATs over the last decade. Here, the MYST family of histone acetyltransferases will be extensively discussed.

1.4.1 MYST Family of HATs

The MYST family is named after its founding members MOZ, Ybf2/Sas3, Sas2, and Tip60 (Utley and Cote 2002; Yang 2004). This large group of proteins is evolutionarily conserved (Figure 1-2), and involved in a variety of cellular processes,
such as transcription activation, DNA repair, cell cycle control, and gene silencing (Carrozza et al. 2003). Members of the MYST family share a highly conserved ~370 amino acid residues MYST domain (36-77 % identity and 54-84 % similarity), which is homologous to the acetyl-CoA binding domain in GNATs family (Roth et al. 2001; Yang 2004). This unique MYST domain contains an acetyl-CoA binding motif and a C2HC zinc finger (except for Esa1) and is essential for the HAT activity of many MYST family members (Takechi and Nakayama 1999; John et al. 2000; Akhtar and Becker 2001; Sutton et al. 2003; Shia et al. 2005). In addition to this conserved domain, the presence of a functionally distinct motif, either a chromodomain (in Esa1, MOF, and Tip60), plant homeodomain (PHD) finger (in Enok, MOZ, and MORF), or second zinc finger (in HBO1), has been reported in several MYST HATs (Yang 2004). Therefore, outside the conserved MYST domain, the MYST family of proteins appears to have diverse domain organizations.
Proteins in the MYST family are often found as the catalytic subunit in a multiprotein HAT complex. For example, Tip60-containing HAT complexes (Tip60 complex) exist in both human and Drosophila. Additionally, the MSL complex in

Figure 1-2: MYST proteins and their domain organizations from (A) S. cerevisiae (B) Drosophila (C) Human.

Chromo, chromodomain; Ser, serine-rich domain; CH, cysteine/histidine-rich motif; H15, linker histones H1- and H5-like domain; NEMM, N-terminal part of Enok, MOZ or MORF; PHD, PHD zinc finger; ED, glutamate/aspartate-rich region; SM, serine/methionine-rich domain. The SM domain of MOZ has an insertion of a proline/glutamine-stretch (labeled P). Bars below the N-terminal and SM domains of MORF denote its transcriptional repression and activation domains, respectively. Numbers on the right correspond to the total residues that each protein has. (Adapted from Yang, 2004)
Drosophila utilizes MOF as the catalyst for its histone acetylation function. This finding is also true in yeast, as Esa1 and Sas3 and their corresponding histone acetyltransferase complexes have been purified and characterized (Esa1 in NuA4 and Sas3 in NuA3) (Utley and Cote 2002; Kusch et al. 2004). Besides the intrinsic histone acetylation activity, these HAT complexes all possess distinct functions reflecting their diversity in domain structure.

1.4.1.1 NuA4 in Yeast

The yeast NuA4 contains Esa1 (Essential sas-related acetyltransferase 1), the only essential HAT identified in yeast, as the catalytic subunit. NuA4 is a large protein complex consisting of 12 subunits with a total molecular weight of 1.3 MDa (Grant et al. 1997). The HAT activity of the Esa1 protein alone is limited to core histones. It is capable of acetylating nucleosomes, preferentially at histone H4, however, when assembled into NuA4 (Allard et al. 1999; Clarke et al. 1999). The importance of Esa1 in cell cycle progression and DNA repair has been demonstrated (Smith et al. 1998; Clarke et al. 1999; Bird et al. 2002).

The NuA4 complex can be specifically targeted to nucleosomes through interaction between the Tra1 subunit and acidic activators to promote transcription activation (Utley et al. 1998). Tra1 also exists in the Gcn5-containing SAGA complex. Its function in activator-targeting in SAGA is similar to that in NuA4, however, the acetylation properties in SAGA and NuA4 appear to be different (Brown et al. 2001). It has been clearly shown that, upon recruitment, SAGA acetylates nucleosomes near the
targeting site while NuA4 acetylation appears to spread bi-directionally from the activator-bound region for a few kilobases (Vignali et al. 2000b).

Interestingly, Esa1, Yng2 (Yeast homolog of mammalian Ing1), and Epl1 (Enhancer of polycomb-like 1) can form a nucleosome-specific HAT complex termed Piccolo NuA4 (Boudreault et al. 2003; Selleck et al. 2005). Unlike NuA4, this highly active Piccolo complex does not interact with activators. It has thus been proposed that the transcription effect of NuA4 is targeted to genes by an activator; on the other hand, the nontargeted Piccolo NuA4 is responsible for the genome-wide acetylation function of Esa1.

1.4.1.2 Tip60 in Human and Drosophila

The human Tip60 complex is considered the homolog of the yeast NuA4 complex based on the similarity in protein composition. More than half of its subunits are homologous to NuA4 subunits, such as Tip60 to Esa1 and TRRAP to Tra1. In addition to the Tip60-dependent transcription activation, the human Tip60 complex is also thought to participate in DNA repair, apoptosis, and c-Myc-dependent oncogenic transformation (Ikura et al. 2000; Park et al. 2001; Frank et al. 2003; Murr et al. 2006). In Drosophila, the Tip60 complex (dTip60) has recently been purified and directly linked to DNA repair. Two enzymatic activities have been shown to associate with dTip60: histone acetylation by Tip60 and ATP-dependent histone replacement by Domino/p400. In response to DNA damage, the histone variant H2AvD is immediately phosphorylated at sites of DNA double strand breaks. Subsequently, dTip60 is able to acetylate the phopho-H2AvD
followed by exchanging it with unmodified H2AvD (Kusch et al. 2004), suggesting a specific role of dTip60 in the DNA repair pathway.

1.4.1.3 MOF in Human and Drosophila

Another MYST-type HAT in Drosophila, MOF (Male-absent On the First), forms a complex termed MSL (Male-Specific Lethal) with MSL1, MSL2, MSL3, MLE (MaleLEss), and two non-coding RNA molecules, roX1 and roX2 (RNA on the X) (Akhtar 2003). The significant impact of the MSL complex on fly dosage compensation has been well documented. Male flies double the transcription activity at their single X chromosome to compensate for the fact that female flies carry two copies of the X chromosome (Straub et al. 2005). This enhancement of transcription in the male X chromosome is achieved specifically by the MSL complex, which specifically acetylates histone H4 at lysine 16 (H4 K16) through its MOF subunit (Akhtar and Becker 2000). In female flies, the assembly of MSL is impaired due to the absence of MSL2, which results in hypoacetylation of H4 K16. In male flies, MSL binds to the X chromosome at hundreds of loci, and has a binding pattern similar to that of H4 AcK16 as confirmed by polytene chromosome immunostaining (Bone et al. 1994). Interestingly, the HAT activity of MOF is required for the spreading of MSL across the X chromosome (Gu et al. 2000). Once the MSL complex acetylates H4 K16, the X chromosome adopts a “diffuse” appearance, which is reminiscent of decondensed chromatin (Bashaw and Baker 1996). Therefore, acetylation at H4 lysine 16 by the MSL complex appears to “open up” the Drosophila male X chromosome to make it more accessible to transcription, which is an
important part of the unique dosage compensation mechanism. The functions of human MOF have recently been characterized. The H4 K16 specific acetyltransferase MOF can be co-purified with H3 K4 methyltransferase MLL1 (Mixed-Lineage Leukemia-1) and both activities are required for optimal transcription activation (Dou et al. 2005). In addition, human MOF protein functionally associates with ATM (Ataxia-Telangiectasia-Mutated), suggesting a role of human MOF in DNA damage response during cell cycle progression (Gupta et al. 2005; Taipale et al. 2005). The human homolog of the Drosophila MSL complex is responsible for the bulk acetylation of H4 K16. Interestingly, neither MLL nor ATM protein is found in this MOF-containing hMSL complex (Smith et al. 2005). Collectively, these discoveries suggest that MOF might exist in several functionally distinct complexes in human cells.

1.4.1.4 NuA3 in Yeast

The yeast NuA3 (Nucleosome Acetyltransferase of histone H3) complex is a histone H3 specific, trimeric HAT complex consisted of Sas3 (Something about silencing 3), Taf14 (TBD associated factor 14), and Yng1 (Yeast homolog of mammalian Ing1) (John et al. 2000). It is capable of acetylating histone H3 and the synthetic lethality caused by $gcn5\Delta sas3\Delta$ double knockout suggests an important role of NuA3 in maintaining the cellular H3 acetylation level (Howe et al. 2001). A recent study showed that NuA3 can be targeted to nucleosomes through interaction with methylated histone H3 for its in vivo acetylation function (Martin et al. 2006). Additionally, the interaction between NuA3 and Spt16 (Suppressor of Ty), a subunit in FACT (FAcilitates Chromatin
Transcription) complex, suggests that the NuA3 complex might be involved in regulating transcription elongation (John et al. 2000).

1.5 The Yeast SAS (Something About Silencing) Complex

As mentioned earlier, the silencing defects at HM loci caused by deletion of SIR1 is mild (Pillus and Rine 1989). The yeast SAS2 gene was first identified in a genetic screen looking for the enhancer of sir1Δ silencing defects at HML locus (Reifsnyder et al. 1996). Like SIR1, deletion of SAS2 does not disrupt HML silencing, however, the sir1Δsas2Δ mutant shows severe silencing defects at HML locus. Interestingly, the HMR silencing defects caused by mutation at HMR-E silencer can be restored by deleting SAS2 (Ehrenhofer-Murray et al. 1997). It should be noted that deletion of the SAS2 gene does not affect HMR silencing in cells with the wild-type HMR-E silencer. These seemingly conflicting silencing defects were also observed at other silent loci where telomere silencing is disrupted and rDNA silencing is enhanced upon the deletion of SAS2 (Reifsnyder et al. 1996; Meijsing and Ehrenhofer-Murray 2001). Later studies using similar genetic screens have isolated two other genes that shared identical silencing effects with SAS2, and were thus named SAS4 and SAS5 (Xu et al. 1999a; Xu et al. 1999b). These three genes seem to regulate silencing at all loci similarly and are considered positive regulators for HML and telomere silencing, but negative regulators for HMR and rDNA silencing. Interestingly, double or triple knockout of these three genes did not cause more severe silencing defects than the individual deletions (Xu et al.
Based on these genetic studies, it was proposed that SAS2, SAS4, and SAS5 might function in the same pathway to regulate transcriptional repression at all silent loci.

Indeed, previous work from our laboratory and others has confirmed the interactions between Sas2, Sas4, and Sas5 by biochemical methods. Furthermore, we were able to purify a multisubunit protein complex, termed SAS complex, containing at least Sas2, Sas4, and Sas5 from yeast cells (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001). Sas2 is one of the founding members of the MYST family. Unlike the other two MYST proteins in yeast (Esa1 in NuA4 and Sas3 in NuA3), the nucleosomal HAT activity of Sas2 in the context of the SAS complex has not yet been described, even though it also contains a highly conserved MYST domain. However, mutations within the MYST domain result in silencing defects, implying a functional link between the putative histone acetylation activity and the silencing regulation function of Sas2 (Osada et al. 2001; Osada et al. 2005). In addition, Sas2 can also interact with Cac1 (Chromatin assembly complex subunit 1), a subunit in histone chaperone CAF-1 (Chromatin Assembly Factor-I) complex (Verreault et al. 1996; Kaufman et al. 1997; Meijsing and Ehrenhofer-Murray 2001). Sas4 is the connective core subunit and bridges Sas2 and Sas5 in the SAS complex (Schaper et al. 2005). It has a cullin-homology domain, which is often found in E3 ubiquitin ligases. Previous studies have demonstrated that the SAS complex is also functionally associated with the histone deposition protein Asf1 (Antisilencing function 1) through the Sas4 subunit (Munakata et al. 2000; Osada et al. 2001; Sutton et al. 2001). One interesting feature of Sas5 is the functionally uncharacterized YEATS (Yaf9-ENL-AF9-Taf14-Sas4) domain. In yeast, all three proteins containing this unique domain are found in chromatin-related complexes. Besides Sas5 in the SAS
complex, Yaf9 is found in NuA4 and SWR complex; Taf14 is a subunit in NuA3, TFIID, TFIIF, SRB-mediator complex, and SWI/SNF (Henry et al. 1994; Poon et al. 1995; Cairns et al. 1996; John et al. 2000; Osada et al. 2001; Le Masson et al. 2003). Although none of them are essential, sas5Δtaf14Δyaf9Δ triple knockout yeast is not viable (Zhang et al. 2004), thus suggesting the importance of the YEATS domain in yeast.

Until recently, the role of SAS in the acetylation of nucleosomes has been poorly understood. The association of SAS with histone chaperone Asf1 and CAF-I leads to the suggestion that SAS might acetylate newly assembled nucleosomes to re-establish the epigenetic acetylation pattern (Ehrenhofer-Murray 2004). Genetically, both Asf1 and CAF-I have the same silencing function with the SAS complex at the HM loci (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001). A recent report has shown Asf1-dependent SAS occupancy at the silenced HML locus (Osada et al. 2005). The histone deposition mechanism of Asf1 and CAF-I is similar. They are both capable of depositing newly synthesized histone H3 and H4 on DNA immediately following replication in a non-overlapping pathway (Verreault et al. 1996; Tyler et al. 1999; Sharp et al. 2001; Tyler et al. 2001; Krawitz et al. 2002). It had been previously shown that the newly synthesized histones are acetylated at lysine 14 of H3 and lysine 5 and 12 on H4 by cytoplasmic HATs (Sobel et al. 1995). The duplicated chromatin has to undergo the removal or addition of acetylation marks at different lysines to maintain epigenetic patterns (Brownell and Allis 1996). For example, acetylation of histone H4 at lysine 16 is the global mark for euchromatin and this specific lysine residue has to become acetylated in the duplicated chromatin to protect the euchromatic regions from being silenced (Kimura et al. 2002; Suka et al. 2002). The fact that the SAS complex interacts with Asf1
and CAF-I makes SAS a likely candidate for the HAT that acetylates nucleosomes following DNA replication and the chromatin assembly.

It has become more obvious that histone H4 lysine 16 might be the specific target for the acetylation function of SAS based on several discoveries, although direct evidence for this role is still missing. First, a point mutation at histone H4 lysine 16 phenocopies \( sas2\Delta \) phenotypes at all silent loci (Meijssing and Ehrenhofer-Murray 2001). Secondly, the bulk acetylation level at histone H4 lysine 16 is dramatically and specifically reduced in \( sas2\Delta \) mutants, while Esa1 shows impacts on the other three lysines at histone H4 N-terminal tail (Kimura et al. 2002). In addition, H4 lysine 16 acetylation levels are lowered near telomeres when \( SAS2 \) is absent (Kimura et al. 2002; Suka et al. 2002). Furthermore, a number of telomere-proximal genes are down-regulated in either \( sas2\Delta \) or H4K16R mutant yeast (Kimura et al. 2002; Dion et al. 2005). Taken together, these findings strongly suggest that the SAS complex is an H4 lysine 16 specific HAT that prevents heterochromatin propagation at telomeres.

1.6 Thesis Overview

The research presented in this dissertation focuses on functional characterization of the yeast SAS complex. In our previous work, we purified the yeast SAS complex using an overexpression system and showed that the SAS complex contains at least three subunits, Sas2, Sas4, and Sas5, and is about 450 kDa in size. However, we failed to detect the HAT activity and identify any additional subunits beside the three known Sas proteins. In cooperation with Dr. Sternglanz’s laboratory (Appendix 1), we showed that
the recombinant SAS complex, consisting of Sas2, Sas4, and Sas5, has the ability to acetylate core histones, but not nucleosomes. We sought to purify the native SAS complex at endogenous expression levels and to examine whether it is able to acetylate core histones and nucleosomes. As summarized in Chapter 2, we have confirmed the composition of the SAS complex as a trimeric protein complex. One copy of Sas2, Sas4, and Sas5 forms the 125 kDa SAS complex. More importantly, the native SAS complex is able to acetylate both core histones and nucleosomes with substrate preference for lysine 16 of histone H4. We also investigated the biological relevance of the specific HAT activity of SAS, and showed that the SAS-mediated acetylation at histone H4 lysine 16 plays an essential role in establishing the anti-silencing mechanism at telomeres (Chapter 3). The acetylation at histone H4 lysine 16 is required for the incorporation of histone variant H2A.Z. As a result, the presence of H4 AcK16 and H2A.Z together block the spread of silenced heterochromatin. We will discuss the current and future directions regarding the interplay of SAS and H2A.Z in this anti-silencing mechanism later in Chapter 4.
Chapter 2

Characterization of the Yeast Trimeric-SAS Acetyltransferase Complex

2.1 Abstract

The yeast \textit{SAS2} (Something \textit{A}bout \textit{S}ilencing \textit{2}) gene encodes a member of MYST protein family of histone acetyltransferases (HATs) and is involved in transcriptional silencing at all silent loci (\textit{HML}, \textit{HMR}, telomeres, and rDNA) in \textit{Saccharomyces cerevisiae}. Sas2 is the catalytic subunit of a yeast histone acetyltransferase complex termed SAS complex. The enzymatic activity of SAS complex on free histones has been reported, but nucleosomal HAT activity has not yet been documented. Here we show that the native yeast SAS complex is a small trimeric protein complex composed solely of Sas2, Sas4, and Sas5 with a molecular weight of about 125 kDa. It is capable of acetylating both free histones and nucleosomes, although the nucleosomal HAT activity of SAS complex is very weak when compared to that of NuA4, the other member of MYST HAT complex. We also demonstrate that the putative acetyl CoA binding motif in Sas2 is essential for both the \textit{in vivo} silencing function and the enzymatic activity of SAS complex. Unlike NuA4, which acetylates all four available lysines at the N-terminal tail of histone H4, SAS complex exclusively acetylates lysine 16 of histone H4 \textit{in vitro} and is required for bulk H4 lysine 16 acetylation \textit{in vivo}. This specific lysine preference corresponds to the role of SAS complex in antagonizing the spreading of Sir proteins at silent loci in \textit{Saccharomyces cerevisiae}. 
2.2 Introduction

In eukaryotic nuclei, chromosomal DNA is packaged into a compact structure with histones to form chromatin. Chromatin can be an effective silencer of gene expression. Specifically, heterochromatin consists of highly condensed chromatin and is thought to contain primarily transcriptionally silenced genes. In yeast *Saccharomyces cerevisiae*, this type of transcriptional silencing occurs at several genomic locations including the *HML* and *HMR* mating-type loci, telomeres, and rDNA (Sherman and Pillus 1997).

Many proteins contribute to the establishment and maintenance of silenced chromatin. Among these proteins, Sir proteins 1-4 (*Silent Information Regulator*) have been shown to play crucial roles at all silent loci (Gasser and Cockell 2001). The *Something About Silencing 2 (SAS2)* gene was first isolated in a genetic screen scoring for a mutant which can enhance the epigenetic silencing defects at *HML* in *sir1Δ* background (Reifsnyder et al. 1996). It has also been reported that *SAS2* regulates silencing differently at different loci. *SAS2* functions as a positive regulator at *HML* and telomeres (Reifsnyder et al. 1996); its deletion leads to loss of silencing at these loci. Oppositely, deletion of *SAS2* enhances rDNA silencing (Meijsing and Ehrenhofer-Murray 2001) and restores the silencing defects at *HMR* with a mutated silencer (Ehrenhofer-Murray et al. 1997). Notably, loss of *SAS2* does not affect silencing at the wild-type *HMR* mating locus. The exact mechanisms for Sas2-mediated silencing at different loci remain unclear. Genetic evidence showed that two other genes, termed *SAS4* and *SAS5*, share the
same regulatory functions in silencing with SAS2 and may function in the same genetic pathway with SAS2 (Xu et al. 1999a; Xu et al. 1999b).

Sas2 is a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60) protein family of histone acetyltransferases (HATs). Proteins in this family include human MOZ, MORF, TIP60, and HBO1, *Drosophila* MOF and Chm and yeast Esa1, Sas2, and Sas3 (Carrozza et al. 2003). The MYST-related proteins all share a highly conserved acetyl-CoA binding motif which is essential for their HAT enzymatic activities (John et al. 2000). Many members in the MYST family are found to be the catalytic subunit of a multiprotein HAT complex (Sas3p in NuA3 and Esa1p in NuA4) and Sas2 is no exception. Recent biochemical studies indicate that Sas2, Sas4, and Sas5 exist as part of a 230-450 kDa protein complex in yeast (Meijising and Ehrenhofer-Murray 2001; Osada et al. 2001). Unlike Sas2, Sas4 is unique in that it does not share homology to any known protein. Sas5, on the other hand, shares a conserved YEATS (Yaf9-ENL-AF9-Taf14-Sas5) domain with several proteins including human leukemogenic proteins AF9/ENL/Gas41, yeast Taf14 and Yaf9. Although the exact function of the YEATS domain is unknown, the YEATS triple knockout yeast strain (*yaf9Δ, taf14Δ, sas5Δ*) is not viable (Zhang et al. 2004), suggesting that the YEATS domain is essential for viability in *Saccharomyces cerevisiae*. The predicted molecular mass of Sas2 (39 kDa), Sas4 (55 kDa) and Sas5 (29 kDa) add up to only 123 kDa suggesting that the SAS complex may contain additional subunits which have not been identified.

The yeast SAS complex, either native or recombinant, has intrinsic HAT activity on free histones (Sutton et al. 2003), but nucleosomal HAT activity has not yet been reported. In studies with recombinant SAS complex, it is clear that both Sas4 and Sas5
are required for optimal Sas2 HAT activity. It has been demonstrated that mutations in the conserved acetyl-CoA binding domain of Sas2 disrupt silencing at *HML* and telomeres (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001) and that the recombinant SAS complex with mutant Sas2 loses its HAT activity (Sutton et al. 2003). These results suggest a link between Sas2 HAT activity and its regulatory roles in gene silencing. There is also a functional link between SAS complex and chromatin deposition activity. The histone deposition proteins Asf1 (*Anti-Silencing Function 1*) and CAF-I (*Chromatin Assembly Factor-I*) both associate with the SAS complex (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001; Sutton et al. 2001). Asf1 and CAF-I contribute to chromatin assembly by depositing newly synthesized histone H3 and H4 into nucleosomes (Verreault et al. 1996; Tyler et al. 1999; Tyler et al. 2001). How SAS complex-mediated histone acetylation is involved in the chromatin assembly is unclear.

It has been reported previously that a point mutation at lysine 16 in the N-terminal tail of histone H4 phenocopies the silencing defects caused by deletion of *SAS2* (Meijsing and Ehrenhofer-Murray 2001). This H4 K16R mutation weakens the silencing at *HML* and telomere, but enhances the silencing at rDNA and restores the silencing at the mutant *HMR*. We and others have shown that SAS complex is a histone H4 lysine 16 specific histone acetyltransferase complex (Kimura et al. 2002; Sutton et al. 2003). The lysine preference of SAS complex plays a direct role in preventing the further spreading of Sir proteins into subtelomeric regions by antagonizing the deacetylation function of Sir2 (Kimura et al. 2002; Suka et al. 2002). The same anti-silencing function of SAS complex has also been observed at *HMR* silent locus at where SAS complex assists a unique tRNA<sup>Thr</sup> gene to establish a heterochromatic barrier (Donze and Kamakaka 2001; Oki et
al. 2004). Together, these data suggest that SAS complex plays an important role in blocking the propagation of heterochromatin.

In this report we show that the native yeast SAS complex is a small trimeric protein complex consisting solely of Sas2, Sas4, and Sas5. Contrary to previous findings, we show that the molecular weight of SAS complex is about 125 kDa. In addition, we demonstrate that SAS complex has novel *in vitro* nucleosomal HAT activity which is dependent on the acetyl CoA binding motif of Sas2. Unlike NuA4, SAS complex exclusively acetylates lysine 16 of histone H4. All three subunits of the complex are required to maintain the overall levels of acetylation at H4 lysine 16 in yeast cells.

### 2.3 Experimental Procedures

#### 2.3.1 Yeast Strains and Plasmids

The genotypes of all strains used in this study are described in Appendix A. Chromosomal-tagged or deleted strains were generated by one-step PCR-based strategy as described previously (Longtine et al. 1998; Puig et al. 2001). Standard yeast manipulations were performed as described (Guthrie 1991). All knockout strains were confirmed by PCR. The expressions of tagged proteins were verified by Western blots. Plasmids for expression of galactose-induced Sas proteins (pS116, pS128, and pS129) were published previously (Osada et al. 2001). To make Sas2-TAP expression vectors, pRS426/TAP (pS223) was first made by subcloning a PCR fragment containing *HindIII-TAP-SpeI* into pRS426 (pS23). Wild-type or mutant *SAS2* ORFs with 1 kb upstream
sequence were amplified by PCR from pS126, pS136, pS137, and pS138 (Osada et al. 2001). The PCR products containing a SAS2 gene with a 5’ XhoI site and a 3’ HindIII site were then subcloned into pRS426/TAP (pS223) to generate plasmids pS224 to pS227. All plasmids generated were further verified by sequencing.

2.3.2 Protein Expression and Purification

Whole cell extracts (WCE) were prepared by following a previously published procedure (Eberharter et al. 1998). Briefly, cells were grown to approximately 2x10^7 cells/ml in yeast extract-peptone-dextrose (YPD) and then washed in 10 ml of extract buffer (40 mM HEPES [pH 7.4], 350 mM NaCl, 10 % glycerol, 0.1 % Tween 20, 2 µg of pepstatin A/ml, 2 µg of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). WCE was prepared by bead beating in a bead beater (BioSpec). Cellular debris was removed by spinning the extracts at 12,000xg for 30 min. The extracts were cleared by ultracentrifugation at 100,000xg for 90 min. Purification of SAS complex (from YJW458, YJW475, and YJW476) and NuA4 (from YWJS053) from 12 liter culture by tandem affinity purification method were essentially performed as described elsewhere with minor changes (Lee et al. 2004). The clarified WCE was incubated with 500 µl of immunoglobulin G-Sepharose (Amersham Biosciences) beads overnight at 4°C. The beads were washed with 20 ml of extract buffer followed by 10 ml of TEV cleavage buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol [DTT]) and then were resuspended in 1 ml of TEV cleavage buffer. TEV cleavage was performed by the use of 10 µl (100 Units) of TEV protease.
(Invitrogen) overnight at 4°C. TEV-cleaved products were added to 3 ml of calmodulin binding buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, 0.1 % NP-40, 10 % glycerol, 0.5 mM DTT) along with 3 µl of 1 M CaCl₂ for each milliliter of TEV elution. Calmodulin-Sepharose (Amersham) purification was performed by binding of the eluate to 500 µl of beads overnight at 4°C. Proteins bound to calmodulin beads were eluted in 0.25-ml fractions by the use of calmodulin elution buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 0.1 % NP-40, 10% glycerol, 0.5 mM DTT). TAP-purified complexes were resolved in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and visualized by silver staining. WCE (5 mg) and SAS complex (purified from 4 liter culture) from YJW458 were fractionated in a Superose 6 HR 10/30 gel-filtration column using ÄKTApurifier system (Amersham Biosciences) (Osada et al. 2001). TAP-purified SAS complex (purified from 2 liter of YJW458) was also fractionated by 10-40 % glycerol gradient sedimentation in TAP Elution Buffer at 300,000xg for 5 hours. SAS complex was loaded on a 4.5 ml gradient and collected in 100 µl fractions. Gel filtration calibration kits (Amersham Biosciences) were used to estimate the molecular weight of protein complex. To check the overall in vivo histone H4 acetylation level (Figure 2-7A and 2-8A), whole cell extracts were prepared as described (Kimura et al. 2002).
2.3.3 MudPIT

MudPIT mass spectrometry analyses were carried out as described previously with usage of the three-phase MudPIT column (Washburn et al. 2001; Wolters et al. 2001; McDonald 2002; Lee et al. 2004). Data analysis commenced with the application of the software algorithm 2 to 3 to determine the charge state and to delete spectra of poor quality. Matches of MS/MS spectra to peptides from a *S. cerevisiae* protein sequence database (National Center for Biotechnology Information website) were determined using the program SEQUEST. Peptide/spectrum matches were sorted using the program DTAselct. Peptide hits from multiple runs were compared with CONTRAST software.

2.3.4 Immunoprecipitations, Western Blots and Antibodies

For immunoprecipitations of Myc epitopes (Figure 2-4), 1 mg of whole cell extracts from each strain were mixed with 1 µl of α-cMyc (clone 9E10, ROCHE) at 4°C overnight. 10 µl of washed, packed Protein G Sepharose beads (Amersham Biosciences) were added to the mixture and incubate at 4°C for 2 hours. The beads were washed 4 times with the extraction buffer, eluted by boiling with SDS-PAGE loading buffer, and then run in 10 % SDS-PAGE for Western blot analysis using peroxidase-conjugated α-cMyc (clone 9E10, ROCHE) and α-FLAG M2 (SIGMA). Fractions obtained from size-exclusion chromatography and glycerol gradient centrifugation were assayed by Western blots using Peroxidase-Anti-Peroxidase (SIGMA), peroxidase-conjugated α-cMyc (clone 9E10, ROCHE), and α-TAP (Open Biosystems). To check the overall *in vivo* acetylation
level (Figure 2-7A and 2-8A), whole cell extracts corresponding to \(1 \times 10^6\) cells were resolved in 18% SDS-PAGE and assayed by Western blots with \(\alpha\)-PGK (3-phosphoglycerate kinase) (Molecular Probes), \(\alpha\)-H4 AcK5 (Chemicon), \(\alpha\)-H4 AcK8 (Upstate), \(\alpha\)-H4 AcK12 (Upstate), and \(\alpha\)-H4 AcK16 (Serotec). In Figure 2-8B, HAT assay results were subjected to 18% SDS-PAGE and analyzed by Western blots with \(\alpha\)-H4 (Upstate), \(\alpha\)-H4 AcK5, \(\alpha\)-H4 AcK8, \(\alpha\)-H4 AcK12, and \(\alpha\)-H4 AcK16.

### 2.3.5 HAT Assays

For the experiments in Figure 2-6A, 2-7B, and 2-8B, HAT assays were performed in 15 µl reactions at 30°C for 1 hour in the presence of 40 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10 mM Sodium Butyrate, 1 mM PMSF, and 1.5 µM acetyl CoA ([\(^3\)H] labeled in Figure 2-6A and 2-7B, non-radioactive in Figure 2-8B). Substrates were as follows: 1 µg of HeLa core histones, 1 µg of HeLa nucleosomes, and 1 µg of recombinant yeast histone H4. For Figure 2-6A and 2-7B, the procedure was as described (Eberharter et al. 1998). For Figure 2-8B, the reactions were loaded on 18% SDS-PAGE and assayed for acetylated histone H4 by Western blots. For the experiments in Figure 2-6B, nucleosome arrays were assembled using recombinant yeast histones (Mizuguchi et al. 2004) as histone sources and were used as substrates in HAT assays. Approximately 2 pmole of SAS complexes were used in all HAT assays described above.
2.4 Results

2.4.1 The Native Yeast SAS Complex is a 125 kDa, Trimeric Complex

Many proteins in the MYST family of HATs are components of multiprotein complexes (Howe et al. 1999; Brown et al. 2000; Utley and Cote 2002). In budding yeast *Saccharomyces cerevisiae*, two well-studied examples from this family are Esa1 in NuA4 and Sas3 in NuA3. We and others have previously shown that Sas2, Sas4, and Sas5 form the SAS histone acetyltransferase complex and that Sas2 is the catalytic subunit (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001; Sutton et al. 2003). Size-exclusion chromatography analysis indicated that the SAS complex was between 230-450 kDa in size based on fractionation of whole cell extracts and complex purified from cells overexpressing Sas2. This molecular weight of the SAS complex, coupled with mass spectrometry analysis suggested additional subunits besides the three known components. We were unable, however, to identify any *bona fide* additional subunits from the SAS complex purified from cells overexpressing Sas2. Thus, we sought to purify the native complex from cells with endogenous levels of Sas2.

In order to purify the native and endogenously expressed SAS complex, we took advantage of the tandem affinity purification method (Puig et al. 2001). We generated a yeast strain with a TAP-tag in *SAS2* and a Myc-tag in *SAS4*. Both tags were integrated into the 3’ end of the gene of interest to ensure that expression was driven by the endogenous promoter. Yeast whole cell extracts were prepared from these double-tagged strains and subjected to the two-step affinity purification procedure to isolate SAS complex (Figure 2-1). We then fractionated both whole cell extracts and TAP-purified
SAS complex by Superose 6 size-exclusion chromatography. Fractions were analyzed by Western blots probing for Sas2-TAP in whole cell extracts and Sas4-Myc in purified complex (Figure 2-2). In both cases, we observed one protein peak at fraction 16 corresponding to 450 kDa in size, suggesting that we did not disrupt the integrity of the SAS complex during the purification procedures. This result using native SAS complex is identical to our previously published findings (Osada et al. 2001) using overexpressed SAS complex. Therefore, it remained a possibility that there were additional proteins in the complex besides Sas2, Sas4, and Sas5.
Yeast strain used: SAS4-Myc, SAS2-TAP

Figure 2-1: Tandem affinity purification of the native SAS complex.
Silver stained SDS-PAGE shows the native SAS complex purified from YJW458 (SAS4-Myc, SAS2-TAP). Proteins indicated in red are identified based on their sizes. Several unknown proteins (blue dots) are co-purified with Sas2, Sas4, and Sas5 from this strain.
To gain further insight into the detailed protein composition of SAS complex, we analyzed the TAP-purified SAS complex by MudPIT mass spectrometry analysis (Table 2-1). A mock TAP purification from a yeast strain expressing Sas4-Myc was performed as a negative control. The three known subunits, Sas2, Sas4, and Sas5, were identified as the top three proteins in MudPIT analysis based on the percent coverage and the number of unique peptide hits. Additional proteins identified in the mass spectrometry analysis fell into two groups. Those also found in the negative control sample and those found only in the purified SAS complex sample. Among the proteins that were recovered only from TAP-purified SAS complex, however, none had significant coverage or peptide hits (compared to Sas2, Sas4 and Sas5) to suggest that they were bona fide components of the SAS complex. We also tested if any of these

Figure 2-2: Gel filtration chromatography analysis suggests that the native yeast SAS complex is about 450 kDa in size.

The native yeast SAS complex was TAP-purified from the strain (YJW458) expressing both carboxyl-terminal Myc-tagged Sas4 and TAP-tagged Sas2. Both whole cell extracts and TAP-purified SAS complex were subjected to gel-filtration chromatography on a Superose 6 column using ÄKTApurifier system. All fractions were analyzed by Western blots probing for Sas2-TAP or Sas4-Myc. Molecular mass standards were used to estimate the size of the protein complex.

To gain further insight into the detailed protein composition of SAS complex, we analyzed the TAP-purified SAS complex by MudPIT mass spectrometry analysis (Table 2-1). A mock TAP purification from a yeast strain expressing Sas4-Myc was performed as a negative control. The three known subunits, Sas2, Sas4, and Sas5, were identified as the top three proteins in MudPIT analysis based on the percent coverage and the number of unique peptide hits. Additional proteins identified in the mass spectrometry analysis fell into two groups. Those also found in the negative control sample and those found only in the purified SAS complex sample. Among the proteins that were recovered only from TAP-purified SAS complex, however, none had significant coverage or peptide hits (compared to Sas2, Sas4 and Sas5) to suggest that they were bona fide components of the SAS complex. We also tested if any of these
proteins interact with the SAS complex *in vivo* by coimmunoprecipitation experiments; none of these proteins coimmunoprecipitated with the SAS complex. In addition, we also purified the SAS complex from strains with single TAP tag on either *SAS2* or *SAS5*. We visualized the purification results on a silver stained gel and observed only three proteins corresponding to Sas2, Sas4, and Sas5 based on their sizes in both cases (Figure 2-3). Based on these findings, we conclude that Sas2, Sas4, and Sas5 are the sole components of the SAS complex. These data presented us with an interesting paradox since our size-exclusion chromatography analysis indicated a 450 KDa complex; a number that is not the additive molecular weights of Sas2, Sas4 and Sas5.
**Table 2-1: Extensive mass spectrometry analysis of the yeast SAS complex.**

SAS complex TAP-purified from YJW458 (*SAS4-Myc, SAS2-TAP*) was analyzed by MudPIT. Tandem affinity purification from YJW228 (*SAS4-Myc*) serves as a mock purification. Data for TAP-purified SAS complex came from two independent purifications and analyses. The molecular weight (MW), number of unique peptide hits, and percent coverage of each protein identified by MudPIT were shown in the table.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>% Coverage (Number of Unique Peptide Hits)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAS complex</strong> <em>(SAS4-Myc, SAS2-TAP)</em></td>
<td><strong>MOCK</strong> <em>(SAS4-Myc)</em></td>
<td><strong>MW</strong> (kDa)</td>
</tr>
<tr>
<td>59.7 (84)</td>
<td>29</td>
<td>Sas5</td>
</tr>
<tr>
<td>57.8 (273)</td>
<td>55</td>
<td>Sas4</td>
</tr>
<tr>
<td>44.1 (88)</td>
<td>39</td>
<td>Sas2</td>
</tr>
<tr>
<td>13.1 (3)</td>
<td>23</td>
<td>Taf10</td>
</tr>
<tr>
<td>7.2 (6)</td>
<td>58</td>
<td>Taf6</td>
</tr>
<tr>
<td>4.5 (1)</td>
<td>25</td>
<td>Rpl10</td>
</tr>
<tr>
<td>3.5 (3)</td>
<td>72</td>
<td>Plb1</td>
</tr>
<tr>
<td>3.1 (3)</td>
<td>82</td>
<td>Ybr007c</td>
</tr>
<tr>
<td>2.9 (1)</td>
<td>31</td>
<td>Bmh2</td>
</tr>
<tr>
<td>13.3 (20)</td>
<td>5.2 (5)</td>
<td>94</td>
</tr>
<tr>
<td>7.8 (5)</td>
<td>2.6 (1)</td>
<td>50</td>
</tr>
<tr>
<td>6.2 (2)</td>
<td>3.1 (1)</td>
<td>52</td>
</tr>
<tr>
<td>5.3 (2)</td>
<td>2.0 (2)</td>
<td>49</td>
</tr>
<tr>
<td>4.4 (2)</td>
<td>2.8 (1)</td>
<td>61</td>
</tr>
<tr>
<td>2.1 (1)</td>
<td>1.4 (1)</td>
<td>100</td>
</tr>
</tbody>
</table>
One possible answer to this problem may be that the SAS complex contains multiple copies of one or more subunits. To test this hypothesis, we transformed a plasmid expressing Sas2-FLAG into a yeast strain with chromosomal-tagged SAS2-Myc. If there was more than one copy of Sas2 in the SAS complex, coimmunoprecipitations from whole cell extracts with α-Myc antibody should be able to pull down FLAG-tagged Sas2. In fact, we were not able to detect any Sas2-FLAG signal in the α-Myc IP although the expression of Sas2-FLAG was robust (Figure 2-4, top panel, Lane 6). Myc-
immunoprecipitations were determined to be efficient based on the fact that Sas2-Myc was dramatically reduced in supernatant (Figure 2-4, Lane 5 compared to Input Lane 4), and enriched on the beads (Figure 2-4, Lane 6). We performed the same experiments with Sas4 and Sas5 with similar results (Figure 2-4, middle and bottom panel). As a negative control, immunoprecipitations were carried out in the absence of Myc-tagged SAS subunit (Figure 2-4, Lanes 1-3). Reciprocal immunoprecipitations with α-FLAG were performed with the same outcome. Collectively, these data indicate that the native yeast SAS complex is a trimeric protein complex consisting of single copies of Sas2, Sas4, and Sas5 respectively.
In gel-filtration chromatography analysis, protein complexes will migrate according to their respective molecular weights only if they are globular. Therefore, this method should not be applied to determine the molecular weight of non-globular proteins. Accordingly, another possible explanation of our paradox is that the SAS

Figure 2-4: SAS complex is a trimeric protein complex.

Yeast strains with or without chromosomal Myc-tagged SAS subunit were transformed with plasmid expressing FLAG-tagged SAS subunit (Top-YJW274: W303a + pSAS2-FLAG, YJW428: SAS2-Myc + pSAS2-FLAG; Middle- YJW481: W303a + pSAS4-FLAG, YJW479: SAS4-Myc + pSAS4-FLAG; Bottom- YJW482: W303a + pSAS5-FLAG, YJW480: SAS5-Myc + pSAS5-FLAG). Yeasts were grown in minimal medium with 2 % galactose to induce the expression for FLAG-tagged subunit. Whole cell extracts were made and 1 mg of whole cell extracts was immunoprecipitated with α-Myc antibody followed by Western blots against either Myc-tagged or FLAG-tagged proteins (Lane 1 and 4: 5 % Input, Lane 2 and 5: 5 % Supernatant, Lane 3 and 6: 20 % Bead). Lane 1 to 3 serves as a negative control for Myc-immunoprecipitation. Lane 6 shows no FLAG-tagged subunit can be pulled down by the same subunit with Myc tag.

In gel-filtration chromatography analysis, protein complexes will migrate according to their respective molecular weights only if they are globular. Therefore, this method should not be applied to determine the molecular weight of non-globular proteins. Accordingly, another possible explanation of our paradox is that the SAS
complex forms a non-globular shape. To test this theory, we fractionated the SAS complex by glycerol gradient sedimentation which separated proteins primarily on the basis of their mass and to a lesser extent on their density and shape (Siegel and Monty 1966). The SAS complex purified from \(SAS2\)-\textit{TAP}, \(SAS4\)-\textit{Myc} double-tagged strain was fractionated by 10-40 % glycerol gradient sedimentation. Fractions were then analyzed by Western blots probing for Sas2-CBP and Sas4-Myc (Figure 2-5). The SAS complex signal peaked at fractions No.9 and 10 in our analyses. After calibrating the fraction profile with molecular mass standards, we estimated that the native yeast SAS complex was actually 150 kDa in size; approximately the sum of Sas2-CBP (44 kDa), Sas4-Myc (75 kDa), and Sas5 (29 kDa). If we subtract the contribution of the CBP (5 kDa) and Myc (20 kDa) tags from this value it indicates a molecular mass of the untagged complex of 125 kDa, very near the sum of the predicted molecular mass of Sas2, Sas4 and Sas5 (123 kDa). Therefore, we concluded that the SAS complex is a small (125 kDa), trimeric (Sas2, Sas4, and Sas5) protein complex consisting of single copies of each subunit.
2.4.2 SAS Complex Has Weak, But Real, Nucleosomal HAT Activity

Several groups have suggested that the HAT activity of the SAS complex is responsible for counteracting the Sir2-mediated deacetylation at telomeres and HMR mating locus, thereby preventing the propagation of Sir proteins into euchromatin (Donze and Kamakaka 2001; Kimura et al. 2002; Suka et al. 2002; Oki et al. 2004). Thus, one possible function for the SAS complex in vivo is nucleosome acetylation. We had previously reported, however, that recombinant SAS complex was able to acetylate free histones, but not nucleosomes in vitro. A very similar acetylation pattern was obtained when we tested the HAT activity of the native SAS complex (Sutton et al. 2003). It efficiently acetylated both the recombinant yeast histones and core histones purified from HeLa cells, suggesting that the preexistent modifications on HeLa core histones didn’t

Figure 2-5: SAS complex is a 125 kD, non-globular protein complex.

SAS complex purified from YJW458 (SAS4-Myc, SAS2-TAP) by tandem affinity purification method was fractionated by 10-40 % glycerol gradient centrifugation. Fractions were then assayed by Western blots against either Sas2-CBP or Sas4-Myc. Fractions not shown in this figure didn’t illustrate the presence of SAS complex. The molecular weight of the purified SAS complex was estimated based on the fractionation of molecular mass standards.

2.4.2 SAS Complex Has Weak, But Real, Nucleosomal HAT Activity

Several groups have suggested that the HAT activity of the SAS complex is responsible for counteracting the Sir2-mediated deacetylation at telomeres and HMR mating locus, thereby preventing the propagation of Sir proteins into euchromatin (Donze and Kamakaka 2001; Kimura et al. 2002; Suka et al. 2002; Oki et al. 2004). Thus, one possible function for the SAS complex in vivo is nucleosome acetylation. We had previously reported, however, that recombinant SAS complex was able to acetylate free histones, but not nucleosomes in vitro. A very similar acetylation pattern was obtained when we tested the HAT activity of the native SAS complex (Sutton et al. 2003). It efficiently acetylated both the recombinant yeast histones and core histones purified from HeLa cells, suggesting that the preexistent modifications on HeLa core histones didn’t
affect the HAT activity of the native SAS complex (see Appendix B, Figure 6B). Although the nucleosomal HAT activity of the native SAS complex was above background, the results were not significant. Since those studies were published we have optimized our in vitro HAT assays by adding HDAC inhibitors, lowering salt content to 80 mM total, and using more concentrated, TAP-purified SAS complex. These new conditions allowed us to detect significant SAS nucleosomal HAT activity for the first time (Figure 2-6A, Lane 5). Using similar amounts of histones, we found stronger SAS HAT activity on free histones when compared to that on nucleosomes (Figure 2-6A, compare Lane 2 and 5). We also compared the nucleosomal HAT activity of SAS complex to NuA4 and found that even with excessive amount of SAS complex (2 pmole of SAS vs. 0.1 pmole of NuA4), nucleosomal HAT activity of SAS complex is still much weaker than NuA4 (Figure 2-6A, compare Lane 5 and 6). In order to rule out the possibility that the SAS nucleosomal HAT activity was contributed by the contaminated free histones in the reaction, we used immobilized nucleosomal arrays as substrates which allowed us to wash away any free histones in the reaction. Again, the nucleosomes were significantly acetylated by SAS complex (Figure 2-6B). Therefore, we conclude that the native SAS complex has real, although weak, nucleosomal HAT activity.
Figure 2-6: SAS complex has weak, but real, nucleosomal HAT activity.

(A) *In vitro* HAT assay using TAP-purified SAS (Lane 2 and 5, from YJW458) and NuA4 (Lane 3 and 6, from YWJS053) as histone acetyltransferases, HeLa core histones (Lane 1 to 3) and HeLa nucleosomes (Lane 4 to 6) as substrates. Half of the reactions were spotted on Whatman P-81 filters for quantification. The other half was loaded on a 18% SDS-PAGE to separate individual histones. Gel was then stained with Coomassie blue and subjected for fluorography (Lane 1 to 3: 2 days exposure; Lane 4 to 6: 7 days exposure).

(B) Immobilized nucleosome arrays assembled from recombinant yeast histones were incubated with SAS complex. After stringent washing, acetylated nucleosomes were directly subjected for quantification by liquid counting assay. Data shown are the average of at least three separate experiments. Error bars represent standard deviation.
Members of the MYST family share a conserved acetyl CoA binding motif. Mutations at the acetyl CoA binding domain of Sas3 cause the loss of HAT activity in NuA3 (John et al. 2000). We have previously shown that the mutations in the Sas2 acetyl CoA binding domain (M1: 219GLG to AAA, M2: 216QR to AA, M3: 224LI to AA) resulted in the disruption of silencing at telomere and the HML mating locus. Among those mutations tested, SAS2-M1 mutation showed the most severe silencing defects (Osada et al. 2001). In addition, recombinant SAS complex containing Sas2-M1 lost its ability to acetylate histones (Sutton et al. 2003). It has been demonstrated that loss of SAS2 will cause a dramatic reduction of in vivo acetylation at histone H4 lysine 16 (Kimura et al. 2002). To further clarify the importance of the acetyl CoA binding domain of Sas2 to its enzymatic activity, we checked the overall acetylation level at histone H4 lysine 16 in the presence of mutant SAS2 and found that bulk H4 lysine 16 acetylation levels were indeed lowered in vivo (Figure 2-7A). Next, we TAP-purified the native SAS complex with either wild-type SAS2 or mutant SAS2 (M1). The purification results were visualized by silver staining and no difference could be observed between wild-type and mutant SAS complex. However, the mutant SAS complex was no longer able to acetylate nucleosomes (Figure 2-7B). We also obtained the same results using free histones as substrates. Our data indicate that the acetyl CoA binding domain of SAS2 is not only essential for its silencing function, but also crucial for the HAT activity of the SAS complex. This result also confirms that the detected weak nucleosomal HAT activity was indeed due to Sas2 and not to any minor contaminants in the preparation.
(A) Plasmid with wild-type or mutant SAS2-TAP was introduced into sas2Δ strain. Whole cell extracts were probed with α-3-Phosphoglycerate Kinase (α-PGK) as loading control and α-H4 AcK16 (Lane 1: YJW475 sas2Δ + pSAS2 WT-TAP, Lane 2: YJW476 sas2Δ + pSAS2 M1-TAP, 219GLG to AAA, Lane 3: YJW477 sas2Δ + pSAS2 M2-TAP, 216QR to AA, Lane 4: YJW478 sas2Δ + pSAS2 M3-TAP, 224LI to AA).

(B) SAS complexes were TAP-purified from either YJW475 (Wilt-Type) or YJW476 (M1 mutant). HAT activity of each complex was compared by HAT assay using HeLa nucleosomes as substrates (Lane 1: nucleosomes along, Lane 2: nucleosomes with WT SAS, Lane 3: nucleosomes with M1 SAS). Results were analyzed by either liquid counting or fluorography. The amount of SAS complex used in HAT assays were normalized based on Western against Sas2-CBP. Data shown are the average of three separate experiments. Error bars represent standard deviation.

Figure 2-7: Acetyl-CoA binding motif in Sas2 is essential for the HAT activity of native SAS complex.
2.4.3 Histone H4 Lysine 16 is the Exclusive Histone Substrate for the SAS Complex

We and others have shown that SAS complex specifically acetylates lysine 16 of histone H4 and loss of SAS2 is directly responsible for the reduction in overall acetylation level at histone H4 lysine 16 (Kimura et al. 2002; Sutton et al. 2003). Here we showed that SAS4 and SAS5 are also essential for maintaining the acetylation specifically at lysine 16 of histone H4 (Figure 2-8A). Deletion of either subunit in the SAS complex caused the equal reduction of acetylation at histone H4 lysine 16. Therefore, these results indicate that the SAS complex can function only if all three subunits are present.
Figure 2-8: SAS is an exclusively histone H4 lysine 16 HAT.

(A) Whole cell extracts prepared from WT (YWJS001), sas2Δ (YWJS002), sas4Δ (YWJS004), or sas5Δ (YWJS005) strains were subjected to Western blots probing for mono-acetylated histone H4 as indicated on the left.

(B) In the presence of non-radiolabeled acetyl-CoA, recombinant yeast histone H4 was incubated with or without HAT complex as indicated in the figure. The reactions were then analyzed by Western blots probing for different mono-acetylated isoforms of histone H4 as indicated on the left. The amount of SAS complex and NuA4 used in the assays were normalized by measuring the individual HAT activity on recombinant yeast histone H4 with [3H] acetyl-CoA.
We next examined the *in vitro* substrate specificity of the native SAS complex by using yeast recombinant histone H4 as acetylation substrate. The same experiments were also performed with NuA4 as a comparison. The amount of SAS complex and NuA4 used in the HAT assays were normalized based on the HAT activity obtained from liquid counting assay using [3H] labeled acetyl CoA. After the HAT reaction, we analyzed the results by Western blots probing for various mono-acetylated H4 isoforms (Figure 2-8B). Unlike NuA4, which acetylates all available lysines at H4 N-terminal tail, the native SAS complex exclusively acetylates lysine 16 on H4. This result further demonstrates that the native SAS complex is an H4 lysine 16 specific HAT complex which correlates with its anti-silencing function in antagonizing the Sir2-mediated deacetylation of H4 lysine 16.

2.5 Discussion

We have presented evidence in this report that the yeast SAS complex is a small trimeric histone acetyltransferase complex that is capable of acetylating nucleosomes. Although the composition of the SAS complex is simple (Sas2, Sas4, and Sas5), each subunit is vital for HAT activity both *in vivo* and *in vitro*. Sas2, as the catalytic subunit, is essential for the enzymatic activity. Sas4 does not show significant homology to any known protein, but it may play a role in physically linking the SAS complex to chromatin assembly since it interacts directly with histone deposition protein Asf1 (Sutton et al. 2001). Sas5 is a member of YEATS protein family. In *Saccharomyces cerevisiae*, YEATS-domain containing proteins are all components of chromatin-related complexes (Sas5 in SAS; Yaf9 in NuA4 and SWR complex; Taf14 in NuA3, TFIID, TFIIF, SRB-mediator complex, and SWI/SNF) (Henry et al. 1994; Poon et al. 1995; Cairns et al. 1996; John et al. 2000; Le Masson et al. 2003). Deletion of all three YEATS-containing proteins
(yaf9Δ, taf14Δ, sas5Δ) from yeast results in lethality (Zhang et al. 2004). Sas5 is required for the full HAT activity of the SAS complex (Sutton et al. 2003). Deletion of Yaf9 shows a similar phenotype to deletion of certain NuA4 or SWR components (Doyon and Cote 2004; Zhang et al. 2004). It appears that a major role for the YEATS-containing complexes is anti-silencing. One possible mechanism for YEATS protein function could be to target their respective complex to specific chromatin boundaries, perhaps through histone binding and recognition.

We present here novel nucleosomal HAT activity for the SAS complex. We confirmed that the SAS complex can acetylate nucleosomes after optimizing reaction conditions. There is no question that the SAS complex is responsible for the specific acetylation of histone H4 lysine 16 in living cells. The fact that SAS can easily acetylate free histones, but not nucleosomes made from them, rules out the possibility that other histone post-translational modifications are prerequisite for SAS-mediated acetylation (Jenuwein and Allis 2001). It is still not clear, however, why free histones serve as better in vitro substrates for SAS complex when compared to nucleosomes. One obvious explanation for this discrepancy is that we are missing some associated factors in our in vitro system that are required for full activity of the SAS complex. These factors may not necessarily co-purify with Sas2, but may be required for optimal SAS complex function on nucleosomes. Specifically, nucleosome remodeling may play a role in facilitating the SAS complex function by making histones more accessible for acetylation. Indeed, Gas41, a human homolog of Sas5, was found to interact with the Snf5 subunit of SWI/SNF (Debernardi et al. 2002).

As proposed by Ehrenhofer-Marray (Ehrenhofer-Murray 2004), SAS complex may play a role in re-establishing the euchromatin mark following replication-coupled nucleosome deposition. Histone deposition proteins, Asf1 and/or CAF-I, incorporate newly synthesized histone H3 and H4 on DNA in a replication-coupled manner (Verreault et al. 1996; Le et al. 1997; Tyler et al. 1999). Interestingly, Asf1 and CAF-I are associated with the SAS complex
(Kaufman et al. 1997; Meijsing and Ehrenhofer-Murray 2001; Sutton et al. 2001). It is possible that SAS complex is recruited by Asf1 or CAF-I following histone deposition to acetylate nucleosomes at histone H4 lysine 16 and reestablish the euchromatin mark. As discussed above, other chromatin-modifying complexes may play a role in this process. For example, Asf1 has been shown to interact with Drosophila SWI/SNF (Brahma complex) (Moshkin et al. 2002).

Yeast with sas2Δ or mutated SAS2 all show silencing defects and loss of enzymatic HAT activity, implying a direct link between these two functions. It has been suggested that the SAS complex is involved in the formation of a barrier that prevents spreading of heterochromatin from silenced regions into euchromatin. Specifically, this mechanism has been termed “anti-silencing” and can be found at the telomeres and HMR mating locus boundaries (Donze and Kamakaka 2001; Kimura et al. 2002; Suka et al. 2002; Oki et al. 2004). The acetylation of histone H4 lysine 16 by SAS complex may provide a barrier to Sir proteins which would prevent adjacent euchromatic regions from being transcriptionally inactivated. It would be interesting to discover whether the SAS complex is involved in anti-silencing at other loci.

Recent studies regarding H2A.Z, a histone H2A variant, have revealed an interesting link between H4 lysine 16 acetylation and histone variant replacement suggesting another mechanism for the establishment of silencing barriers by SAS complex. It is believed that SWR complex replaces H2A with H2A.Z at subtelomeric regions (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004) and that this substitution plays a key role in blocking the propagation of Sir proteins. Interestingly, many genes near telomeres are dependent on SAS2 for activation based on microarray analysis (Kimura et al. 2002), and the same results have been reported for many components of the SWR complex, including SWR1, BDF1, HTZ1, and YAF9 (Krogan et al. 2003; Ladurner et al. 2003; Meneghini et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Zhang et al. 2004). This overlap in function suggests a possible cooperation between SAS complex and SWR complex in the establishment of silencing barriers. Perhaps acetylation of histone H4 lysine
16 by the SAS complex not only antagonizes the NAD-dependent deacetylation by Sir2, but also signals the SWR complex for H2A.Z incorporation.

2.6 Acknowledgments

We are grateful to Bing Li for providing recombinant nucleosome arrays used in HAT assays and Samantha Pattenden for critical reading of the manuscript. This work is supported by NIGMS Grant R37-GM-047867.
Chapter 3

SAS-Mediated Acetylation of Histone H4 Lysine 16 is Required for H2A.Z Incorporation at Telomeres in *Saccharomyces cerevisiae*

### 3.1 Abstract

The native yeast SAS complex is a trimeric histone acetyltransferase complex composed solely of Sas2, Sas4, and Sas5. It exclusively acetylates lysine 16 of histone H4. This specific lysine preference corresponds to the role of SAS in antagonizing the spreading of Sir proteins at silent loci in yeast. The histone variant, H2A.Z, also plays an important role in anti-silencing at subtelomeric regions, however, the exact mechanism for this action remains unclear. Here we demonstrate that *SAS2* and *HTZ1* synergistically regulate the transcriptional activation of a subset of genes near telomeres. ChIP-chip experiments revealed that there is a dramatic reduction in acetylation of H4 lysine 16 at subtelomeric regions when *SAS2* is deleted. Interestingly, the H2A.Z occupancy near telomeres is also reduced in the absence of Sas2. A similar loss of H2A.Z is observed upon introduction of a point mutation at histone H4 lysine 16. Moreover, acetylation of H4 lysine 16 by artificially recruited SAS complex boosts the enrichment of H2A.Z. Overall, our data suggest a novel anti-silencing mechanism at telomeres: acetylation of histone H4 lysine 16 by SAS serves as a signal for H2A.Z deposition. As a result, the presence of H4 lysine 16 acetylation and H2A.Z synergistically prevent the ectopic propagation of heterochromatin.
3.2 Introduction

The eukaryotic genome is packaged with histones to form higher order chromatin structure. This compact state of chromatin renders the DNA poorly accessible for many essential cellular processes including transcription, DNA recombination, repair, and replication (Workman and Kingston 1998). To overcome the naturally repressive nucleosome impediment, cells have devised three mechanisms to alter chromatin architecture: post-translational histone modifications, ATP-dependent chromatin remodeling, and histone variant incorporation (Berger 2002; Henikoff et al. 2004; Cairns 2005).

The formation of highly condensed heterochromatin results in transcription repression. In budding yeast, *Saccharomyces cerevisiae*, this type of transcriptional silencing occurs at several genomic locations including the *HML* and *HMR* mating-type loci, telomeres, and rDNA in a gene-independent, position-dependent manner (Rusche et al. 2003). Among the numerous silencing regulatory factors, Sir2 plays a crucial role at all silent loci. It is generally believed that the NAD$^+$-dependent histone deacetylase activity of Sir2 is the driving force behind the formation of silenced heterochromatin (Blander and Guarente 2004). Yet, how the spreading of this heterochromatin is blocked remains poorly understood.

Several hypotheses have been proposed to explain how the propagation of heterochromatin is halted. One model suggests that specific DNA elements function as silencing barriers either by interacting with perinuclear substrates to separate chromosomal regions, or by recruiting histone modification machinery to alter the
underlying chromatin structure (Kimura et al. 2002; Suka et al. 2002; Oki et al. 2004; West et al. 2004; Oki and Kamakaka 2005). At yeast telomeres, there is no clear evidence of such barrier elements; competition between histone acetylation and deacetylation forms the euchromatin-heterochromatin boundary. One very important histone acetyltransferase, the SAS (Something About Silencing) complex, plays a crucial role in anti-silencing in this subtelomeric region (Kimura et al. 2002; Suka et al. 2002).

Sas2 is the catalytic subunit of the yeast histone acetyltransferase SAS complex. The yeast SAS complex is a small trimeric protein complex consisting of Sas2, Sas4, and Sas5 (Osada et al. 2001; Sutton et al. 2003; Shia et al. 2005). Genetic studies suggest that all of the SAS subunits are essential for maintaining the proper silencing at all loci (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997; Xu et al. 1999a; Xu et al. 1999b; Meijsing and Ehrenhofer-Murray 2001). The highly conserved MYST domain in the Sas2 catalytic subunit is absolutely required for both the silencing function and enzymatic activity of the SAS complex (Osada et al. 2001; Shia et al. 2005). The function of the Sas4 and Sas5 subunits has yet to be determined. SAS is a histone H4 lysine 16 specific acetyltransferase complex (Sutton et al. 2003; Shia et al. 2005). Mutation at lysine 16 of histone H4 phenocopies the silencing defects of sas2Δ (Meijsing and Ehrenhofer-Murray 2001), suggesting a functional link between the HAT activity of SAS and its role in regulating transcription silencing. Notably, the unique lysine preference of SAS has been shown to antagonize the deacetylation function of Sir2 at telomeres, thereby preventing Sir proteins from spreading into subtelomeric regions. This dynamic balance between Sas2 and Sir2 is responsible for the establishment of euchromatin-heterochromatin boundaries at telomeres (Kimura and Horikoshi 2004).
In addition to its global anti-silencing effects at telomeres, SAS also plays a role at the *HMR* silent locus. The SAS complex is thought to be targeted to a unique tRNA\textsubscript{Thr} gene close to *HMR* I silencer and may help establish a heterochromatic barrier, presumably through acetylating histone H4 lysine 16 (Donze and Kamakaka 2001; Oki et al. 2004). Together, these observations suggest that the SAS complex plays an important role in blocking the propagation of heterochromatin.

Histone H2A.Z is one of the evolutionarily conserved histone variants found from yeast to humans. Unlike the canonical histone H2A, which is expressed and assembled exclusively in S phase, the variant H2A.Z is constitutively expressed and deposited into chromatin throughout the cell cycle (Kamakaka and Biggins 2005). The H2A.Z containing nucleosomes are only slightly different from H2A nucleosomes in surface charge and structure (Suto et al. 2000; Fan et al. 2004; Park et al. 2004). There is considerable evidence, however, to suggest that incorporation of H2A.Z into nucleosomes has significant effects on cell cycle regulation, chromosome segregation, DNA repair, genomic stability, heterochromatin barrier formation, and transcription (both activation and repression) (Kamakaka and Biggins 2005; Dhillon et al. 2006). H2A.Z is deposited by the SWR1 complex, which specifically exchanges H2A/H2B dimers with variant H2A.Z/H2B dimers in an ATP-dependent manner (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). Studies from different groups have demonstrated a role for several SWR1 subunits, including Bdf1, Swr1, and Yaf9, in anti-silencing at telomeres (Ladurner et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Zhang et al. 2004).
Interestingly, transcription activation of many genes near telomeres is dependent on deposition of H2A.Z, possibly due to the ability of this histone variant to limit the ectopic spreading of Sir proteins into the nearby euchromatin. In the absence of either the SAS complex or histone variant H2A.Z (encoded by *HTZ1*), Sir proteins are capable of spreading further into neighboring euchromatic regions both at *HMR* and telomeres (Kimura et al. 2002; Suka et al. 2002; Meneghini et al. 2003; Kobor et al. 2004; Oki et al. 2004). This finding suggests a possible role for both Sas2 and H2A.Z in an anti-silencing mechanism.

Here we present evidence demonstrating that SAS-mediated acetylation at histone H4 lysine 16 is required for H2A.Z incorporation at telomeres. *SAS2* and *HTZ1* regulate transcriptional activation of a similar set of genes near telomeres. In the absence of SAS, H4 lysine 16 acetylation and H2A.Z occupancy are reduced specifically at the subtelomeric genome in a SAS-dependent manner. Consistent with these observations, a point mutation at histone H4 lysine 16 results in the loss of histone variant H2A.Z. Moreover, when recruited to a genomic locus where H4 AcK16 and H2A.Z are not detectable, the SAS complex can boost the acetylation at H4 lysine 16, which subsequently leads to enrichment of H2A.Z. Overall, our findings suggest a novel, SAS-dependent, signaling pathway for H2A.Z incorporation. The co-ordinate presence of H4 lysine 16 acetylation and H2A.Z establishes a mechanism for anti-silencing at telomeres.
3.3 Experimental Procedures

3.3.1 Yeast Strains and Plasmids

The strains used in this study are listed in Appendix A. All genomically tagged or deleted strains were generated by a one-step PCR-based integration method (Longtine et al. 1998; Puig et al. 2001) and confirmed by PCR or western blots. Sequences of primers used in strain construction can be found in Table 3-1. YWJS069 was generated through a cross between YWJS038 and YWJS056. YWJS075 was generated through a cross between YWJS060 and YWJS056. Strains YWJS098-100 were generous gifts from M. Smith (Megee et al. 1990). Plasmids pS-15, pS-126, and pS-236 were published previously (Osada et al. 2001). Plasmids pS-236 and pS-237 were generous gifts from R. Kamakaka (Oki et al. 2004). To generate pS-238, SAS2 M1 (Osada et al. 2001) fragment with AscI and NotI restriction sites was PCR amplified followed by subcloning into pS-237. pS-235 (pBS-LoxP-URA3-LoxP-UASgal1) was constructed as follows: LoxP-URA3 (with BamHI and EcoRI sites) and LoxP-UASgal1 (with EcoRI and HindIII sites) was PCR amplified from pRS406-URA3 and yeast genomic DNA respectively followed by subcloning into BamHI/HindIII digested pBluescript. Using pS-235 as templates, LoxP-URA3-LoxP-UASgal1 fragment was then PCR amplified and integrated to a genomic locus of our interest to generate YWJS141.
3.3.2 ChIP Assay and DNA Microarray Analysis

ChIP analysis was performed as described (Li and Reese 2001). Antibodies used in ChIPs include α-Histone H3 (Abcam), α-Histone H4 AcK16 (Serotec), α-Sas2 (rabbit, generated against N-terminus of Sas2, a.a. 1-20), α-cMyc (clone 9E10, ROCHE), and rabbit IgG (SIGMA). Sequences of primers used in ChIP assays can be found in Table 3-2. Reactions were resolved on 1.5% agarose gels, scanned by Typhoon 9400 (Amersham), and quantified by ImageQuant TL software. Fold enrichment was determined as the ratio of normalized ChIP DNA to the input DNA using PRP8 ORF as an internal control.
DNA microarray analysis was done as previously described (Iyer et al. 2001; Li et al. 2005). For expression microarray in Figure 3-1 to 3-5, arrays used contain approximately 6,300 spots (70-mer oligos) including all ORFs in *Saccharomyces cerevisiae* genome. For ChIP-chip experiments, arrays used contain approximately 14,000 spots (PCR products) including all ORFs, intergenic regions, mitochondrial DNA, and other noncoding regions of special interest, such as rDNA, tRNA, snoRNA, Ty transposons, LTRs, centromeres, and some introns. After chromatin immunoprecipitation, DNA amplification and labeling procedures are essentially adopted from earlier publications (Iyer et al. 2001). Briefly, in Round A amplification, a degenerated primer with an universal oligo tag at the 5’ end are used in the two-round primer extension reaction catalyzed by T7 sequenase version 2.0 (USB); 1/6 of Round A product was then subjected to Round B amplifications with ExTaq (Takara) using the universal primer; 1/10 of Round B product was then transferred into Round C where aminoallye-dUTP (Ambion) was incorporated onto DNA. Reactive Cy5 and Cy3 dye were then individually added to the purified DNA. Finally, unincorporated dyes were removed from labeled DNA using Qiagen PCR purification kit (Qiagen). Normally the base:dye ratio is about 80-150. 40-80 pmol of each dye were then mixed and competitively hybridized with DNA microarray at 65°C overnight. Slides were washed before scanned with a GenePix 4000B scanner (Axon) and analyzed with GenePix 5.0 software (Axon). All data sets are generated from at least three independent biological samples and microarray hybridizations including one dye-swap experiment. Using Acuity 4.0 software (Axon), the raw data were normalized such that the median ratio of intensities from two channels is 1. The normalized data were then filtered by using the following criteria: the signal is
>150 units over background, correlation between two channels is >0.5, and the spot
quality is passed by spot-to-spot visual inspection. The median value of all experiments is
used to create the final data set.

### Table 3-2: Sequences of Primers Used in ChIP Assays.

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<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
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</table>

#### 3.3.3 Mating and Spotting Assays

Mating assays were performed essentially as described (Sprague 1991), with the
exception that strains were mated in liquid culture prior to plating. Briefly the *MATa* type
mutant strains were grown into logarithmic phase and mixed with α tester strain (YJW251). After mating, approximately $0.5 \times 10^6$ cells from each mutant and $0.2 \times 10^6$ cells of α tester were spotted on rich medium as a growth control and on selective medium (lacking histidine, leucine, tryptophan, and uracil) for mating efficiency test. Two URA3 insertion strains (TEL VII-L $adh4\Delta::URA3$ and TEL V-R:$URA3$) were used as the parental strains in spotting assays for telomeric silencing (Gottschling et al. 1990; Renauld et al. 1993). Transcriptional silencing of the URA3 reporter gene in mutant strains was tested by spotting serial dilutions (4-fold) on 5-FOA plates. Similar spotting assays were also applied to experiments in Figure 3-7 where cells were grown on different temperatures (37°C, 30°C, and 16°C), various carbon sources (dextrose, galactose, and raffinose), and with treatment of 1 µg/ml sulfometuron methyl (SM), 0.03 % methyl methanesulfonate (MMS), 1 µJm² UV light exposure, or 100 mM hydroxyurea (HU) (Hampsey 1997).

### 3.3.4 Protein Purification and Nucleosome Reconstitution

SAS was purified from YJW458 by tandem affinity purification method as described in Chapter 2. Yeast recombinant histones H2A, H2A.Z, H2B, H3, and H4 were purified as described (Luger et al. 1997; Suto et al. 2000). Immobilized nucleosome array was assembled as described (Mizuguchi et al. 2004).
3.3.5 HAT Assays

HAT assays were carried out as previously described (Shia et al. 2005). Nucleosome array was used as substrate and approximately 2 pmole of SAS was used as the enzyme.

3.4 Results

3.4.1 SAS2 and HTZ1 Synergistically Regulate Transcription of Telomere-Proximal Genes

The yeast SAS complex has long been implicated in the maintenance of transcription silencing in yeast. At telomeres, SAS counteracts the deacetylation function of Sir2 and consequently blocks the spread of silenced heterochromatin. Interestingly, the histone H2A variant, H2A.Z, exerts a similar anti-silencing function through SWR1-mediated deposition. We sought to investigate a possible interplay between histone acetylation and variant incorporation in the regulation of silencing at telomeres.

It has been shown that many genes near telomeres are dependent on SAS or H2A.Z for transcription activation (Kimura et al. 2002; Meneghini et al. 2003). In order to determine whether SAS and H2A.Z regulate the same set of telomere-proximal genes, we performed microarray analysis on htz1Δ, sas2Δ, and sas2Δhtz1Δ yeast strains. The whole-genome expression profiles of these three strains are shown in Figure 3-1, Figure 3-2, and Figure 3-3 respectively. Our results indicated that very few genes were activated in the absence of SAS2 and/or HTZ1. We did observe, however, that genes
requiring *SAS2* or *HTZ1* for activation clustered at subtelomeric regions (Figure 3-4). Interestingly, the telomere bias for transcription regulation was exceedingly obvious in *sas2Δhtz1Δ* double knockout yeast, especially for genes residing within 20 kb from the chromosome ends (Figure 3-5A). In fact, more than 30% of genes within this range were highly repressed when both *SAS2* and *HTZ1* were deleted. Upon comparison of the transcription profiles of telomere-proximal genes in all three strains, we noted that transcription was similarly regulated by *SAS2* and *HTZ1*. A slight repression of telomere-proximal genes was noted when either *SAS2* or *HTZ1* was deleted, but these same genes became highly repressed in the *sas2Δhtz1Δ* double deletion mutant strain (See example, Figure 3-5B). We concluded that *SAS2* and *HTZ1* synergistically regulated transcription activation of a subset of genes near telomeres.
**htz1Δ**

Figure 3-1: Expression profile from microarray analysis of *htz1Δ* yeast strain.

Shown is the expression profile in whole yeast genome upon the deletion of *HTZ1*. The data set was generated by comparing *htz1Δ* (YJW491) to wild-type (YJW100) yeast strain. The color coding for the gene expression is shown in the bottom. Genes that are activated in *htz1Δ* yeast strain are shown in red; in contrast, the repressed genes are in green.
**sas2Δ**

Figure 3-2: Expression profile from microarray analysis of sas2Δ yeast strain.

Shown is the expression profile in whole yeast genome upon the deletion of SAS2. The data set was generated by comparing *sas2Δ* (YJW253) to wild-type (YJW100) yeast strain. The color coding for the gene expression is shown in the bottom. Genes that are activated in *sas2Δ* yeast strain are shown in red; in contrast, the repressed genes are in green.
Figure 3-3: **Expression profile from microarray analysis of sas2Δhtz1Δ yeast strain.**

Shown is the expression profile in whole yeast genome upon the double deletion of SAS2 and HTZ1. The data set was generated by comparing sas2Δhtz1Δ (YJW493) to wild-type (YJW100) yeast strain. The color coding for the gene expression is shown in the bottom. Genes that are activated in sas2Δhtz1Δ yeast strain are shown in red; in contrast, the repressed genes are in green.
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Figure 3-4: Relationship of genes activated by SAS2 and/or HTZ1.

Venn diagram showing the number of genes that exhibited a significant decrease (> 1.5-fold) in expression in mutant versus wild-type strains. Shown on the left is the relationship for the whole genome (6172 genes) and on the right for genes within 50 kb of a telomere (747 genes).
Figure 3-5: Microarray analysis using *htz1Δ* and/or *sas2Δ* yeast strains.

(A) Histogram showing the number of H2A.Z and/or Sas2-activated genes (> 1.5-fold) plotted as a function of their distance to the nearest telomere. Genes were categorized at 5 kb intervals for up to 50 kb from telomeres. The inset table shows the percentage of genes that require H2A.Z and/or Sas2 for activation in either genome-wide scale, 50 or 20 kb region from telomeres. Strains used were YJW100, YJW253, YJW491, and YJW493.

(B) Expression profiles for telomere-proximal genes (50 kb from both chromosome ends) at chromosome XII. The color coding for the gene expression is shown in the bottom. These results are representative of the whole genome.
Additionally, we sought to establish the genetic interaction between HTZ1 and SAS2. It has been previously reported that the HML silencing is slightly impaired in either sir1Δ or sas2Δ mutants, but severely disrupted in sir1Δsas2Δ double knock-out yeast cells by measuring the mating efficiency to α haploid cells (Reifsnyder et al. 1996). The additional deletion of HTZ1 in these mutants, however, only showed little effect on the HML silencing phenotype (Figure 3-6A). Similar results were observed when we tested the telomeric silencing upon the deletion of HTZ1. To measure the telomeric silencing, we used a yeast strain which has a reporter URA3 gene integrated 15 kb away from the left telomere of chromosome VII (Renauld et al. 1993). The silencing efficiency was measured by 5-fluoro-orotic acid (5-FOA) sensitivity. 5-FOA is toxic to Ura+ cells. Lack of telomeric silencing, and thus expression of the URA3 gene, results in sensitivity to 5-FOA, whereas strains that repress URA3 transcription are resistant to 5-FOA. The wild-type strain grew on 5-FOA plates because the URA3 gene was repressed. In contrast, cells were sensitive to 5-FOA in the absence of SAS2 as previously described (Reifsnyder et al. 1996). However, deletion of HTZ1 in wild-type or sas2Δ mutant didn’t seem to have any impact on URA3 transcription (Figure 3-6B, top). This was further confirmed when we performed the same experiments in a strain which has the URA3 marker inserted 1 kb away from the right telomere of chromosome V (Gottschling et al. 1990) (Figure 3-6B, bottom). The growth phenotypes in htz1Δmutant has been determined previously, including nucleic acid metabolism defects when treated with methyl methanesulfonate (MMS), hydroxyurea (HU), and UV light, and sensitivity to low and high temperatures (Jackson and Gorovsky 2000; Santisteban et al. 2000; Mizuguchi et al. 2004). Under these conditions, the deletion of SAS2 itself didn’t show any growth defects. In addition,
we didn’t observe any difference in \(htz1\Delta\) and \(sas2\Delta htz1\Delta\) mutants (Figure 3-7). We also tested the sensitivity to sulfometuron methyl (SM), which inhibited isoleucine and valine biosynthesis, and various carbon sources, but we did not find any growth defects in \(sas2\Delta\), \(htz1\Delta\), or \(sas2\Delta htz1\Delta\) mutants. Collectively, we did not observe significant genetic interactions of \(SAS2\) and \(HTZ1\) in our analysis, but we certainly should not exclude this possibility.
Figure 3-6: Silencing defects in \textit{sas2}\Delta and \textit{htz1}\Delta mutants.

(A) The absence of \textit{HTZ1} didn’t affect \textit{HML} silencing. The \textit{MATa} strains used in mating assay were YJW100, YJW252, YJW253, YJW258, YJW491, YJW492, YJW493, and YJW494. After mating with the mating tester YJW251 (\textit{\alpha his4}), the yeasts were spotted on a plate lacking histidine, leucine, tryptophan, and uracil for selection.

(B) The absence of \textit{HTZ1} didn’t affect TEL silencing. Silencing of a \textit{URA3} gene inserted near either the left telomere (15 kb) of chromosome VII or the right telomere (1 kb) of chromosome V was tested in serial dilution assays (4-fold) on 5-FOA-containing medium. Strains used included YJW287, YJW289, YJW495, YJW496, YWJS150, YWJS156, YWJS159, and YWJS160.
Figure 3-7: Deletion of SAS2 didn’t have any influence on the htz1Δ mutant phenotypes.

Yeast strains with indicated deletions were serially diluted (4-fold) and grown on different temperatures (37°C, 30°C, and 16°C), carbon sources (dextrose, galactose, and raffinose), and with various treatments (1 µg/ml SM, 0.03 % MMS, 1 µJ m² UV light exposure, or 100 mM HU). Strains used included YJW100, YJW253, YJW491, YJW493.
3.4.2 Acetylation of H4 Lysine 16 at Telomeres is Dramatically Reduced in the Absence of SAS

SAS is a histone H4 lysine 16 specific acetyltransferase complex; however, it is not the only HAT that is capable of acetylating H4 lysine 16. In order to determine where in the genome SAS specifically acetylated this residue, we took advantage of the genome-wide ChIP-chip technique using an antibody against acetylated lysine 16 of H4. Our results indicated that H4 lysine 16 acetylation was randomly distributed throughout the genome in wild-type yeast (Figure 3-8), but was lost specifically near telomeres in sas2Δ cells (Figure 3-9). We then merged our data sets from both cell types to determine the genomic loci where the H4 lysine 16 acetylation was lost in the mutant. To our surprise, SAS-mediated acetylation was extremely specific to the regions neighboring telomeres (Figure 3-10A). Among the genomic loci where we observed over 2-fold reduction in H4 lysine 16 acetylation, more than two thirds were located within 20 kb from chromosome ends (Figure 3-10B). It should be noted that the loss of acetylation was not limited to either open reading frames or intergenic regions. This restricted and limited acetylation pattern of SAS coincides with its function in transcription. In fact, previous studies reported that mutation at H4 lysine 16 specifically results in the repression of telomere-proximal genes (Dion et al. 2005). Given the fact that Sas2 and H2A.Z also show a synergistic effect on transcription activation near telomeres, these findings suggest possible co-operation between SAS and H2A.Z in anti-silencing function at telomeres.
Figure 3-8: ChIP-chip analysis for H4 AcK16 in wild-type yeast strain.

Shown is the ChIP result reflecting the genome-wide H4 AcK16 level in wild-type (YWJS001) yeast strain. The data set was normalized to its input material. Each bar represents either an ORF or intergenic locus. The color coding for the enrichment of H4 AcK16 is shown in the bottom.
Figure 3-9: ChIP-chip analysis for H4 AcK16 in sas2Δ yeast strain.

Shown is the ChIP result reflecting the genome-wide H4 AcK16 level in sas2Δ (YWJS002) yeast strain. The data set was normalized to its input material. Each bar represents either an ORF or intergenic locus. The color coding for the enrichment of H4 AcK16 is shown in the bottom.
Figure 3-10: ChIP-chip analysis for H4 AcK16 in wild-type and sas2Δ yeast strains.

(A) Shown is the result reflecting the genome-wide difference in H4 AcK16 level between wild-type (YWJS001) and sas2Δ (YWJS002) yeast strains. Each data set was first normalized to its individual input. Data from sas2Δ strain was then merged over wild-type strain to generate the final result. Each bar represents either an ORF or intergenic locus. The color coding for the enrichment of H4 AcK16 is shown in the bottom. The loci where H4 AcK16 is decreased upon the deletion of SAS2 are in green color; in contrast, red loci have more H4 AcK16 in sas2Δ strain.

(B) Diagram showing the number of ORF or intergenic loci, either in whole genome scale or within 20 kb from telomeres, whose loss of H4 AcK16 is greater than 2-fold in the absence of Sas2. The dark blue bars represent data gathered from whole yeast genome and the light blue bars represent data gathered specifically from loci within 20 kb from telomeres.
In addition to chromosome ends, we also examined the effect of SAS on other silent loci (Figure 3-11). At HML loci, H4 lysine 16 acetylation was weak in wild-type but slightly increased upon the deletion of SAS2 suggesting a possible spreading of Sir2 from the HML locus. These data are consistent with observations from the sas2Δ mutant phenotype in which HML silencing is disrupted (Ehrenhofer-Murray et al. 1997). We did not detect a significant difference in acetylation at the HMR locus, which agreed with genetic studies showing that SAS does not affect HMR silencing (Ehrenhofer-Murray et al. 1997). Loss of SAS, however, did lead to an increase in rDNA silencing. We observed significant reduction of H4 lysine 16 acetylation over the entire rDNA array, which was also consistent with previous genetic studies (Meijsing and Ehrenhofer-Murray 2001). Therefore, our ChIP-chip data for H4 AcK16 agree with phenotypic studies in sas2Δ at all silent loci. It remains to be addressed, however, whether the HAT activity of SAS is directly involved in anti-silencing function at HML, HMR, and rDNA loci.
3.4.3 Acetylation of H4 Lysine 16 at Telomeres is Required for H2A.Z Incorporation

Figure 3-11: ChIP-chip analysis for H4 AcK16 in Wild-Type and sas2Δ.
A closer look from Figure 3-10 at silent loci HML, HMR, and rDNA. Shown are the individual data sets and the merged result (sas2Δ over wild-type). Each bar represents either an ORF or intergenic locus. The color coding for the enrichment of H4 AcK16 is shown in the bottom.
We next investigated whether there was a relationship between SAS-mediated H4 lysine 16 acetylation and SWR1-mediated H2A.Z incorporation in telomeric anti-silencing. *In vitro* HAT assays demonstrated that the SAS complex showed similar HAT activity on wild-type and H2A.Z-containing nucleosome substrates, indicating that acetylation by SAS was not simply influenced by the incorporation of H2A.Z into chromatin (Figure 3-12). We then carried out ChIP experiments to examine whether H2A.Z incorporation influenced the *in vivo* H4 lysine 16 acetylation near a telomere (Figure 3-13A). Again, H4 lysine 16 acetylation was unaffected by HTZ1 deletion (Figure 3-13B). Collectively, these results rule out the possibility that H2A.Z incorporation is the upstream signal for SAS acetylation.
Figure 3-12: Influence of H2A.Z presence to HAT activity of the SAS complex.

(A) Diagram of nucleosome assembly followed by HAT assay.

(B) Recombinant yeast histones H2A (or H2A.Z), H2B, H3, and H4 were used for immobilized nucleosome array assembly. After incubated with SAS (TAP-purified from YJW458), the nucleosome arrays were washed stringently and subjected for quantification of $[^3]$H on beads by liquid counting assay. Data shown are the average of three independent experiments. Error bars represent standard deviation.
Figure 3-13: SAS-mediated acetylation of H4 lysine 16 is required for H2A.Z incorporation at right telomere of chromosome VI.

(A) Location of PCR primer sets used in ChIP assay (B to E). Each primer set is about 2.5 kb apart interspersed along the 20 kb region from right telomere of chromosome VI.

(B) α-H4 AcK16 antibody was used in ChIP assays to determine the level of H4 AcK16. Strains used were YWJS001, YWJS002, and YWJS046.

(C) and (D) Rabbit IgG was used to pull-down TAP-tagged H2A.Z to determine its enrichment by ChIP assays. Strains used in (C) included YWJS056, YWJS069, YWJS075, and YWJS139 to examine the effects upon deletion of genes indicated. Strains used in (D) included YWJS101, YWJS102, and YWJS103 to demonstrate the rescue by SAS2 plasmid.

(E) H2A.Z occupancy was determined by ChIP assays using α-cMyc antibody in H4 mutant yeasts. Strains used were YWJS132, YWJS133, and YWJS134.

Data shown are the average of three independent experiments. Error bars represent standard deviation.
It has been proposed that histone acetylation targets the Bdf1 subunit of SWR1 to chromatin, leading to H2A.Z deposition. Perhaps H4 lysine 16 acetylation by SAS directly affects the presence of H2A.Z at subtelomeric regions. ChIP assays on the right telomere of chromosome VI were used to monitor TAP-tagged H2A.Z occupancy in vivo. In wild-type cells, the amount of H2A.Z gradually increased from telomere end toward the euchromatin (Figure 3-13C), which correlated with the pattern of H4 lysine 16 acetylation. Following deletion of SWR1, H2A.Z was no longer detected, due to the lack of deposition machinery. Interestingly, we observed a dramatic reduction in the amount of H2A.Z in sas2Δ cells (Figure 3-13C). These results were reproducible when we examined the left telomere of chromosome XV (Figure 3-14) suggesting a universal role for SAS at telomeres. This loss of H2A.Z was restored by expression of a plasmid containing wild-type Sas2 plasmid, but not the HAT-deficient Sas2 mutant (Figure 3-13D). Notably, further spreading of Sir proteins in sas2Δ yeast did not interfere with the H2A.Z incorporation since no noticeable difference in H2A.Z amount was detected when we compared sas2Δ and sir2Δsas2Δ yeast strains. We also examined the distribution of H2A.Z in H4 mutant cells using yeast strains that expressed either wild-type or mutant H4 solely from a plasmid (Figure 3-13E). In these strains, the H4 K16R mutation, but not the H4 K5R mutation, caused a loss of H2A.Z similar to that seen in SAS2 deletion strains indicating that lysine 16 acetylation was specifically required for H2A.Z incorporation at telomeres.
Interestingly, the ChIP results revealed a unique feature of H2A.Z exchange. The enrichment of H2A.Z beyond the 15 kb region did not seem to be affected by either SAS or H4 lysine 16 acetylation. However, we could not detect any H2A.Z in the absence of either SAS or H4 lysine 16 acetylation.

Figure 3-14: The loss of H2A.Z in the absence of SAS is not chromosome specific.

(A) Location of PCR primer sets used in ChIP assay. Each primer set is about 2.5 kb apart interspersed along the 20 kb region from left telomere of chromosome XV.

(B) Rabbit IgG was used to pull-down TAP-tagged H2A.Z to determine its enrichment by ChIP assays. Strains used included YWJS056, YWJS069, and YWJS075 to examine the effects upon deletion of genes indicated.

Interestingly, the ChIP results revealed a unique feature of H2A.Z exchange. The enrichment of H2A.Z beyond the 15 kb region did not seem to be affected by either SAS or H4 lysine 16 acetylation. However, we could not detect any H2A.Z in the absence of...
SWR1 beyond this 15 kb region from the telomere. These findings suggested that at least two mechanisms exist for triggering H2A.Z exchange by SWR1: the SAS-mediated acetylation of H4 lysine 16 at subtelomeric regions, and another, undetermined mechanism in other areas of the genome.

We next established a direct connection between SAS-mediated acetylation and H2A.Z incorporation by integrating a UASgal1 sequence containing four Gal4 binding sites into a genomic locus where our ChIP-chip data indicated that both H4 lysine 16 acetylation and H2A.Z were barely detectable (Figure 3-15) (Li et al. 2005). By expressing Gal4-fused Sas2 in these cells, we were able to artificially recruit SAS to the Gal4 binding sites and determine whether SAS-mediated acetylation would result in the enrichment of H2A.Z. Indeed, ChIP analysis confirmed that SAS was recruited to this locus and that its acetylation of histone H4 at lysine 16 led to an enrichment of H2A.Z (Figure 3-16). In contrast, recruitment of the HAT-deficient Sas2 (SAS2 M1) failed to elevate the H2A.Z incorporation at this locus. H3 ChIP showed that overall histone amounts remained unchanged in these yeast strains. Thus, we provided direct evidence that SAS-mediated acetylation of H4 lysine 16 could facilitate H2A.Z incorporation.
Figure 3-15: **Locus selection for artificial recruitment of SAS.**

A UASgal1 site is integrated to a genomic locus (1239 kb at chromosome IV) where both H4 AcK16 and H2A.Z signals are low based on our ChIP-chip data shown here. Each bar represents either an ORF or intergenic locus. The color coding for the enrichment of H2A.Z or H4 AcK16 is shown in the bottom.
Figure 3-16: Recruitment of SAS results in the H4 lysine 16 acetylation and the subsequent H2A.Z enrichment.

(A) ChIPs using antibodies indicated on the left. PCR primers are specific to a genomic locus (1239 kb at chromosome IV) where both H4 AcK16 and H2A.Z signals are low. Strains used were YWJS148, YWJS149, and YWJS161.

(B) Quantification for ChIP results from (A). Data shown are the average of three independent experiments. Error bars represent standard deviation.
3.5 Discussion

Biochemical and genetic studies have implicated either acetylation of H4 K16 by SAS or deposition of the histone variant H2A.Z in anti-silencing. Here we present evidence that H4 lysine 16 acetylation by the SAS complex is required for H2A.Z incorporation, and that this synergistic mechanism is key for anti-silencing in the sub-telomeric region. Based on these findings, we propose that the presence of acetylated H4 lysine 16, coupled with subsequent H2A.Z deposition establishes a barrier that prevents heterochromatin spreading from the telomere.

Our microarray analyses show that both H4 K16 acetylation and H2A.Z are required for the optimal boundary effect. In either the sas2Δ or htz1Δ single knockout strain, we observed marginal repression of transcription at telomere-proximal genes. These genes were highly repressed, however, when both Sas2 and H2A.Z were absent. This greater-than-additive repression on telomere-proximal genes in sas2Δhtz1Δ double knockout yeast indicates that the presence of both H4 AcK16 and H2A.Z in the subtelomeric region is necessary to prevent heterochromatin spreading. A recently published study indicated that formation of telomeric heterochromatin boundaries required acetylated H2A.Z (Babiarz et al. 2006). Since nucleosomes containing H2A.Z are structurally similar to canonical nucleosomes (Suto et al. 2000), the physical presence of unmodified H2A.Z might not be sufficient to prevent heterochromatin spreading. Instead, H2A.Z that has been modified by acetylation appears to play a crucial role in telomeric boundary formation. In fact, the N-terminal of H2A.Z shows significant similarity to the N-terminal tail of histone H4 (Schaper et al. 2005). It has been shown
that, after its deposition into chromatin, NuA4 acetylates H2A.Z at lysine 14 (Babiarz et al. 2006; Keogh et al. 2006; Millar et al. 2006), which is the consensus residue to lysine 16 of histone H4. This fact raises the possibility that Sir proteins might need to overcome the acetylation of both H4 lysine 16 and H2A.Z lysine 14 in order to propagate toward euchromatin.

We demonstrated that acetylation of H4 K16 by SAS was a prerequisite for H2A.Z incorporation (Figure 3-13, 3-14, and 3-16). This requirement of SAS for H2A.Z incorporation appears to be specific to telomere-proximal genes. Histone acetylation has been proposed to target the SWR1 complex to chromatin, leading to H2A.Z deposition (Zhang et al. 2005). Our artificial recruitment assays demonstrated that a euchromatin locus acetylated at H4 lysine 16 by SAS subsequently became enriched with H2A.Z (Figure 3-16). Our ChIP-chip data (Figure 3-13) showed that outside the subtelomeric region, the acetylation of H4 lysine 16 was randomly distributed and did not correlate well with H2A.Z occupancy. Taken together, these findings suggest that H2A.Z deposition by the SWR1 complex requires both histone acetylation and the physical presence of SAS. Without the SAS complex, H4 lysine 16 acetylation (as seen throughout euchromatin), might not be sufficient for SWR1 targeting. Several lysines in other histones have shown much higher genome-wide correlation with H2A.Z (Raisner et al. 2005; Zhang et al. 2005). Besides H4 lysine 16, the SWR1 complex may target other histones since the Bdf1 subunit responsible for targeting is associated with other acetylated lysine residues (Kurdistani et al. 2004). Therefore, a distinct mechanism appears to be responsible for H2A.Z deposition outside the subtelomeric regions. Recently, studies from several groups regarding the global localization of H2A.Z all
concluded that H2A.Z preferentially occupied promoters of inactive genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005). It will be interesting to determine the underlying deposition mechanism of H2A.Z at promoters as it relates to its function in transcription regulation of yeast genes.

The question of the origin of the telomeric anti-silencing mechanism remains to be addressed. The recruitment of Sir proteins to telomeric silencers by Rap1 and Ku70 initiates heterochromatin formation. The deacetylase activity of Sir2, as well as the binding ability of Sir3 and Sir4 to hypoacetylated histones, then enables the silent chromatin to propagate (Rusche et al. 2003). We have shown that SAS and H2A.Z co-operate to form a barrier to Sir spreading. Nevertheless, it remains to be shown how SAS is specifically targeted to the subtelomeric regions. Given that the newly deposited histone H4 is not acetylated at lysine 16 (Sobel et al. 1995), it has been proposed that SAS is recruited to newly replicated DNA through the interaction with histone chaperon Asf1 or CAF-I, thus maintaining the epigenetic mark (Ehrenhofer-Murray 2004). Understanding whether this replication and chromatin assembly-coupled model for SAS recruitment applies specifically to subtelomeric regions will certainly better our knowledge of this important anti-silencing mechanism.

3.6 Acknowledgments

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Chapter 4

Summary and Future Directions

The purpose of this study was to better our understanding of the role of the SAS complex in yeast cells. The yeast SAS complex was originally identified in our laboratory as a putative histone acetyltransferase complex (Osada et al. 2001). Work from this dissertation not only provides evidence demonstrating that the yeast SAS complex is a HAT complex, but also shows how its HAT activity regulates specific cellular processes.

We were able to purify a native and active SAS complex from yeast. This trimeric SAS complex consists of Sas2, Sas4, and Sas5 and has a molecular weight of 125 kDa. We confirmed that this complex is enzymatically active and is capable of acetylating core histones and nucleosomes. The most intriguing discovery was that histone H4 lysine 16 acetylation depends heavily on the SAS complex. This unique substrate preference was further verified \textit{in vitro} by several biochemical experiments.

Our findings also establish the biological relevance of the HAT activity in the SAS complex. After DNA replication and chromatin assembly, telomeric silencing is initiated by spreading of Sir proteins toward euchromatin. We showed that SAS is responsible for the acetylation of histone H4 at lysine 16 specifically in subtelomeric regions. This specific telomere-proximal acetylation by SAS can somehow signal for the histone variant H2A.Z exchange by the SWR1 complex. Consequently, the presence of H4 AcK16 and H2A.Z forms the optimal silencing barrier to protect euchromatin from being transcriptionally silenced.
Recently, several independent studies found that the histone variant H2A.Z is acetylated by NuA4 following deposition by the SWR1 complex, and that the acetylation of H2A.Z is necessary for blocking heterochromatin spreading (Babiarz et al. 2006; Keogh et al. 2006; Millar et al. 2006). This discovery further extends our current understanding of how the spreading of silent chromatin is terminated. Together, these findings lead us to propose that the dual-acetylation of H4 and H2A.Z provides a strong resistance to transcriptional silencing at telomeres (Figure 4-1).
After DNA replication and chromatin assembly, Sir proteins spread toward euchromatin from the telomere. The SAS-mediated acetylation at histone H4 lysine 16 temporarily halts the propagation of heterochromatin and signals the SWR1 complex to exchange for the histone variant H2A.Z. After deposition, H2A.Z is acetylated at lysine 14 by the histone acetyltransferase NuA4 complex. This dual-acetylation of H4 and H2A.Z provides the optimal anti-silencing environment that blocks the spreading of silenced chromatin.

Figure 4-1: Model for anti-silencing at telomeres.
Our findings, together with others, have lead to a hypothesis for an anti-silencing mechanism at telomeres. Still, many interesting questions regarding this model will need to be addressed in the future in order to fully comprehend how the anti-silencing mechanism might work.

The establishment of telomeric silencing has been well documented (Rusche et al. 2003). The Sir proteins are recruited to the telomeric silencer by Rap1 and Ku70, followed by spreading away from the telomere. However, little is known about the recruitment of SAS to chromatin, especially to the telomere-proximal regions for anti-silencing function. One obvious possibility is through the interaction with histone chaperones Asf1 or CAF-I immediately after DNA replication (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001). The Asf1-dependent occupancy of SAS at the $HML$ locus has recently been demonstrated, suggesting a possible role of Asf1 in recruiting SAS to regulate the $HML$ silencing (Osada et al. 2005). It is possible that SAS is targeted to subtelomeric regions by a similar mechanism to initiate its anti-silencing function.

Another interesting question involves the location in the genome of the initial SAS-binding site(s). Our ChIP-chip data suggests that SAS is exceedingly specific to the subtelomeric regions within 15-20 kb from most of the yeast chromosome ends. Like the barrier element found near the $HMR-I$ silencer (Donze et al. 1999), there might be a general cis-acting element residing about 15-20 kb from telomeres that provides a binding site for SAS to initiate its anti-silencing function. Answers to these questions will definitely benefit our understanding in how the anti-silencing mechanism is initiated.
We provide evidence that the specific acetylation of histone H4 lysine 16 by SAS signals for the H2A.Z incorporation. We do not, however, know how this acetylation mark is recognized by the SWR1 complex. The presence of Bdf1 in the SWR1 complex provides an ideal explanation. Bdf1 contains two bromodomains and preferentially binds to acetylated histone H4 (Ladurner et al. 2003; Matangkasombut and Buratowski 2003). We will use two in vitro systems to examine whether H4 AcK16 can target the SWR1 complex to chromatin, more importantly, whether this interaction is Bdf1-dependent. Histone H4 N-terminal tail peptide with acetylation at lysine 16 will be used to pull-down native SWR1 complex to examine the direct interaction between H4 AcK16 and SWR1. Alternatively, we can also acetylate an immobilized nucleosome array using SAS and determine whether these pre-acetylated (presumably H4 K16 only) nucleosomes are suitable substrates in an H2A.Z exchange assay catalyzed by SWR1. The SWR1 complex containing a Bdf1 bromodomain deletion will also be included in both assays to determine whether wild type Bdf1 directly contacts acetylated histone H4.

Sas2 and Sir2 are all histone H4 lysine 16 specific enzymes. Surprisingly, the N-termini of histone H3, H4, variant H2A.Z, and Sas2 share a high level of sequence similarity and one of the consensus residues is the lysine 16 of histone H4 (Figure 4-2A). We have data showing that in vitro SAS can weakly acetylate Sas2 itself and H2A.Z as well (Figure 4-2B), presumably on this conserved lysine residue (K32 of Sas2 and K14 of H2A.Z). The acetylation of H2A.Z at lysine 14 by NuA4 has recently been reported to occur downstream of H2A.Z deposition to prevent heterochromatin spreading. Based on these observations and our microarray analysis, we propose that the dual-acetylation of H4 K16 and H2A.Z K14 constitutes the strongest barrier to Sir protein propagation. In
order to verify this hypothesis, the H2A.Z-containing nucleosomes will be assembled and acetylated by NuA4, followed by an HDAC assay using Sir2 as the catalyst to determine whether Sir2 is able to deacetylate H2A.Z in the nucleosome context. In addition, ChIP assays for Sir proteins will be performed in htz1Δsas2Δ mutant to determine whether the Sir proteins occupancy reflects the high transcriptional repression observed in this yeast strain.
Other histone modifications may also be involved in the anti-silencing mechanism at telomeres. Besides acetylation, methylation of histone H3 lysine 4 (by Set1) and lysine 79 (by Dot1) both have been previously shown to function as silencing barriers near telomeres (Singer et al. 1998; Krogan et al. 2002; Ng et al. 2002; Sun and Allis 2002). It

Figure 4-2: SAS complex is capable of acetylating histone variant H2A.Z in vitro.

(A) Amino acid sequence alignment of the N-termini of Sas2, histone H3, H4, and H2A.Z. The consensus residues are shown in red. Lysine 16 of histone H4 and the corresponding lysine in all other proteins are highlighted with pink background.

(B) In vitro HAT assays were performed with TAP-purified SAS complex and recombinant yeast histones as indicated. After incubation, reactions were run on an 18% SDS-PAGE gel followed by Coomassie staining and fluorography to visualize the acetylation results.
is important to know whether there is crosstalk between various histone modifications in regulation of transcriptional silencing. Ultimately, we should also make an effort toward understanding how heterochromatin is restricted at other silent loci.
Bibliography


Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. 1999. Exit from mitosis is triggered by
Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**: 233-244.


Wirbelauer, C., Bell, O., and Schubeler, D. 2005. Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active


# Appendix A

## Yeast Strains Used in This Thesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJW100</td>
<td>w303-1a ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100</td>
<td>A. Sachs</td>
</tr>
<tr>
<td>YJW228</td>
<td>a SAS4-13Myc:kanMX6</td>
<td>Osada et al., 2001</td>
</tr>
<tr>
<td>YJW251</td>
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Blue: Isogenic to YJW100        Red: Isogenic to YWJS001
Appendix B

Sas4 and Sas5 Are Required for the Histone Acetyltransferase Activity of Sas2 in the SAS Complex

Sas4 and Sas5 Are Required for the Histone Acetyltransferase Activity of Sas2 in the SAS Complex*

Ann Sutton‡, Wei-Jong Shia§, David Band‡, Paul D. Kaufman§, Shigeihiro Osada¶, Jerry L. Workman§, and Rolf Sternglanz**

From the ‡Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, New York 11794-5215, the §Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802-4500, the ¶Lawrence Berkeley National Laboratory and Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California 94720, and the Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

The SAS2 gene is involved in transcriptional silencing in Saccharomyces cerevisiae. Based on its primary sequence, the Sas2 protein is predicted to be a member of the MYST family of histone acetyltransferases (HATs). Sas2 forms a complex with Sas4 and Sas5, which are required for its silencing function. Here we show that recombinant Sas2 has HAT activity that absolutely requires Sas4 and is stimulated by Sas5. The recombinant SAS complex acetylates H4 lysine 16 and H3 lysine 14. Furthermore, a purified SAS complex from yeast shows similar activity and specificity. In contrast to other MYST HATs, neither the recombinant nor the native SAS complex acetylated nucleosomal histones under conditions that were optimum for acetylating free histones. Finally, although the SAS subunits interact genetically and physically with Asf1, a histone deposition factor, association of H3 and H4 with Asf1 blocks their acetylation by the SAS complex, raising the possibility that the SAS HAT complex may acetylate free histones prior to their deposition onto DNA by Asf1 or CAF-I.

In the yeast Saccharomyces cerevisiae, as in higher eukaryotes, transcriptional silencing results from the formation of highly condensed chromatin called heterochromatin. Many factors have been identified that influence the formation and maintenance of heterochromatin. Some of these proteins either physically interact with or modify the histone N termini, while others are required for deposition of histones onto DNA (1). In S. cerevisiae, silenced chromatin regions are found at the two silent mating loci, HML and HMR, at telomeres, and at rDNA (2).

The SAS2 gene was identified in two screens for genes involved in transcriptional silencing (3, 4). sas2 mutants are defective in silencing at telomeres, and at HML in a Δsir1 background, but display improved silencing at a mutated HMR locus (3, 4) and at rDNA (5). Genetic evidence suggested that SAS2 functions in a pathway with two other genes, SAS4 and SAS5 (6) and recent biochemical evidence demonstrated that the three proteins exist as a complex in yeast termed SAS (5, 7).

SAS2 encodes a putative member of the MYST family of histone acetyltransferases (HATs), which also includes human MOZ, MORF, TIP60, and HBO1; Dro sophila MOF and Chm; and S. cerevisiae Sas3 and Esal. Although many of these proteins have been shown to possess HAT activity (8), no enzymatic activity had previously been detected for Sas2. However, recent work suggests that acetyltransferase activity may be important for Sas2 function. We and others (5, 7) have shown that the conserved acetyl-CoA binding domain of Sas2 is required for HML and telomeric silencing, as mutations in this motif cause the same silencing defects as does deletion of SAS2 (5, 7). Furthermore, a point mutation (K16R) in the histone H4 N-terminal tail phenocopies the effects of sas2 mutants on silencing (5). The H4-K16R mutation causes complete loss of telomeric silencing and improves silencing at a mutated HMR to the same extent as deletion of SAS2. Combination of a SAS2 gene deletion with the H4-K16R mutation leads to no additional increase in HMR silencing (5). Very recently, two groups (9, 10) have demonstrated that lysine 16 of histone H4 is hypoacetylated in sas2 mutants. Together, these data are consistent with the possibility that histone H4 lysine 16 is a direct substrate for acetylation by Sas2.

The SAS complex has also been functionally linked to the histone deposition proteins Cac1 and Asf1. Sas4 was isolated in a two-hybrid screen with Asf1 as bait (11), and Cac1 was isolated in a two-hybrid screen with Sas2 as bait (5). Co-immunoprecipitation analyses further showed that the SAS complex associates with both Asf1 (7) and Cac1 in yeast (5). Cac1 is a subunit of the yeast CAF-I complex (12). Both Asf1 and CAF-I bind to histones H3 and H4 and are suggested to function in non-overlapping pathways for histone deposition and chromatin assembly (13–18). asf1 mutants display the same effects on silencing at HML and HMR as sas2, 4, or 5 mutants (5, 7), but unlike sas mutants, asf1 mutants have little or no defect in telomeric silencing (19). cac mutants, like sas mutants, have defects in telomeric and HML silencing although the magnitudes of these defects differ (12, 20). Furthermore, cac mutations cause silencing defects at HML and HMR that are only partially epistatic with sas mutations (5). Together, these data suggest that the interactions among the SAS complex, CAF-I, and Asf1 may be complex and locus-specific.

In this study we show that co-expression of Sas2, Sas4, and Sas5 in Escherichia coli leads to formation of a stable SAS

* This work was supported by grants from the National Institutes of Health (to R. S. and J. L. W.) and from the National Science Foundation (to P. D. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HAT, histone acetyltransferase; IPTG, isopropyl β-D-thiogalactoside; TAP, tandem affinity purification; CBP, calmodulin binding protein.
complex that acetylates histones. Sas4 is essential for the acetyltransferase activity of Sas2, and Sas5 is important. The preferred in vitro substrates for the recombinant SAS complex are lysine 16 of histone H4 and lysine 14 of histone H3. In contrast to the Sas3-containing NuA3 and the Esa1-containing NuA4 complexes, the SAS complex shows no activity toward nucleosomes or (H3-H4) tetramers deposited onto DNA under conditions of our assays. We have also purified enzymatically active SAS complex from yeast, which contains Sas2, Sas4, and Sas5. This native enzyme shows the same substrate specificity as the recombinant enzyme, including the preference for free histones.

**Experimental Procedures**

**Yeast Strains—**Yeast strains used in this study were derived from YJW228 (SAS4–13myc;chanMX5) described previously (7). Strains YJLA48 and YJW459 were constructed by integration into YJW228 of the sequences encoding the tandem affinity purification (TAP) tag immediately before the stop codon of the SAS2 and SAS5 genes, respectively, as described elsewhere (21). Standard yeast manipulations were performed as described (22).

**Plasmids—**Plasmids for expression of Sas proteins in E. coli were created as follows. pAS131 (Sas2-His6) was made by PCR modification of the SAS2 open reading frame (ORF) from yeast genomic DNA. The primers were designed so that the SAS2 sequence contained a 5′ NcoI site and a 3′ EcoRI site. The PCR product was cloned into pTE28b (Novagen) so that Sas2 was under control of an inducible T7 promoter and was in-frame with sequences for a C-terminal His6 tag. pDB16 (sas2M1–His6) was made exactly as described for pAS131 except that pS139 was used for the template for the PCR reaction. pS139 was previously described (7) and encodes a mutated version of Sas2 in which amino acids 219–221 are changed to alanines. pDB5 (Sas4) was made by cloning a PCR fragment containing the SAS4 gene with a 5′ NcoI site and a 3′ BamHI site into pET28b. pDB6 (Sas5) was made by cloning a PCR fragment containing the SAS5 ORF with a 5′ NcoI site and a 3′ BamHI site into pET28b. pDB7 (Sas4/Sas5) was made by cloning a BglII-BamHI fragment containing SAS4 from pDB5 into the BglII site of pDB6. Both SAS4 and SAS5 are transcribed in the same direction, each under control of a separate T7 promoter. PCR was used to amplify a fragment from pAS131, which contained SAS2-His6, the T7 promoter and terminator, and Spel restriction sites on each end. The fragment was cloned into the Spel site of pDB7 to make pAS132 (Sas2-His6;Sas4;Sas5), into pDB5 to make pDB18 (Sas2-His6;Sas4), and into pDB6 to make pDB17 (Sas2-His6;Sas5). A similar Spel fragment was made using pDB16 as template and cloned into pDB7 to make pAS134 (sas2M1–His6;Sas4;Sas5). All coding regions were sequenced to check for PCR errors.

**Protein Expression and Purification in E. coli—**Sas proteins were expressed in E. coli strain BL21 (DE3) codon plus by a 3-h induction with 0.4 mM IPTG. The proteins were purified from 100-mL cultures using Novagen His Bind Affinity Resin according to the manufacturer’s instructions. Purified proteins were dialyzed against 50 mM Tris, pH 8 and frozen at −80 °C in 50 mM Tris, pH 8, 10% glycerol. Esa1 was induced from plasmid pLP831 (His8-Esa1) in strain BL21 (DE3) with 1.0 mM IPTG. Protein was purified as described above for the SAS complex. (H3–H4) tetramers were prepared as described (23). Aef1 and Aof1 (H3–H4)2 complexes were purified as described (12). HeLa histones for Fig. 5 were purified as described (24). (H3–H4)2 tetramers were assembled onto plasmid DNA as described (25). The tetramer-DNA complexes were further purified by collecting them in the void fraction of a S200 gel filtration column to ensure that no free histones remained.

**Protein Expression and Purification in Yeast—**Whole cell extracts were prepared from a 12-liter culture as previously described (26). The native SAS complex was purified by the tandem affinity purification method (21). After purification, 20% of the purified SAS complex was separated on a polyacrylamide gel and silver stained.

**HAT Assays—**HAT assays using recombinant SAS were done as above except that the reaction volume was 15 µl, and the substrates were as follows: 0.5 µg of HeLa nucleosomes, HeLa core histones, and recombinant yeast core histones and 40 µM H4 N-terminal peptides. The amount of enzyme used was 1% of that obtained from a 12-liter preparation (5.0% of that shown on the silver-stained gel in Fig. 6A). Reactions were incubated at 30 °C for 45 min and then spotted onto Whatman p81 cation exchange paper and air-dried. The dried papers were washed three times for 5 min each with 50 mM sodium carbonate, pH 9.0 and once with acetone. Radioactivity was quantitated in a liquid scintillation counter.

**SDS-PAGE of Acetylated Histones—**For the experiment in Fig. 3A, chicken histones were acetylated with SAS complexes or Esa1 as described above except that the final concentration of histones was 0.4 mg/ml. For the experiment in Fig. 6A, reactions were carried out exactly as described above for the native SAS enzyme. Reactions were terminated by addition of 5 µl of 5× protein loading dye. Samples were heated to 100 °C for 5 min, centrifuged at 16,000 × g for 2 min, and resolved on 15% SDS-polyacrylamide gels. Proteins were stained with Coomassie Blue, destained, saturated with ENHANCE (PerkinElmer Life Sciences), dried under vacuum, and exposed to film.

For Fig. 5, reactions were performed with 150 nCi of [3H]acetyl coenzyme A (57 mCi/mmol). Reactions were performed in a 20-µl reaction volume in the same buffer described above, plus 10 mM sodium butyrate and 1 mM phenylmethylsulfonyl fluoride. Reactions were assembled on ice, incubated at 30 °C for 30 min, and stopped with the addition of 5 µl of 5× SDS-PAGE loading buffer. Reactions were heated at 100 °C for 1 min and loaded onto 15% SDS-PAGE gels, which were silver-stained, photographed, treated with 1 M sodium salicylate/30% MeOH, dried, and subjected to fluorography.

**Amino Acid Sequence Analysis—**HAT assays were done as described above using the recombinant SAS complex and either the H3 or H4 peptide. Four reactions were pooled, applied to ProSorb membranes (PerkinElmer Life Sciences) and processed for sequencing according to instructions from the manufacturer. The samples were subjected to Edman degradation. Eluates from each cycle were collected, dried, resuspended in 50 mM sodium acetate, pH 5.2, and counted in a liquid scintillation counter.

**Results**

Recombinant preparations of most MYST family proteins identified to date function as HATs in vitro in the absence of other factors (8). Although Sas2 displays a high degree of homology to MYST family proteins, we and others (5) have been unable to detect HAT activity using recombinant Sas2. Because Sas2 is part of a complex with Sas4 and Sas5 in vitro, and because mutation of SAS4 or SAS5 causes the same phenotypes as mutation of SAS2, we reasoned that Sas2 might require these additional subunits for activity. Furthermore, Sas2 expressed in E. coli is to a large extent insoluble (data not shown). It has been shown that, in some cases, co-expression of several subunits of a multicomponent complex can increase the solubility of the individual proteins (28, 29). Therefore, we designed a plasmid for co-expression of SAS2, SAS4, and SAS5 in E. coli. Plasmid pAS132 contains SAS2 (including sequences for a C-terminal His6 tag), SAS4 and SAS5, with each gene under control of an inducible T7 promoter. Upon induction, all three proteins were expressed, although only a small fraction of Sas2-His6 and Sas5 was soluble (data not shown). When the soluble fraction was purified using nickel-affinity chromatography by virtue of the His tag on Sas2, substantial amounts of Sas4 and Sas5 coeluted with Sas2, indicating that the proteins formed a complex in E. coli (Fig. 1B). Remarkably, this complex had robust HAT activity using chicken histones as substrate (Fig. 1A). In contrast, equivalent amounts of recombinant Sas2-His6 expressed and purified in the same manner in the absence of Sas4 and 5 displayed no activity (Fig. 1, A and B).

To determine whether all three Sas proteins were required for HAT activity, or whether the activity resulted from either Sas2,4 or Sas2,5 subcomplexes, additional expression plasmids were constructed that encoded Sas2-His6 and either Sas4 or Sas5. In both cases, equivalent amounts of the Sas4 or Sas5 proteins copurified with Sas2-His6 as when all three proteins
of Sas2 is essential for function in vivo activity, optimal HAT activity requires the Sas2,4,5 complex. While the Sas2,4 complex has C-terminal proteins, against either chicken histones or a H4 peptide (Fig. 2A). Both the Sas2,4 and Sas2,5 complexes had activity, but the specific activity of the Sas2,4 complex was consistently 2-fold higher than that of the Sas2,5 complex. However, histone H3 is also acetylated by this enzyme (Fig. 3A). This acetylation pattern is similar to that of Esa1, another member of the MYST family of acetyltransferases (Fig. 3A). The Sas2,4 complex showed a similar substrate specificity but had lower specific activity (data not shown). We conclude that the Sas5 subunit does not alter substrate specificity but had lower specific activity (data not shown). We conclude that the Sas5 subunit does not alter histone polypeptides are acetylated by Sas2.

To determine which residues within the histone H3 and H4 tails are acetylated by the SAS complex, we used as substrates synthetic peptides corresponding to the first 28 amino acids of H4 or the first 21 amino acids of H3. Both peptides were efficiently acetylated when incubated with the SAS complex, but not with Sas2 alone (Figs. 1 and 2 and data not shown). In vitro 3H-acetylated peptides were subjected to N-terminal sequencing, and the amount of radioactivity in each residue was determined. We observed that the primary site of acetylation on histone H4 was lysine 16 and that the primary site of acetylation on the H3 peptide was lysine 14 (Fig. 3B).

Interestingly, when the products of the HAT assays using the wild-type SAS complex were analyzed on a protein gel, Sas2, as well as histones H3 and H4, was radioactively labeled (Fig. 3A). Because the labeling of Sas2 survived SDS-PAGE and because no radioactivity was incorporated into the sas2Δ1 mutant protein (Fig. 3A), the labeling most likely resulted from autoacetylation of Sas2 rather than non-covalent trapping of acetyl-CoA. Similar autoacetylation was observed for Esa1 (Fig. 3A), but not for Hat1, a histone acetyltransferase that is not a member of the MYST family (data not shown). Interestingly, the autoacetylation of Sas2 was dependent upon the presence of histones; when histones were omitted from the reaction, very little radioactivity was incorporated into Sas2 (data not shown). Whether this autoacetylation is intermolecular or intramolecular, and whether it is important for the function of the enzyme, is not known.

Most MYST family members studied to date exist in multi-protein complexes and interact with chromatin in order to acetylate histones (8). In S. cerevisiae, these include Sas3, a component of the NuA3 complex (30), and Esa1, the HAT for the NuA4 complex (31). Both the NuA3 and NuA4 complexes acetylate histones in a nucleosomal context. Therefore, we hypothesized that SAS would display a similar activity. Consistent with this idea, a Gal4-Sas2 fusion protein, when tethered
via Gal4 binding sites placed at \textit{HMR}, functioned as an effective barrier to the spread of silencing (32). This was proposed to result from Sas2-mediated histone acetylation counteracting Sir2-mediated histone deacetylation. These data suggested that when artificially tethered, Sas2 can acetylate histones in nucleosomes.

To test whether the SAS complex acetylates nucleosomal histones, nucleosomes and core histones derived from human HeLa cells were compared as substrates of the SAS complex. Strikingly, we detected no acetylation of nucleosomes using assay conditions that were optimum for acetylating free histones (Fig. 4). However, the SAS complex did acetylate free histones derived from these nucleosomes (Fig. 4), demonstrating that these HeLa histones were not already fully acetylated on histone H3 lysine 14 and H4 lysine 16. Furthermore, the Sas3-containing NuA3 complex, which acetylates H3 lysine 14 on nucleosomes, had robust activity using the HeLa nucleosomes as substrate (Fig. 4). We also purified nucleosomes from wild-type and \textit{sas2} yeast strains and tested those nucleosomes as substrates for the SAS complex. The assays were done using several different buffer conditions that are optimal for other HATs, with a salt range from 0–150 mM. The SAS complex showed no activity on nucleosomes from either strain (data not shown). In contrast, the Esa1-containing NuA4 complex was active on both (data not shown). We also tested whether SAS could acetylate histones H3 and H4 in the form of (H3/H4)\textsubscript{2} tetramers deposited onto DNA, in the absence of H2A/H2B dimers. Again, no modification of the histones was observed, although the same preparation of histones used to form the tetramer-DNA complexes was efficiently acetylated (Fig. 5). Thus, under our reaction conditions, free histones H3 and H4, but not those deposited onto DNA, are substrates of the recombinant SAS complex \textit{in vitro}.

Neither Esa1 nor Gcn5 acetylate nucleosomes \textit{in vitro} unless they are part of a multiprotein complex (NuA4 for Esa1, Ref. 31 and SAGA or ADA for Gcn5, Ref. 33). Therefore, the inability of the recombinant SAS complex to acetylate nucleosomes may result because a targeting subunit is missing. To address this, we purified active SAS complex from yeast. We created strains in which either Sas2 or Sas5 contained at their C terminus a tag for TAP (21). This tag enabled us to purify native SAS complex consisting of Sas2, Sas4, and Sas5 (Fig. 6A). We tested these purified complexes for HAT activity using HeLa nucleosomes, HeLa core histones and recombinant yeast histones. Just as for the recombinant SAS complex, the native yeast complex could acetylate free histones, but not the same histones when incorporated into nucleosomes (Fig. 6B). The native complex, like the recombinant SAS complex, acetylated histones H3 and H4, with a stronger preference for H4 than for H3 (Fig. 6B).

To further characterize the specificity of the yeast enzyme, we used as substrates a series of peptides corresponding to amino acids 1–20 of the histone H4 N terminus. These peptides were either unacetylated or tri- or tetra-acetylated at lysines 5, 8, 12, and 16. Only peptides in which lysine 16 was not acetylated were substrates for the native SAS complex (Fig. 7). Therefore, under our reaction conditions the recombinant and native SAS complexes show similar substrate specificities; both acetylate free histone H4 at lysine 16, and to a lesser extent histone H3, and neither acetylates histones that are packaged into nucleosomes.
SAS Complex Has HAT Activity

Sas2 was predicted to be a histone acetyltransferase because of its sequence similarity to members of the MYST family. However, until this report, no in vitro activity was detected either from recombinant Sas2 or from Sas2 purified from yeast. In this analysis, we show that recombinant Sas2 can acetylate histones, but only when associated with the Sas4 subunit. Although the in vitro specificity of other HATs is altered by associated proteins (8), this is the first case in which enzymatic activity of a histone acetyltransferase absolutely depends upon additional subunits. Furthermore, maximal activity of Sas2,4 required the Sas5 subunit. Sas5 may be required to help stabilize the complex, or to help in substrate recognition. Sas5 has homology to tf2f domain-containing proteins including yeast TAFII30, which is a component of a number of transcription/chromatin remodeling complexes (34–36). Perhaps the tf2f domain of Sas5 is involved in histone binding. Because sas5 mutants are as defective in silencing as sas2 mutants, Sas5 may be crucial for HAT activity in cells in a manner not reflected in our in vitro assays.

Previous attempts by this group and others (5, 7) to purify enzymatically active SAS complex from yeast were unsuccessful, although the complexes that were purified contained Sas2, Sas4, and Sas5. In this report, we show that using the tandem affinity purification method (21) to isolate the SAS complex results in active enzyme. Perhaps the previously isolated complexes co-purified with an inhibitor, or the purification schemes used inactivated the enzyme. For all substrates tested, the recombinant enzyme and yeast native enzyme show similar substrate specificities. Both enzymes acetylate histone H4 and to a lesser extent, histone H3. Furthermore, both enzymes acetylate free histones, but not those in nucleosomes.

We showed that both the recombinant and native enzymes acetylate lysine 16 of histone H4. Previous genetic data showed that mutating histone H4 lysine 16 to a nonacetylatable residue (K16R) confers silencing defects similar to those of sas2 null cells (5) and that H4 lysine 16 is underacetylated in sas2 mutants (9, 10). Our data strongly suggest that these genetic effects are a direct consequence of acetylation of histone H4 lysine 16 by the SAS complex. Both the recombinant and native enzymes acetylated lysine 16 of histone H4.

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enzyme complexes also acetylate histone H3 in vitro, and we determined that the recombinant enzyme that the target was lysine 14. It has not been determined whether histone H3 lysine 14 is also an in vitro substrate for the SAS complex. Acetylation of H3 lysine 14 is reduced in a sas2 mutant, but this effect may be indirect (10). Since the histone H4 K16R mutation causes the same reduction in silencing as deletion of SAS2 at telomeres and at HML (in a Δsir1 strain), it may be that acetylation of histone H3 lysine 14 by Sas2 either does not occur in vivo or is not important for silencing.

Almost all HAT enzyme complexes studied to date except for Hat1 acetylate both free and nucleosomal histones. However, neither the recombinant nor native SAS complex could acetylate histones in HeLa nucleosomes; whereas using the same assay conditions, both enzymes could acetylate free histones derived from these nucleosomes. Furthermore, the recombinant enzyme had no activity on nucleosomes purified from wild-type and sas2 mutant cells. We considered four possible explanations for these results. First, it is possible that a subunit required to target SAS to nucleosomal histones was lost during the purification of the enzyme from yeast. Second, SAS may only work on histones that have been previously modified in some way, and these modified histones are not present in the HeLa or yeast nucleosomes we used. If true, then this modification must only be required when histones are part of nucleosomes, since once released from the HeLa nucleosomes the HeLa histones were good substrates for the SAS enzyme. Third, acetylation of nucleosomes by SAS may require assay conditions different from those that we used. A fourth explanation is that in vitro SAS does acetylate only free histones and not those in nucleosomes. Sas proteins physically interact with subunits of the Asf1 and CAF-I histone deposition factors. Furthermore, asf1 mutations and sas mutations cause similar effects on silencing at the HM loci, and cac mutants and sas mutants both display reduced telomeric silencing. Asf1 and CAF-I bind to free histone H3 and H4, and are believed to function in histone deposition. SAS may function in these two pathways to participate in the deposition process. However, we have demonstrated that recombinant SAS does not acetylate H3 or H4 when they are bound to DNA as tetramers or associated with Asf1. A model to explain the biochemical data and genetic interactions between SAS and Asf1 (and CAF-I) would be that SAS acetylates newly synthesized H3 and H4 and then passes these histones on to Asf1 (or CAF-I) for deposition onto chromatin. SAS has been implicated in formation of a barrier between silenced and unsilenced regions of the chromosome (32, 9, 10). Perhaps Asf1 and CAF-I function downstream of SAS in the formation of these barriers by depositing acetylated histones adjacent to regions of silent chromatin.

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Publications


