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Role of the Conserved Sir3-BAH Domain in Nucleosome Binding and Silent Chromatin Assembly

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SUMMARY

Silent chromatin domains in *Saccharomyces cerevisiae* represent examples of epigenetically heritable chromatin. The formation of these domains involves the recruitment of the SIR complex, composed of Sir2, Sir3, and Sir4, followed by iterative cycles of NAD-dependent histone deacetylation and spreading of SIR complexes over adjacent chromatin domains. We show here that the conserved bromo-adjacent homology (BAH) domain of Sir3 is a nucleosome- and histone-tail-binding domain and that its binding to nucleosomes is regulated by residues in the N terminus of histone H4 and the globular domain of histone H3 on the exposed surface of the nucleosome. Furthermore, using a partially purified system containing nucleosomes, the three Sir proteins, and NAD, we observe the formation of SIR-nucleosome filaments with a diameter of less than 20 nm. Together, these observations suggest that the SIR complex associates with an extended chromatin fiber through interactions with two different regions in the nucleosome.

INTRODUCTION

The formation of silent chromatin or heterochromatin plays important roles in the regulation of gene expression and maintenance of chromosome stability in eukaryotes. Silent domains are often associated with repetitive DNA sequences that surround centromeres or form telomeres and are required for the functions of these chromosome structures (Grewal and Moazed, 2003; Karpen and Allshire, 1997; Richards and Elgin, 2002). Additionally, genes that regulate cellular identity and differentiation, such as the metazoan homeotic genes or the mating-type loci of fungi, are maintained in their silenced state by assembly into repressive structures that resemble heterochromatin (Paro and Hogness, 1991; Ringrose and Paro, 2004). Studies in yeast, flies, and mammals have revealed a divergence in the mechanisms by which cells establish, maintain, and regulate heterochromatin. However, despite differences, the general mechanisms by which heterochromatic structures are assembled in these distant organisms appear to remain strikingly analogous (Grewal and Moazed, 2003; Moazed, 2001; Richards and Elgin, 2002).

Eukaryotic nuclear DNA is packaged with histones and other proteins into chromatin (Kornberg and Lorch, 1999; Luger et al., 1997). The basic unit of chromatin folding is the nucleosome, in which 147 base pairs of DNA are wrapped around an octamer composed of the histones H2A, H2B, H3, and H4. The highly conserved N-terminal tails and globular domains of histones provide binding sites for numerous chromatin-associated proteins (Hecht et al., 1995; Jenuwein and Allis, 2001). This binding is regulated by posttranslational histone modifications, including the reversible acetylation, methylation, and ubiquitination of specific lysine residues (Kouzarides, 2007). In most cases, the assembly of silent chromatin involves the recruitment of silencing complexes, which contain histone-binding and -modifying proteins, to specific nucleation sites on DNA, called silencers. This recruitment then leads to a pattern of histone modifications specific to silenced chromatin regions, possibly accompanied by an alteration in higher-order chromatin folding.

Silent chromatin regions of budding yeast are found at telomeres, at the two silent mating-type cassettes (HM loci, *HML* and *HMR*), and at the ribosomal DNA (rDNA) repeats (Rusche et al., 2003). Silencing of the HM loci and telomeres requires the Sir2, Sir3, and Sir4 proteins, as well as a set of common and locus-specific DNA-binding proteins (Aparicio et al., 1991; Klar et al., 1979; Rine and Herskowitz, 1987; Rusche et al., 2003). The Sir proteins form a silencing complex called the SIR complex (Moazed et al., 1997; Moretti et al., 1994; Strahl-Bolsinger et al., 1997), composed of a stable Sir2/Sir4 heterodimer as well as dimers and higher-order oligomers of Sir3 (Liou et al., 2005; Rudner et al., 2005; Tanny et al., 2004). The Sir2 subunit of this complex is the founding member of a conserved family of proteins that couple

deacetylation to NAD hydrolysis and synthesis of *O*-acetyl-ADP-ribose (OAADPR, or acetoxy ADP-ribose [AAR])(Imai et al., 2000; Landry et al., 2000; Sauve et al., 2001; Tanner et al., 2000; Tanny and Moazed, 2001). The Sir3 and Sir4 subunits of the complex bind to deacetylated histone H3 and H4 N-terminal peptides in vitro (Hecht et al., 1995), and the deacetylation of histone H4 lysine 16 (H4K16) in the H4 N terminus appears to be particularly crucial for Sir3 binding and silencing (Johnson et al., 1990; Liou et al., 2005). Current models of silencing propose that the SIR complex is recruited by DNA-binding proteins to specific sites of initiation. The Sir2 subunit then deacetylates the N-terminal tail of histone H4 in silencer-proximal nucleosomes, promoting further SIR complex binding, followed by iterative cycles of deacetylation and binding that result in the spreading of Sir2, Sir3, and Sir4 along adjacent chromatin (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). AAR, together with a deacetylated histone H4 tail, promotes the oligomerization of Sir3 in the SIR complex in vitro and has therefore been proposed to regulate SIR complex spreading in vivo (Liou et al., 2005).

In addition to acetylation, the assembly and spreading of silent chromatin are regulated by other antisilencing mechanisms, involving the histone H2A.Z variant, Htz1, and methylation of specific lysine residues in histone H3. Htz1 acts as an antisilencing protein that limits the spreading of Sir proteins and is excluded from silent domains (Meneghini et al., 2003). Methylation of H3K4 is frequently associated with transcriptionally active genes (Fischle et al., 2003), and this site has been found to be hypomethylated at regions of silent chromatin. Deletion of *SET1*, which encodes the H3K4 methyltransferase (HMT) enzyme, results in loss of silencing, at least in part due to mislocalization of Sir3 (Bernstein et al., 2002; Fingerman et al., 2005; Katan-Khaykovich and Struhl, 2002; Nislow et al., 1997; Santos-Rosa et al., 2004). Moreover, lysine 79 in the globular domain of histone H3 (H3K79) is methylated by another HMT enzyme, Dot1, and either deletion or overexpression of the *DOT1* gene, or a mutation of H3K79 to alanine, results in defects in silencing (Ng et al., 2002; van Leeuwen et al., 2002). Additional support for the importance of the region surrounding H3K79 comes from genetic screens that have identified histone H3 residues 68–83 as necessary for silencing (Park et al., 2002). Finally, establishment of silencing through posttranslational modifications appears to occur in two major steps. Rapid deacetylation is followed by a decrease in methylation that occurs over multiple generations (Katan-Khaykovich and Struhl, 2002). The molecular mechanism by which Htz1 and the methylation of histone H3K4 or K79 antagonize silencing is unknown.

The mechanism of association of the SIR complex with specific nucleosomes, perhaps the most important step in silent chromatin assembly, is still poorly understood. While full-length Sir3 purified from yeast binds selectively to histone H4 peptides that contain deacetylated histone H4K16 (Liou et al., 2005), Sir3 synthesized in heterologous systems (*E. coli*, reticulocyte lysate, or insect cells) displays little specificity for the acetylation state of H4K16 in peptide or nucleosome-binding experiments (Carmen et al., 2002; Georgel et al., 2001; Hecht et al., 1995). Moreover, while previous binding studies have pointed to the C terminus of Sir3 as the histone N-terminal tail-binding domain (Hecht et al., 1995), other studies suggest that a conserved domain in the N terminus of Sir3, called the bromo-adjacent homology (BAH) domain, may also play a role in nucleosome or DNA binding. In particular, mutations that suppress the silencing defect of histone H4 N-terminal tail mutations map to the BAH domain (Johnson et al., 1990) and, though nonspecific, bacterially produced BAH has DNA and nucleosome-binding activity in vitro (Connelly et al., 2006).

In this study, we examine the association of Sir3 and its subfragments, produced in yeast, with chromatin fragments from yeast cells that are either wild-type or carry mutations in specific histone residues or modifying enzymes. Surprisingly, we find that, in this system, the BAH domain is primarily responsible for the association of Sir3 with chromatin. This association is direct, as it could be demonstrated between the purified BAH domain and histone H4 N-terminal peptides or nucleosomes and is regulated by the modification state of lysine 16 in the N terminus of histone H4 and lysine 79 in histone H3. Furthermore, in examining the association of purified Sir proteins with purified yeast chromatin fragments using electron microscopy (EM), we observed the formation of SIR-nucleosome filaments. The formation of these filaments displays requirements that closely mirror those observed for the formation of silent chromatin in vivo, and their diameter is consistent with the association of the SIR complex with an extended chromatin fiber.

RESULTS

The BAH Domain of Sir3 Is a Nucleosome-Binding Domain

Previous experiments had identified the C-terminal region of Sir3 as a histone H3 and H4 N-terminal tail-binding region (Carmen et al., 2002; Hecht et al., 1995). In order to identify the domains of Sir3 involved in binding to nucleosomes, a more physiological substrate, we constructed yeast cells expressing C-terminally TAP-tagged full-length Sir3 (amino acids 1–978), an N-terminal fragment of Sir3 containing the BAH domain (amino acids 1–214), and a C-terminal fragment of Sir3 lacking the BAH domain (amino acids 214–978) (Figure 1A). The cells were lysed, and the TAP-tagged Sir3 constructs were analyzed for their ability to bind to chromatin fragments present in whole-cell extracts. As expected, immunoprecipitation by the TAP tag showed an interaction between full-length Sir3 and histones, as observed by Coomassie staining (Figure 1B, lane 6) and by western blotting for histone H3 (Figure 1C, lane 6). The presence of all four histones (Figure 1B, lane 6), as well as the sensitivity of binding to treatment with ethidium bromide (see Figure S1 available

online), which disrupts protein-DNA interactions (Lai and Herr, 1992), indicated binding to nucleosomes rather than free histones. Furthermore, because TAP-tagged Sir3 still immunoprecipitated nucleosomes in a *sir2Δ* strain, this binding occurred independently of Sir2 (data not shown). We therefore believe that the binding observed here does not represent the association of Sir3 with silent chromatin domains but rather represents the general nucleosome-binding properties of Sir3. Surprisingly, the BAH domain of Sir3, but not the fragment of Sir3 lacking the BAH domain, immunoprecipitated nucleosomes as efficiently as full-length Sir3 (Figures 1B and 1C, lanes 7 and 8), suggesting that the primary nucleosome-binding capacity resides in the BAH domain.

Association of the Sir3 BAH Domain with Nucleosomes Is Regulated by the NatA N-Terminal Acetyltransferase Complex and Histone Modifications

In order to further study the binding properties of Sir3 to nucleosomes, we mutated enzymes that have previously been shown to affect Sir3 function or localization in vivo (Briggs et al., 2001; Katan-Khaykovich and Struhl, 2005; Kimura et al., 2002; Ng et al., 2002, 2003; Santos-Rosa et al., 2004; Suka et al., 2002; van Leeuwen et al., 2002). N-terminal acetylation of Sir3 by the NatA complex is required for Sir3 function in silencing, and deletion of the gene encoding the catalytic subunit, *ARD1*, leads to a silencing defect (Connelly et al., 2006; Wang et al., 2004). In agreement with the genetic data, we found that Sir3 or the BAH domain alone from *ard1Δ* cells was unable to bind to nucleosomes (Figures 1D and 1E, lane 6).

We next analyzed the effect of mutating histone-modifying enzymes on the interaction between Sir3 and nucleosomes. Because silent chromatin regions have been found to be hypoacetylated and hypomethylated (Braunstein et al., 1993; Katan-Khaykovich and Struhl, 2005; Santos-Rosa et al., 2004), acetylation and methylation have been proposed to limit the binding and spreading of Sir3 on chromatin. We reasoned that deletion of the enzymes that posttranslationally modify histones would allow Sir3 to bind to a larger proportion of nucleosomes present in the lysate. Compared to Sir3 from wild-type cells, nucleosomes lacking histone H3K79 methylation (*dot1Δ*) or decreased histone H4K16 acetylation (*sas2Δ*) bound to Sir3 with greater efficiency (Figure 1F, lanes 15–17). The Sir3 BAH domain alone showed a similar increase in nucleosome binding in these mutant strains (Figure 1F, lanes 19–21). Surprisingly, full-length Sir3 in a strain in which the histone H3K4 methyltransferase gene, *SET1*, was deleted failed to immunoprecipitate nucleosomes (Figure 1F, lane 18), while the BAH domain alone bound to the H3K4-unmethylated nucleosomes to the same degree as those from a *dot1Δ* or *set1Δ* strain (Figure 1F, lane 22). The reason for the inability of full-length Sir3 to bind to nucleosomes from *set1Δ* cells is presently unclear, but it is consistent with the silencing defect observed in *set1Δ* cells and may be related to the presence of the previously defined histone H3 and H4 N-terminal tail-binding domain in the C terminus of Sir3 (Briggs et al., 2001; Hecht et al., 1995; Krogan et al., 2002; Nislow et al., 1997; Santos-Rosa et al., 2004). More importantly, however, Sir3 lacking the BAH domain failed to bind to nucleosomes even in strains lacking histone-modifying enzymes (Figure 1F, lanes 24–26), suggesting that the binding to unmodified nucleosomes requires the BAH domain.

We next wanted to more directly determine the histone regions that affect Sir3 binding to nucleosomes by using strains in which specific histone residues were mutated. We found complete loss of Sir3 binding to nucleosomes in strains with histone H4K16 mutated to either Q or G, mimicking a neutral, acetylated lysine residue (Figure 2A, lanes 14 and 16), while a mutation of the same residue to R, mimicking a deacetylated lysine, showed greater binding to Sir3 (Figure 2A, lane 15). In contrast, mutation of H4K5 to Q did not affect Sir3 binding to nucleosomes (Figure 2A, lane 13). These binding results are in agreement with previously observed in vivo silencing defects (Johnson et al., 1990; Megee et al., 1990) as well as surface plasmon resonance (SPR) binding data (Liou et al., 2005). The increase in Sir3-nucleosome interaction observed in *dot1Δ* cells suggests that methylation of H3K79 by Dot1 normally inhibits the association of Sir3 with nucleosomes (Figure 1F, lanes 16 and 20), which has also been observed in previous ChIP studies (Ng et al., 2002; van Leeuwen et al., 2002). Consistent with these data, Sir3 bound less efficiently to nucleosomes containing histone H3 with a lysine 79-to-alanine substitution (H3K79A) (Figure 2A, lane 17), while it strongly associated with H3K79R-containing nucleosomes (Figure 2A, lane 20). Together, these observations suggest that histone H4K16 is the most critical residue for the binding of Sir3 to nucleosomes. Because, under our experimental conditions, the BAH domain is the primary nucleosome-binding domain in Sir3 (Figures 1C–1F), we further tested the effect of these histone mutations on binding of the truncated Sir3 protein containing only the BAH domain to nucleosomes. Like the full-length Sir3 protein, the BAH domain bound both wild-type and H4K5Q-containing nucleosomes (Figure 2B, lanes 12 and 13) and did not bind H4K16Q- or H4K16G-containing nucleosomes (Figure 2B, lanes 14 and 16). Interestingly, the Sir3 BAH domain bound strongly to nucleosomes containing an H4K16R mutation (Figure 2B, lane 15), suggesting that this domain efficiently recognizes the charged, unacetylated state of H4K16. The BAH domain alone also showed little binding to nucleosomes containing H3K79A (Figure 2B, lane 17) and efficient binding to nucleosomes containing H3K79R (Figure 2B, lane 20). These results suggest that the positively charged surface of the nucleosome containing H4K16 and H3K79 (Luger et al., 1997; Schalch et al., 2005) forms a binding site for the BAH domain. These observations also provide an explanation for previous genetic studies, which identified mutations in W86 and D205 amino acids of Sir3, located near each

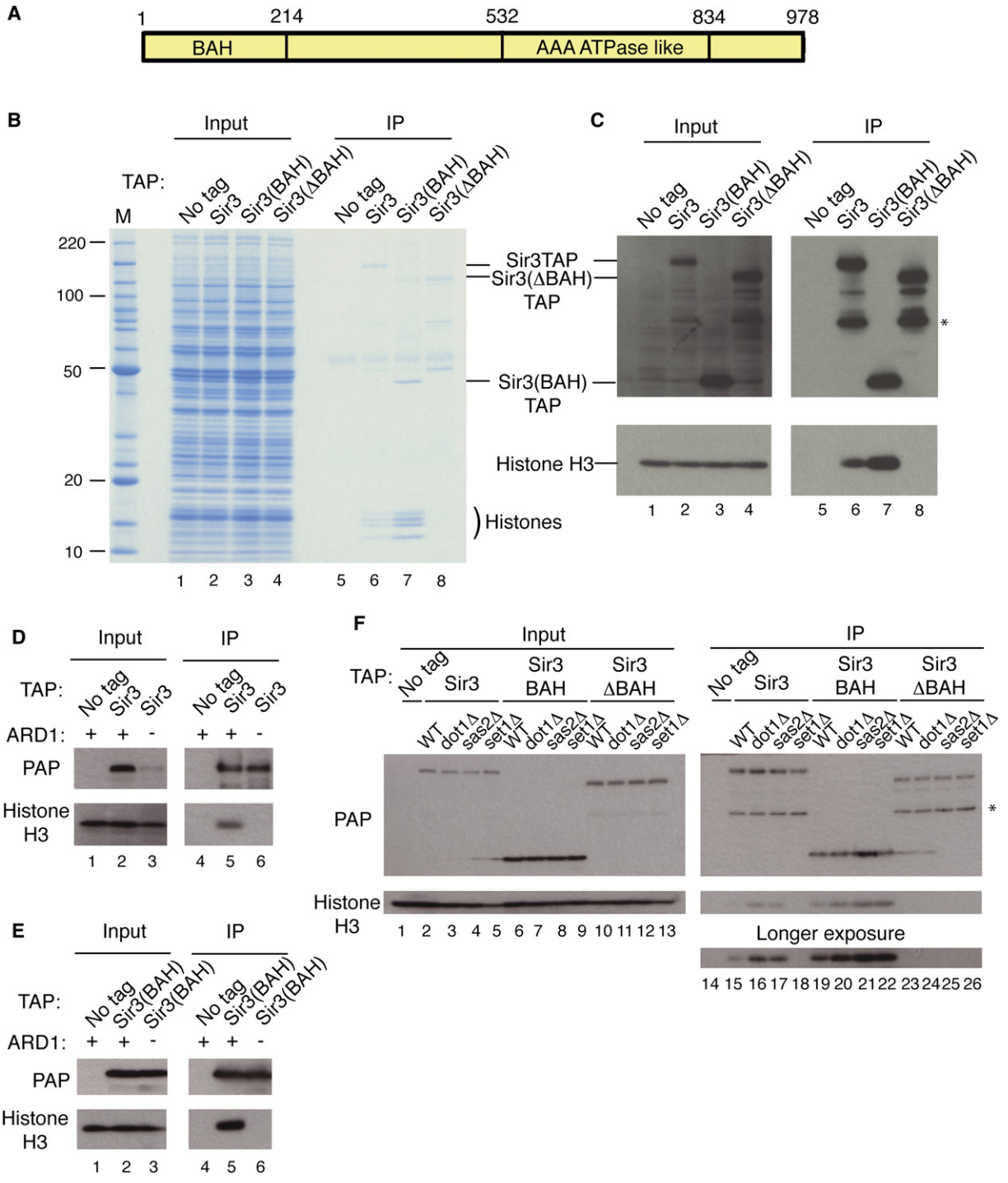


Figure 1. The BAH Domain Is Necessary for Sir3 Binding to Nucleosomes

(A) Sir3 contains an N-terminal BAH domain and an AAA ATPase-like domain.

(B) Immunoprecipitated TAP-tagged full-length Sir3, Sir3 BAH domain (Sir3[BAH]), and Sir3 lacking the BAH domain (Sir3[ΔBAH]) on an SDS-PAGE gel, stained with Coomassie.

(C) Immunoprecipitations, as in (B), probed with peroxidase anti-peroxidase (PAP) (top panel) or histone H3 antibody (bottom panel) by western blotting. Asterisk denotes Sir3 degradation product.

(D) Full-length Sir3-TAP immunoprecipitated from WT (+) or *ard1Δ* (–) cells. Sir3-TAP in mutant strain backgrounds is unstable.

(E) Sir3(BAH)-TAP immunoprecipitated from WT (+) or *ard1Δ* (–) cells.

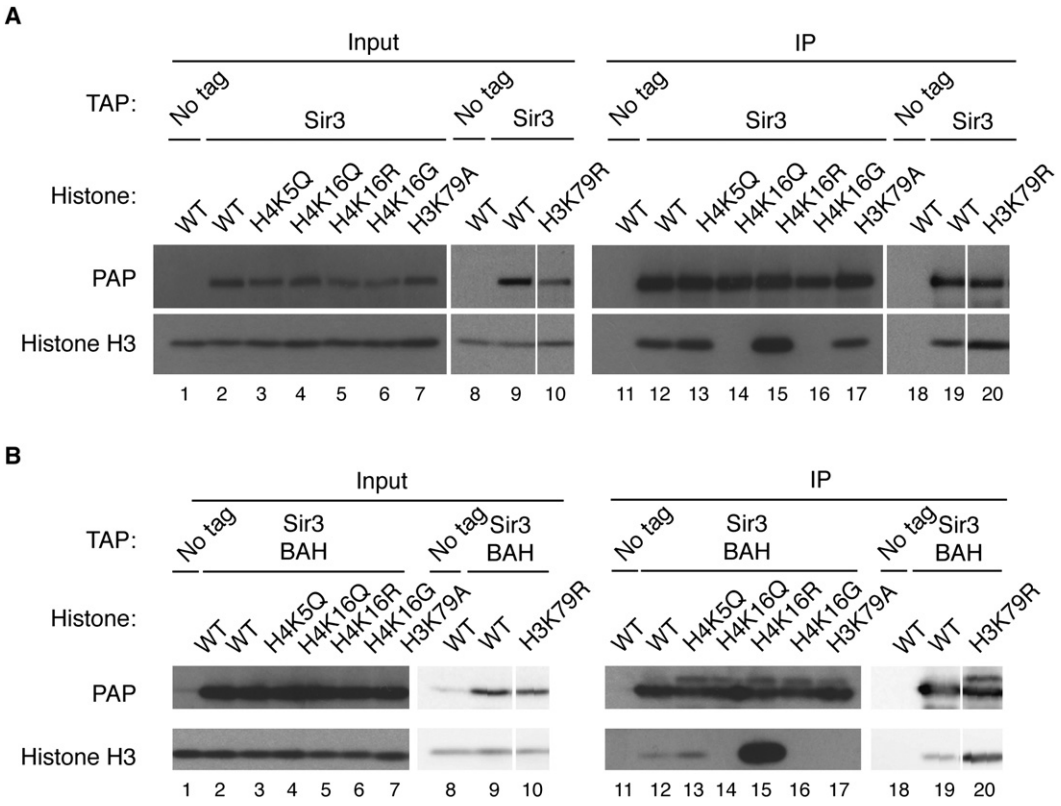


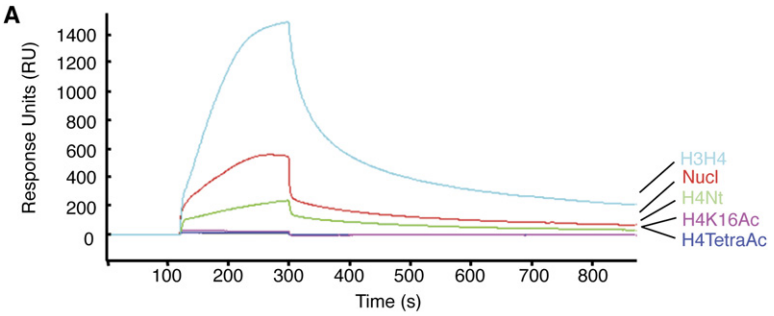
Figure 2. Histone H4K16 Is a Critical Residue for Sir3 and Sir3-BAH Binding to Nucleosomes
(A) Full-length Sir3-TAP immunoprecipitated from strains expressing wild-type (WT) or mutant histones.
(B) Sir3(BAH)-TAP immunoprecipitated from strains expressing wild-type (WT) or mutant histones.

other in the BAH domain (Figure 6A) (Connelly et al., 2006; Hou et al., 2006), as suppressors of the silencing defect of histone H4 N-terminal tail mutations (Johnson et al., 1990).

The BAH Domain Binds Directly to Nucleosomes and the Histone H4 N Terminus

To determine whether the BAH domain can associate with nucleosomes directly, we used either full-length Sir3 or the BAH domain in SPR spectroscopy experiments. As expected (Liou et al., 2005), we observed that full-length Sir3 bound strongly to an unacetylated histone H4 N-terminal peptide and recombinant histone H3/H4 tetramers, as well as to purified native yeast nucleosomes, but not to an acetylated histone H4 N-terminal peptide (Figure 3A). In addition, the BAH domain displayed histone H3/H4 tetramer- and nucleosome-binding properties that were similar to those of full-length Sir3, but these interactions were more sensitive to mutations in the N terminus of histone H4, especially that of histone H4K16 to Q (Figure 3B). This is likely to be due to the presence of the second histone-tail-binding domain in the C terminus of Sir3 (Hecht et al., 1995). We next tested whether the BAH domain bound to histone H4 N-terminal peptides. While full-length Sir3 was able to bind to histone H4 N-terminal peptides that were either 20 or 34 amino acids long, the BAH domain bound only to the longer, 34 amino acid N-terminal tail, indicating that the BAH domain requires histone H4 residues beyond amino acid 20 for binding to nucleosomes (Figure 3C). This result suggests that the histone-binding properties of the BAH domain are distinct from the binding properties of the previously mapped histone-interacting domain at the C terminus of Sir3. In this regard, we note that genetic studies have mapped the silencing domain of histone H4 to a region encompassing amino acids 16–29 (Johnson et al., 1992). Together with data in Figures 1 and 2, these results demonstrate that the BAH domain can directly associate with nucleosomes via interactions with the N-terminal region of histone H4 containing deacetylated K16 and extending beyond amino acid 20. The association of the BAH domain with the H4 N terminus and bacterially produced histone H3/H4 tetramers also indicates that BAH-chromatin association does not require the assembly of histones with DNA into nucleosomes.

(F) Sir3-TAP, Sir3(BAH)-TAP, and Sir3(ΔBAH)-TAP immunoprecipitated from wild-type (WT), *dot1Δ*, *sas2Δ*, or *set1Δ* cells. The middle panel shows a shorter western blot exposure, while the bottom panel shows a longer exposure of the same blot. Asterisk denotes Sir3 degradation product.



B

Interaction	k_a (1/Ms)	k_d (1/s)	K_D (M)
Sir3-Nucl	(9.5 +/- 1.9) x 10^4	(6.8 +/- 1.4) x 10^{-4}	(7.6 +/- 2.7) x 10^{-9}
BAH-Nucl	(5.6 +/- 1.2) x 10^5	(1.4 +/- 0.2) x 10^{-3}	(2.5 +/- 0.3) x 10^{-9}
Sir3-NuclK5,12Q	(5.4 +/- 1.9) x 10^5	(9.6 +/- 3.4) x 10^{-4}	(2.0 +/- 1.5) x 10^{-9}
BAH-NuclK5,12Q	(5.0 +/- 2.8) x 10^3	(2.6 +/- 2.3) x 10^{-3}	(4.0 +/- 2.8) x 10^{-7}
Sir3-NuclK16Q	(2.7 +/- 1.7) x 10^3	(6.3 +/- 1.6) x 10^{-3}	(2.8 +/- 1.1) x 10^{-6}
BAH-NuclK16Q	NM	NM	NM
Sir3-H3H4	(1.1 +/- 0.1) x 10^5	(1.4 +/- 0.2) x 10^{-4}	(1.3 +/- 0.2) x 10^{-9}
BAH-H3H4	(6.4 +/- 0.5) x 10^4	(5.4 +/- 1.6) x 10^{-4}	(8.5 +/- 2.7) x 10^{-9}

C

Interaction	k_a (1/Ms)	k_d (1/s)	K_D (M)
Sir3-H4Nt(1-34aa)	(9.7 +/- 3.6) x 10^4	(1.5 +/- 0.1) x 10^{-3}	(1.7 +/- 0.7) x 10^{-8}
BAH-H4Nt(1-34aa)	(5.7 +/- 0.3) x 10^2	(2.2 +/- 0.1) x 10^{-3}	(3.8 +/- 0.4) x 10^{-6}
Sir3-H4Nt(1-20aa)	(1.4 +/- 0.9) x 10^5	(2.7 +/- 0.6) x 10^{-3}	(2.7 +/- 1.6) x 10^{-8}
BAH-H4Nt(1-20aa)	NM	NM	NM

Figure 3. The Sir3 BAH Domain Binds to Nucleosomes and the Histone H4 N-Terminal Tail

(A) Surface plasmon resonance (SPR) sensorgrams showing the interaction of various ligands with Sir3 immobilized on a CM5 chip. H3H4, recombinant H3-H4 tetramers; Nucl, purified native yeast nucleosomes; H4Nt, unacetylated histone H4 N-terminal tail; H4K16Ac, histone H4 N-terminal tail acetylated at K16; H4TetraAc, histone H4 N-terminal tail acetylated at K5, 8, 12, and 16.

(B) Summary of the k_a, k_d, and calculated K_D values based on SPR experiments. The protein in bold was immobilized on the chip.

(C) Summary of SPR experiments using short (1–20 amino acids) or long (1–34 amino acids), unacetylated histone H4 N-terminal peptides.

Because the BAH domain has been identified in other chromatin-regulating proteins, including Orc1, Rsc1, and Rsc2 in *S. cerevisiae* as well as Polybromo and Dnmt1 in multicellular eukaryotes (Cairns et al., 1999; Callebaut et al., 1999; Nicolas and Goodwin, 1996), we wanted to determine whether the BAH domain has general nucleosome-binding properties. Thus, the BAH domain of yeast Orc1 (amino acids 1–228) and the tandem BAH domains of human Dnmt1 (amino acids 634–980) were tagged and expressed in yeast and immunoprecipitated. The human Dnmt1 BAH domains did not bind to yeast nucleosomes (Figure S2A, lane 12), possibly because yeast nucleosomes either contain inhibitory modifications or lack a required modification. Interestingly, the Orc1 BAH domain, which has 50% identity to the Sir3 BAH domain and can partially replace the Sir3 BAH domain in silencing function (Bell et al., 1995), bound strongly to nucleosomes (Figure S2A, lane 11) yet displayed distinct sensitivity to histone mutations (Figure S2B, lanes 17, 18, and 21–24).

The BAH Domain Is Required for Silencing

The importance of the BAH domain in silencing has been established in several previous studies (Connelly et al., 2006; Johnson et al., 1990), but the silencing phenotype of cells with Sir3 lacking the BAH domain has not been reported. Consistent with these studies and the biochemical data presented above, deletion of the BAH domain resulted in a complete loss of silencing of reporter genes at the telomere of chromosome VII and at the *HMR* locus (Figure 4A). Furthermore, Sir3 localization to silent chromatin domains, as tested by chromatin immunoprecipitation (ChIP), required both the BAH domain and the remaining C-terminal fragment of Sir3 (Figures 4B and 4C, lanes 6–8), indicating that both the BAH domain and the C-terminal region of Sir3 contributed to the specific recruitment of Sir3 to silent chromatin.

Formation of SIR-Nucleosome Filaments In Vitro

We have previously used EM to show that the association of a deacetylated histone H4 peptide together with the AAR product of NAD-dependent deacetylation induces a structural change in the SIR complex (Liou et al., 2005). Having defined conditions for the specific association of Sir3 with nucleosomes, we examined purified SIR-nucleosome complexes (Figures 5A and 5B) to determine if we could observe this structural change using nucleosomes, rather than histone peptides, as substrates. We observed the formation of extended filaments, which were 15–20 nm in diameter and >100 nm in length, in reactions that contained Sir2/Sir4, Sir3, nucleosomes, and NAD (Figures 4D–4I). Importantly, no filaments were observed in reactions that lacked NAD, nucleosomes, or any of the Sir proteins (Figure 5C, Table 1). Since binding of the Sir proteins to the same affinity-purified nucleosomes or to histone peptides does not require NAD (Figure 3, G.-G.L. and D.M., unpublished data), the role of NAD in filament formation is likely to involve AAR synthesis. In fact, replacement of NAD with AAR also resulted in the formation of filaments (Table 1). These filaments are too large to represent single SIR complexes and are longer than expected for a single nucleosome array, suggesting that the formation of long SIR-nucleosome

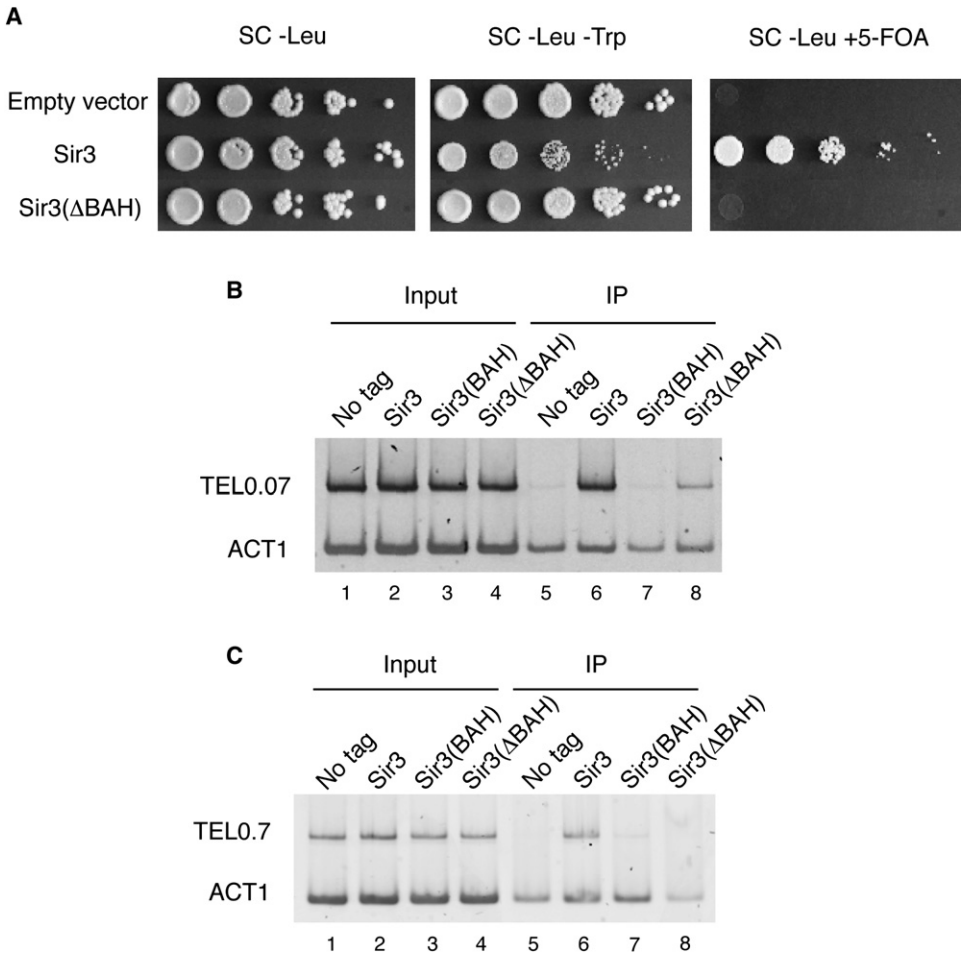


Figure 4. The BAH Domain Is Required for Silencing and Recruitment of Sir3 to Silent Chromatin

(A) Silencing was assayed by monitoring the growth of 10-fold serial dilutions of cells containing a *TRP1* reporter inserted at the *HMR* mating-type locus and a *URA3* reporter gene inserted near a telomere. Sir3(ΔBAH) cells display loss of silencing for both the HMR and telomeric reporters as indicated by increased growth on −Trp and decreased growth on +5FOA media, respectively.

(B and C) ChIP experiments showing that the BAH domain is required for the efficient recruitment of Sir3 to telomeric silent chromatin.

filaments may involve interactions between shorter nucleosome arrays. In this regard, the nucleosomes used in our studies were produced by affinity purification of micrococcal digested yeast nuclei, which produces chromatin fragments with a range of sizes. Initial experiments suggested a positive correlation between the length of the filaments and that of the purified nucleosome arrays. To further analyze this correlation, a sucrose gradient was used to separate mononucleosomes from larger, tetra(+) nucleosome arrays. As shown in Figure 5J, DNA extracted from these nucleosomes migrated at the expected size in agarose gels, and they appeared as mono- and oligonucleosomes on EM grids (Figures 5K and 5L). EM images of complete reactions containing mononucleosomes lacked long filaments (Figures 5M–5O), while those containing oligonucleosomes formed the long filaments (Figures 5P–5R). Thus, longer arrays of nucleosomes are necessary for the efficient formation of extended SIR-nucleosome filaments.

To determine whether the formation of the SIR-nucleosome filaments obeyed the in vivo rules for silent chromatin assembly, we tested the effect of mutations that disrupt nucleosome binding and silencing on filament formation. In agreement with the biochemical data demonstrating the importance of the histone H4 N-terminal tail in the binding of Sir3 to nucleosomes (Figures 3 and 4) (Hecht et al., 1995), filament formation was not observed in reactions using nucleosomes lacking the histone H4 N-terminal tail (Table 1) or a histone H4K16Q mutation (Table 1, Figures S3B and S3C). Remarkably, no filaments were observed using nucleosomes containing the histone H2A variant, Htz1 (Figures S3E and S3F, Table 1), which has antisilencing functions in vivo (Meneghini et al., 2003; Zhang et al., 2004), or using a catalytically inactive Sir2 protein (Tanny and Moazed, 2001) (data not shown). In addition, Sir3 purified from an *ard1Δ* strain (Connelly et al., 2006; Wang et al., 2004), lacking the N-terminal acetylation that is necessary for silencing and

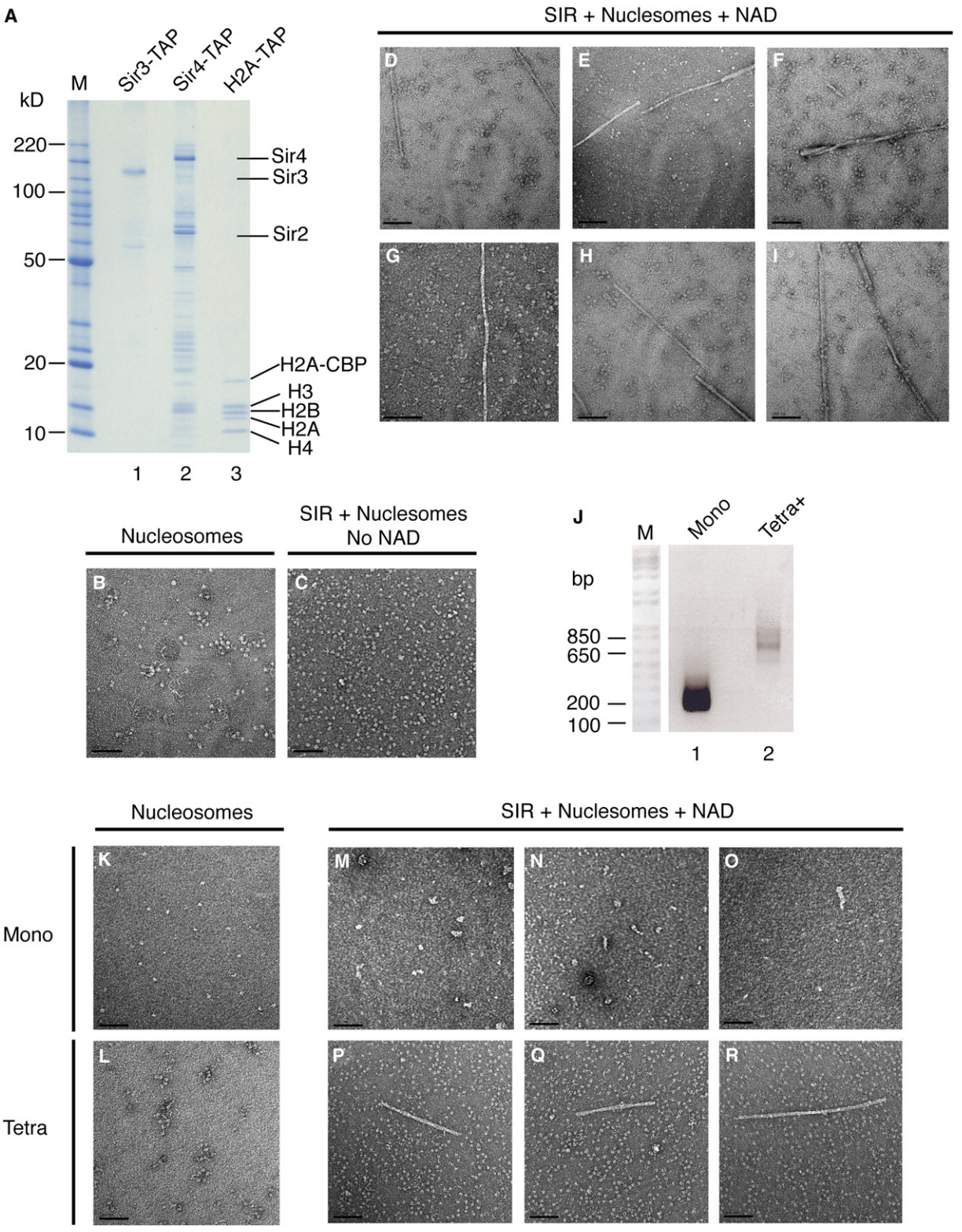


Figure 5. The SIR Complex and Native Yeast Oligonucleosomes Form NAD-Dependent Filaments

(A) Overexpressed Sir3-TAP, Sir4-TAP/Sir2-HA, and native yeast nucleosomes purified by H2A-TAP on a Coomassie-stained SDS-PAGE gel.

(B) EM image of purified nucleosomes.

(C) EM image of assembly reactions containing Sir3-TAP, Sir4-TAP/Sir2-HA, and nucleosomes in the absence of NAD.

Sir3-nucleosome interaction (Figure 1D), typically formed short filaments with a different morphology compared to those formed in the presence of wild-type Sir3 (Figures S3G and S3H, Table 1). Filament formation was also disrupted when wild-type Sir3 was replaced with Sir3 containing mutations in the BAH domain that disrupt silencing (J.R.B., unpublished data), suggesting that BAH-mediated association of Sir3 with nucleosomes is required for filament formation. In contrast, nucleosomes purified from a strain lacking the HMT enzyme, Dot1, still formed filaments to a level comparable to wild-type nucleosomes (Table 1), in agreement with the ability of Sir3 to bind to nucleosomes from *dot1Δ* cells (Figure 1F). Furthermore, filament formation displayed a stringent requirement for nucleosomes, as we observed no filaments when nucleosomes were replaced with DNA, core histones, or histone N-terminal peptides (Table 1), which can serve as a substrate for deacetylation by Sir2 and can also bind to Sir3. Together, these observations suggest that mutations in histones or the Sir proteins that disrupt silencing in vivo either prevent or greatly reduce the frequency of SIR-nucleosome filament formation in vitro. These parallel requirements for the formation of SIR-nucleosome filaments in vitro and silencing in vivo suggest that the filaments observed here represent the in vitro assembly of structures that resemble silent chromatin in vivo.

DISCUSSION

The results presented here show that the conserved BAH domain in Sir3 is the primary nucleosome-binding domain in the SIR complex that also monitors the posttranslational modification state of histones. Our results suggest that the BAH domain binds to both the histone H4 N terminus and the globular domain of histone H3 that lies on the surface of the nucleosome. Furthermore, nucleosome binding in the context of the SIR complex can lead to the formation of extended SIR-nucleosome filaments in vitro. Below, we discuss the implications of these findings for the mechanism of gene silencing.

The Sir3 BAH Domain Is a Conserved Histone- and Nucleosome-Binding Domain

The association of the Sir3 BAH domain with nucleosomes appears to be regulated by distinct histone regions in the nucleosome. The first is composed of the silencing domain of histone H4, which contains amino acids 16–29 (Johnson et al., 1992). Our binding studies (Figures 2A and 2B) indicate that the association of the BAH domain with the nucleosome is tightly controlled by the acetylation state of lysine 16 within this domain. The second domain includes lysine 79 of histone H3 and the residues that surround it (Park et al., 2002). These regions of histones H3 and H4 are located on the same surface of the nucleosome and are close enough to each other that they can simultaneously contact a single BAH domain (Luger et al., 1997; Schalch et al., 2005). We propose that the surface of the nucleosome containing these regions forms a composite binding site for the Sir3 BAH domain (Figure 6B). Within this domain, both H4K16 and H3K79 must be unmodified for efficient binding of the nucleosome to the Sir3 BAH domain, although some binding can still occur when H3K79 is mutated to alanine. These results are consistent with the observation that, during re-establishment of silent chromatin, histone deacetylation and Sir protein binding can occur prior to removal of inhibitory methyl marks (Katan-Khaykovich and Struhl, 2005).

In addition to the BAH domain, Sir3 has a region at its C terminus that has been shown previously to associate with histone H3 and H4 N-terminal peptides (Carmen et al., 2002; Hecht et al., 1995). Although this C-terminal region has a preference for deacetylated histone tails, it does not appear to have the same selectivity for the acetylation state of H4K16 as full-length Sir3 or the BAH domain (Figures 2 and 3), and its possible association with the globular domain of histone H3 has not yet been directly examined. Our results, together with previous observations, suggest that the formation of silent chromatin depends on multiple interaction surfaces in both the nucleosome and the SIR complex. These surfaces include the N termini of histones H3 and H4, the globular domain of histone H3, the BAH and C-terminal domains of Sir3, and an uncharacterized domain in Sir4 (Figures 2–4) (Hecht et al., 1995). Mutations that perturb any of these association domains result in complete or partial loss of silencing, suggesting that cooperative interactions involving each domain may be crucial for binding of the SIR complex to nucleosomes and the formation of silent chromatin domains.

The observation that the BAH domain of Sir3 is a nucleosome and histone H4 N-terminal tail-binding domain suggests that the SIR complex associates with chromatin via an evolutionarily conserved mechanism. The BAH domain is present in chromatin-associated proteins from yeast to human. In particular, the Orc1 subunit of the origin recognition complex, the Rsc1 and Rsc2 subunits of the RSC chromatin remodeling complex, the mammalian DNMT1 DNA methyltransferase, and the Mta1 subunit of the mammalian NURD histone deacetylase complex all contain BAH domains (Nicolas and Goodwin, 1996) and may use this domain to associate with specifically

(D–I) EM images of assembly reactions containing Sir3-TAP, Sir4-TAP/Sir2-HA, and nucleosomes in the presence of NAD.
(J) Agarose gel stained with ethidium bromide of DNA extracted from native yeast nucleosomes separated by sucrose gradient into mononucleosome (mono) and longer nucleosome arrays (tetra+).
(K and L) EM images of purified mono- and oligonucleosomes, respectively, in the absence of Sir proteins.
(M–O) EM images of reactions containing mononucleosomes, Sir3, Sir4/Sir2, and NAD.
(P–R) EM images of reactions containing oligonucleosomes, Sir3, Sir4/Sir2, and NAD. Scale bar, 100 nm.

Table 1. Summary of SIR-Nucleosome Filaments Observed by EM

Reaction	Filaments Observed[a]	Number of Experiments
Sir2/4 + Sir3 + Nucl + NAD	Yes	>20
Sir2/4 + Sir3 + Nucl + AAR	Yes	2
Sir3 + Nucl + NAD	No	2
Sir2/4 + Nucl + NAD	No	2
Sir2/4 + Sir3 + NAD	No	2
Sir2/4 + Sir3 + Nucl	No	2
Nucl + NAD	No	2
Sir3 + NAD	No	2
Sir3 + Nucl	No	5
Sir2/4 + Nucl	No	2
Sir2/4 + Sir3 + Nucl(Htz1) + NAD	No[b]	4
Sir2/4 + Sir3 + Nucl(H4K16Q) + NAD	No	4
Sir2/4 + Sir3 + Nucl(H4ΔNterm) + NAD	No	2
Sir2/4 + Sir3 + Nucl(*dot1Δ*) + NAD	Yes	4
Sir2/4 + Sir3 (*ard1*Δ) + Nucl + NAD	No[b]	4
Sir2/4 + Sir3 + Histones + NAD	No	2
Sir2/4 + Sir3	No	2
Sir2/4 + NAD	No	2
Sir2/4 + Sir3 + DNA from Nucl	No	2
Sir2/4 + Sir3 + DNA from Nucl + NAD	No	2
Sir2/4 + Sir3 + H4Tet + NAD	No	5
Sir2/4 + Sir3 + H4Nt + NAD	No	5
Sir2/4 + Sir3 + H4Nt + AAR	No	2
Sir2/4 + Sir3 + H3Nt + AAR	No	2
Sir2/4 + Sir3 + DNA from Nucl + H4Tet + NAD	No	2
Sir2/4 + Sir3 + DNA from Nucl + H4Tet + AAR	No	2
Sir2/4 + Sir3 + DNA from Nucl + H3Tet + NAD	No	2

Abbreviations include the following: Nucl, affinity-purified native yeast nucleosomes; Nucl(Htz1), affinity-purified native yeast nucleosomes containing histone H2A variant Htz1; Nucl(H4K16Q), affinity-purified native yeast nucleosomes containing histone H4K16Q; Nucl(H4ΔNterm), affinity-purified native yeast nucleosomes containing histone H4 with a deletion of amino acids 4–19; Nucl(*dot1Δ*), affinity-purified native yeast nucleosomes from a *dot1Δ* strain; Sir3(*ard1*Δ), Sir3 purified from an *ard1Δ* strain; histones, bacterially produced recombinant core histones; H3Tet and H4Tet, tetracetylated histone H3 and H4 N-terminal 20 amino acid peptides, respectively; and H3Nt and H4Nt, unacetylated H3 and H4 N-terminal peptides, respectively.

[a] Column indicates whether or not SIR-nucleosome filaments were observed during inspection of EM images. When all three Sir proteins, oligonucleosomes, and NAD were present, we observed up to five filaments per 90 μm × 90 μm EM grid square (~300 grid squares in a 200 mesh grid) in more than 20 assembly experiments.

[b] We observed only short or very short atypical filaments using Sir3 purified from *ard1*Δ cells (Figure S2), suggesting that acteylation of the BAH-containing N terminus of Sir3 is important for both nucleosome binding (Figure 1) and filament formation. In addition, we observed no filaments when we replaced full-length Sir3 purified from yeast with a recombinant Sir3 fragment produced in *E. coli*, which lacks the BAH domain (Sir3, 464–978).

modified chromatin domains. In this regard, the BAH domain may perform a function that is analogous to that of the chromodomains of HP1 and Polycomb proteins, which are involved in the recognition of H3K9 and K27 methylation marks that mediate silent chromatin assembly in fission yeast and metazoans (Grewal and Moazed, 2003; Jenuwein, 2001; Richards and Elgin, 2002). In contrast to the chromodomain, however, the BAH domain, at least in Sir3, prefers unmodified lysines, and its binding site includes residues from both histones H3 and H4.

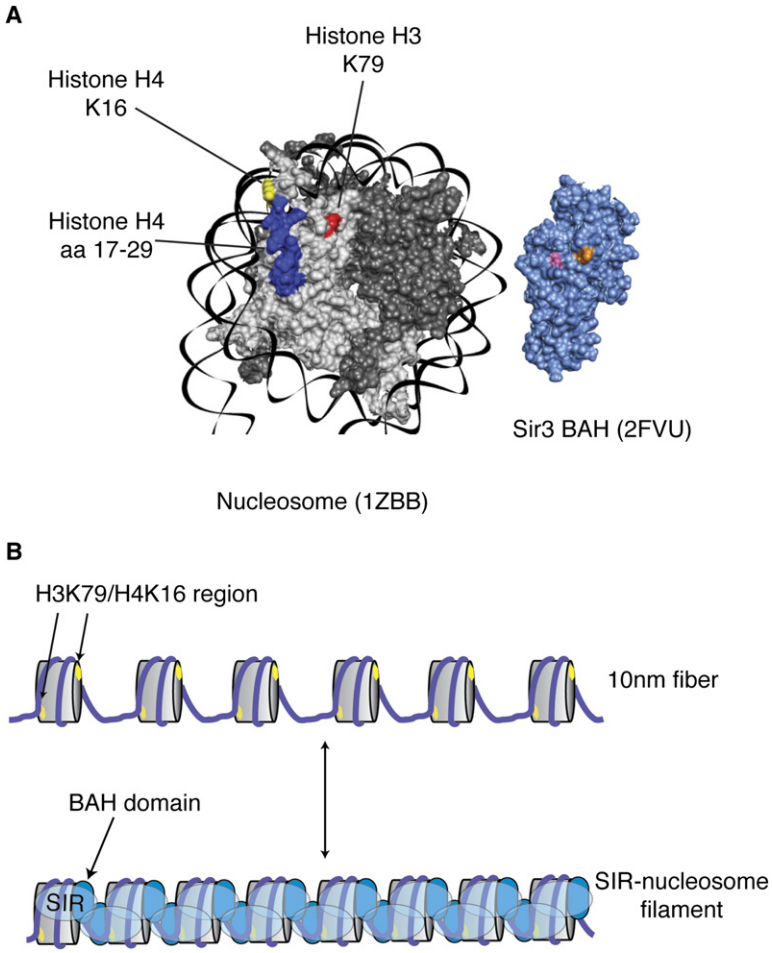


Figure 6. Model for Association of the SIR Complex with Chromatin

(A) Images of the structure of a nucleosome from the *Xenopus laevis* tetranucleosome structure (1ZBB) (Schalch et al., 2005) and the Sir3 BAH domain (2FVU) (Connelly et al., 2006) were visualized by UCSF Chimera. Histones H3 and H4 are colored in light gray, and histones H2A and H2B are colored in dark gray. Within the structure, histone H4 amino acid 16 is colored in yellow, histone H4 amino acids 17–29 are colored in blue, and histone H3K79 is colored in red. The Sir3 BAH domain, in light blue, has residue W86 colored in orange and D205 colored in pink.

(B) Spreading of Sir3 along the chromatin fiber requires interactions with both a deacetylated histone H4K16 in the N terminus of histone H4 and unmethylated H3K79 in the globular domain of H3 located on the surface of the nucleosome (highlighted in [A] and represented in yellow in the diagram). Spreading of the ~10 nm SIR complex (Liou et al., 2005) along 10 nm nucleosome arrays generates ~20 nm filaments.

SIR-Nucleosome Filaments

The association of DNA with histone octamers generates nucleosome arrays that represent the "beads-on-a-string" 10 nm fiber, which can further fold into a more compact 30 nm fiber (Tremethick, 2007). The latter is thought to represent the most prevalent state of chromatin in the cell and is speculated to be unfolded during the transition to a more open form of chromatin that accompanies gene activation. In contrast, gene silencing is usually believed to be associated with further chromatin compaction. Our results, however, suggest that the association of the SIR complex with nucleosomes may occur without chromatin compaction. First, the association of Sir3 with nucleosomes is sensitive to the modification status of H4K16 and H3K79. Both of these nucleosome regions have been implicated in chromatin folding. Acetylation of H4K16 interferes with compaction of nucleosome arrays (Shogren-Knaak et al., 2006), and recent structures of tetra- and oligonucleosome arrays show that the H3K79 region is inaccessible in the 30 nm fiber (Dorigo et al., 2004; Schalch et al., 2005). Thus, condensation into a 30 nm fiber or more compact states may be incompatible with the association of Sir3 with the globular domains of histone H3 in the region surrounding K79 on the exposed surface of the nucleosomes. Second, consistent with the biochemical requirements for the association of Sir3 with nucleosomes, discussed above, the SIR-nucleosome filaments observed in our experiments have a diameter of ~20 nm, which suggests that the SIR complex binds to and spreads along an extended 10 nm chromatin fiber, binding to the flat surface of the nucleosome disk (Figure 6). We believe that these filaments represent a true step in the formation of silent chromatin because their formation requires a similar set of components as silencing in vivo, and the filaments are not observed in the presence of mutations that disrupt silencing. However, the SIR-nucleosome filaments observed in our studies are assembled using a heterogeneous population of nucleosomes that lack silencers, the regions of DNA that initiate silent chromatin assembly in vivo (Brand et al., 1985), and are required for its continued maintenance (Bi and Broach, 1997; Cheng and Gartenberg, 2000). It remains possible that silencer-binding proteins mediate changes in silent chromatin structure that are not observed using the purified components in our experiments. Additional studies are required to fully establish the relationship between SIR-nucleosome filaments and silent chromatin. In particular, the reconstitution of these filaments using chromatin

templates containing defined DNA sequences and histone modifications should allow a dissection of the structure of silent chromatin and the individual reactions that mediate its formation.

EXPERIMENTAL PROCEDURES

Yeast Strains

The yeast strains are listed in Table S1. Epitope-tagged strains were constructed by a PCR-based gene-targeting method (Huang et al., 2006; Longtine et al., 1998; Rigaut et al., 1999; Rudner et al., 2005). Primer sequences are available upon request. Histone mutations were introduced by plasmid shuffle using strains and plasmids as described (Zhang et al., 1998). The Orc1-TAP and Dnmt1-TAP strains were constructed by PCR amplifying the BAH domain from yeast genomic DNA or a HeLa two-hybrid library (kindly provided by Adrian Salic), ligating it with T4 DNA ligase (NEB) to pKG1810 (TAP-Kan). The resulting constructs were PCR amplified and used to modify the Sir3 open reading frame.

Immunoprecipitation

Immunoprecipitations were based on a method described previously (Rudner et al., 2005). Briefly, 0.3–0.8 g of frozen cell pellets were lysed by bead beating (Biospec) in an equal volume of lysis buffer (50 mM HEPES-KOH [pH 7.6]; 10 mM magnesium acetate; 5 mM EGTA; 0.1 mM EDTA; 150 mM potassium chloride; 0.2% NP-40; 5% glycerol; 2 mM phenylmethylsulfonylfluoride; 1 mg/ml of leupeptin, bestatin, and pepstatin; and 1 mM benzamidine). The lysate was bound to 5–10 × 10^6 M-270 Dynabeads (Invitrogen/Dynal) coupled to IgG (Sigma) for 90–120 min. The beads were washed three times with 1 ml of wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% NP-40). The beads were resuspended in SDS-sample buffer. A fraction of the lysate (0.375%–0.5%) and all of the beads were run on 10%–20% polyacrylamide gels. Simply Blue stain (Invitrogen) was used to stain the gels. For western blotting, the gel was transferred onto Immobilion PSQ PVDF membrane (Millipore). Peroxidase anti-peroxidase (Sigma) was used for the TAP epitope (1:10,000 in 5% nonfat dried milk, 20 mM Tris-HCl [pH 7.5], 0.1% Tween-20), while anti-H3 antibody (Abcam) was used to detect histone H3 (1:5000 in 3% bovine serum albumin, 20 mM Tris-HCl [pH 7.5], 0.1% Tween-20).

Silencing Assay

Strain DMY3315 (*TELVII-L*::*URA3 hmrΔE*::*TRP1 sir3*Δ::*KAN*R) was constructed by crossing ADR2829 and ADR3246 (Rudner et al., 2005), and deleting *SIR3* in the resulting strain by transformation with a PCR product that contained *SIR3*-flanking regions on either side of the KanMX6 cassette. DMY3315 was transformed with plasmids pRS315 (empty vector), pDM832 (Sir3-3xFLAG), or pDM988 (Sir3ΔBAH-3xFLAG). For silencing assays, strains were grown to an OD_{600} of 1.0; concentrated 5-fold; plated as 10-fold serial dilutions on SC-LEU, SC-LEU-TRP, or SC-LEU+5-FOA; and incubated at 30°C for 3 days.

Chromatin Immunoprecipitation

ChIPs were performed as described previously (Huang et al., 2006; Rudner et al., 2005). One microliter of a 1:10 dilution of IP or a 1:200 dilution of input DNA was used in a 25 μl PCR reaction using primers described previously (Huang et al., 2006; Rudner et al., 2005) to amplify TEL0.07kb (OAR149 and OAR150), TEL0.7 kb (DM241 and DM242), and *ACT1* (JH301 and JH302). Five microliters of the PCR product was run on a 6% acrylamide gel in 1 × TAE at 100V. The gel was incubated with ethidium bromide and visualized on Fujifilm LAS-3000.

Protein Purification

Sir3-TAP, Sir4-TAP/Sir2-HA, Sir3-Flag, and Sir3(BAH)-Flag were purified as described previously (Huang et al., 2006; Liou et al., 2005). Native yeast nucleosomes were purified by spheroplasting HTA2-TAP cells, lysing the nuclei by Dounce homogenization, treating the nuclei with micrococcal nuclease (200 U/ml, Worthington), and binding to IgG-Sepharose for a standard TAP purification. A 5%–20% sucrose gradient on a Beckman ultracentrifuge (SW28, 25000 rpm, 24 hr) was used to separate mononucleosomes from oligonucleosomes (Mizzen et al., 1999). DNA was extracted by Proteinase K treatment and phenol-chloroform extraction.

Filament Analysis by Electron Microscopy

Purified Sir3-Flag or Sir3-CBP (~0.8 μg) and Sir4-CBP/Sir2-HA (~0.25–0.5 μg) were incubated with purified nucleosomes (~0.2 nM) with or without 10 mM NAD, 50 mM HEPES-KCl (pH 7.6), 300 mM KCl, and 4 mM $MgCl_2$ in a 10 μl reaction volume. These mixtures were incubated at room temperature for 2–4 hr and overnight with rocking at 4°C. EM was performed on these samples as described (Liou et al., 2005).

BIAcore Surface Plasmon Resonance Analysis

Real-time protein-protein interactions were analyzed as described (Liou et al., 2005).

Structural Analysis

Images of the *X. laevis* tetranucleosome crystal structure (1ZBB) (Schalch et al., 2005) and the Sir3 BAH domain (2FVU)(Connelly et al., 2006) structures were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen et al., 2004).

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/28/6/1015/DC1/.

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