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A dual role of H4K16 acetylation in the establishment of yeast silent chromatin

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Discrete regions of the eukaryotic genome assume heritable chromatin structure that is refractory to transcription. In budding yeast, silent chromatin is characterized by the binding of the Silent Information Regulatory (Sir) proteins to unmodified nucleosomes. Using an *in vitro* reconstitution assay, which allows us to load Sir proteins onto arrays of regularly spaced nucleosomes, we have examined the impact of specific histone modifications on Sir protein binding and linker DNA accessibility. Two typical marks for active chromatin, H3K79^{me} and H4K16^{ac} decrease the affinity of Sir3 for chromatin, yet only H4K16^{ac} affects chromatin structure, as measured by nuclease accessibility. Surprisingly, we found that the Sir2-4 subcomplex, unlike Sir3, has higher affinity for chromatin carrying H4K16^{ac}. NAD-dependent deacetylation of H4K16^{ac} promotes binding of the SIR holocomplex but not of the Sir2-4 heterodimer. This function of H4K16^{ac} cannot be substituted by H3K56^{ac}. We conclude that acetylated H4K16 has a dual role in silencing: it recruits Sir2-4 and repels Sir3. Moreover, the deacetylation of H4K16^{ac} by Sir2 actively promotes the high-affinity binding of the SIR holocomplex.

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Introduction

Heterochromatin is a heritable, specialized chromatin structure that silences discrete regions in eukaryotic genomes. Among other features, gene silencing within heterochromatic regions is thought to involve compaction of the chromatin fibre in order to structurally limit DNA accessibility. In budding yeast, silent chromatin requires the binding of Silent Information Regulatory (Sir) proteins to unmodified nucleosomes. Work of many laboratories has identified Sir2, Sir3 and Sir4 proteins as the core components of silent chromatin at telomeres and silent mating type loci (Rine and Herskowitz, 1987; reviewed in Rusche *et al* (2003)). Two-hybrid and protein binding studies suggested that they form a complex, with Sir4 being a scaffold protein that bridges between Sir2 and Sir3 (Moazed *et al*, 1997; Strahl-Bolsinger *et al*, 1997; Rudner *et al*, 2005; Cubizolles *et al*, 2006). Although initial attempts to purify the Sir proteins from yeast yielded only an Sir2-4 heterodimer (Ghidelli *et al*, 2001; Hoppe *et al*, 2002), a stable Sir2-Sir3-Sir4 heterotrimer with 1:1:1 stoichiometry (hereafter SIR complex) was purified from insect cells (Cubizolles *et al*, 2006). A fourth Sir protein, Sir1, is important for the establishment of silencing at the silent mating type loci, but is not required for repression at telomeres (Pillus and Rine, 1989; Aparicio *et al*, 1991).

Sir proteins do not bind DNA in a sequence-specific manner, yet zones of silencing are restricted to specific domains in the yeast genome. To achieve targeted silencing, Sir proteins are recruited by bifunctional DNA binding factors, such as Rap1, Abf1 and Orc1, which bind yeast silencer elements. The SIR complex then spreads from this nucleation site for 3–20 kb, depending on the abundance and balance of available Sir proteins (reviewed in Gasser and Cockell (2001) and Rusche *et al* (2003)). SIR complex association decreases the ability of enzymes, like DNA methylases or endonucleases, to access the DNA (Gottschling, 1992; Loo and Rine, 1994). Transcription in these regions is repressed, most likely by reducing RNA polymerase II occupancy at promoters (Chen and Widom, 2005; Lynch and Rusche, 2009), although other studies suggest that Sir protein binding interferes with RNA polymerase II elongation (Sekinger and Gross, 2001; Gao and Gross, 2008).

All three Sir proteins, Sir2, Sir3 and Sir4, are essential for transcriptional repression. Sir3 and Sir4 are primarily thought to be structural proteins of silent chromatin (Gasser and Cockell, 2001). *SIR3* arose from a duplication of the *ORC1* gene, with which it shares an N-terminal BAH domain and a related AAA+ ATPase domain (Hickman and Rusche, 2010). Sir4 is found only in related ascomycetes species, while Sir2 is a NAD-dependent histone deacetylase conserved from bacteria to man. Its enzymatic activity is required for gene silencing (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000).

The key substrate of Sir2 is histone H4 acetylated on lysine 16 (H4K16^{ac}; Imai *et al*, 2000; Smith *et al*, 2000; Borra *et al*, 2004). This mark is found on transcriptionally active chromatin in most species and marks early firing origins in yeast and flies (Kimura *et al*, 2002; Suka *et al*, 2002; Schwaiger *et al*, 2010). It has been shown that unmodified H4K16 promotes compaction of the chromatin fibre *in vitro* and *in vivo* (Smith *et al*, 2003; Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008). Consistently, in budding yeast H4K16^{ac} is found throughout the genome except at silent loci (Suka *et al*, 2001; Smith *et al*, 2003).

Recombinant fragments of Sir3 and Sir4 were shown to bind to the histone H4 tail *in vitro*, in a manner sensitive to

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mutations near K16 and to lysine acetylation (Hecht *et al*, 1995; Carmen *et al*, 2002). Using recombinant proteins and nucleosomal substrates, it was found that the H4K16A mutation can decrease binding of Sir3 *in vitro* (Johnson *et al*, 2009), while mutations of H4K16 to glycine or glutamate, and to a lesser extent arginine, diminished mating efficiency *in vivo* (Johnson *et al*, 1990; Megee *et al*, 1990; Park and Szostak, 1990). Finally it was shown that the H4K16G phenotype could be suppressed by a compensatory mutation in Sir3, suggesting that Sir3 contacts the H4 tail in an acetylation-sensitive manner (Johnson *et al*, 1990).

In yeast, H4K16 is acetylated primarily by the histone acetyltransferase (HAT) Sas2 (Kimura *et al*, 2002; Suka *et al*, 2002) and secondarily by the essential HAT Esa1, which also targets H4K5 and H4K12 (Suka *et al*, 2001, 2002; Chang and Pillus, 2009). Similar to conservative mutations in H4K16, the deletion of *SAS2* impairs repression of a reporter gene at telomeres or the *HML* locus, although the same mutation favours silencing of a reporter at *HMR*, which has much stronger silencer elements (Reifsnyder *et al*, 1996; Ehrenhofer-Murray *et al*, 1997; Meijsing and Ehrenhofer-Murray, 2001). However, the rate of Sir3 recruitment to *HMR* was slower in cells that lack Sas2 (Katan-Khaykovich and Struhl, 2005), as was the establishment of silencing at *HML* (Osborne *et al*, 2009). This, together with the observation that the catalytic activity of Sir2 is required for silencing (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000; Yang and Kirchmaier, 2006; Yang *et al*, 2008a), suggests that Sir-mediated deacetylation of H4K16^{ac} might have an active role in the formation of silent chromatin.

The H4K16^{ac} mark is also required for efficient methylation of lysine 79 on the histone H3 (H3K79^{me}) by the methyltransferase Dot1 (Altaf *et al*, 2007). *In vivo* H3K79^{me} appears to impair the spreading of the Sir proteins, and is thought to act by reducing association of Sir3 to chromatin (Ng *et al*, 2002, 2003; van Leeuwen *et al*, 2002; Altaf *et al*, 2007; Onishi *et al*, 2007). Consistently, a recent study with recombinant proteins has suggested that both Sir3 and the SIR holocomplex have lower affinities for reconstituted chromatin bearing H3K79^{me} (Martino *et al*, 2009). This shows that, in addition to the histone tails, the Sir3 protein also interacts with the nucleosomal core, a property that has been assigned both to the N-terminal BAH domain (Onishi *et al*, 2007; Buchberger *et al*, 2008; Norris *et al*, 2008; Sampath *et al*, 2009) as well as the Sir3 C-terminal region (Altaf *et al*, 2007).

A further histone modification that interferes with SIR-mediated repression is the acetylation of K56 on histone H3. In budding yeast, H3K56^{ac} is deposited by Rtt109 during S phase before the loading of the histones onto DNA, and therefore serves as a marker for newly assembled nucleosomes (Hyland *et al*, 2005; Masumoto *et al*, 2005; Han *et al*, 2007; Li *et al*, 2008). A large number of studies have also linked H3K56^{ac} to gene transcription from yeast to man (Xu *et al*, 2005, 2007; Schneider *et al*, 2006; Williams *et al*, 2008; Yang *et al*, 2008b; Michishita *et al*, 2009; Xie *et al*, 2009). In yeast, amino-acid substitutions at H3K56 severely disrupt silencing without completely displacing the SIR complex (Xu *et al*, 2007; Yang *et al*, 2008b). Moreover, elimination of the histone deacetylases responsible for removal of H3K56^{ac}, Hst3 and Hst4, disrupts SIR-mediated repression as well (Yang *et al*, 2008b). A recent report has shown that acetylation of H3K56 favours transcriptional elongation through yeast heterochromatin, generating speculation that H3K56^{ac} promotes the displacement of the Sir proteins (Varv *et al*, 2010). However, there is as yet no direct evidence that the affinity of Sir proteins for nucleosomes is lowered by H3K56^{ac}.

To gain insight into the role played by these histone modifications in the assembly of silent chromatin, we reconstituted SIR-bound chromatin *in vitro* using nucleosomes homogeneously modified on only one residue. Our system recapitulates many of the characteristics of silent chromatin (Martino *et al*, 2009) and allows us to probe both Sir protein binding and accessibility of the linker DNA to micrococcal nuclease (MNase). We find that both H3K79^{me} and H4K16^{ac} decrease the affinity of Sir3 for chromatin, while only H4K16^{ac} has an effect on MNase accessibility. Surprisingly, we found that Sir2-4 prefers to bind to chromatin acetylated on H4K16. The binding of Sir2-4, in presence of NAD and Sir3, leads to the removal of the H4K16^{ac} mark and couples stable binding of the Sir2-3-4 complex with a significant decrease in linker DNA accessibility. On the other hand, H3K56^{ac} slightly increases MNase accessibility and reduces the interaction of chromatin with the SIR complex. Importantly, we find that H3K56^{ac} is not a substrate for Sir2-mediated deacetylation. We, thus, show how the anti-silencing properties of different histone modifications differentially affect silent chromatin. Of particular interest are the two contradictory roles played by H4K16^{ac}, which reduces the binding of Sir3 and favours the recruitment of Sir2-4. The acetylation and deacetylation of H4K16 thus appear to orchestrate the sequential binding of Sir proteins in order to establish a stable silent chromatin.

Results

The H4K16^{ac} mark differentially affects the binding of Sir2-4 and Sir3 to chromatin

It is generally accepted that the H4K16^{ac} mark plays an important role in silent chromatin by preventing the ectopic spread of the Sir proteins from the non-acetylated silent domains (Kimura *et al*, 2002; Suka *et al*, 2002; Millar *et al*, 2004; Yang *et al*, 2008a). However, accumulating evidence suggests that not only the absence of the acetyl mark but its Sir2-dependent removal may be required for efficient establishment of silencing (Liou *et al*, 2005; Yang and Kirchmaier, 2006; Yang *et al*, 2008a; Martino *et al*, 2009; Osborne *et al*, 2009). To shed light on this matter, we analysed in detail the binding of SIR subcomplexes to nucleosomal arrays bearing a fully acetylated H4K16.

Nucleosomes were reconstituted with recombinant histones that were either unmodified or fully acetylated on H4K16. These were generated by expressing a truncated version of H4 and adding the N-terminal tail by native chemical ligation (NCL) using a synthetic peptide (Shogren-Knaak *et al*, 2006; Supplementary Figure S1). Nucleosomal arrays were then reconstituted by salt dialysis using a DNA template containing repeated arrays of a 167-bp histone octamer positioning sequence (Widom 601) as described previously (Huynh *et al*, 2005; Martino *et al*, 2009). Recombinant Sir proteins were purified from insect cells (Figure 2F; Cubizolles *et al*, 2006; Martino *et al*, 2009).

We first compared acetylated and non-acetylated arrays of a 6mer of nucleosomes by monitoring the accessibility of linker DNA to MNase in the absence of Sir proteins. Previous studies have shown that acetylation of H4K16 inhibits chromatin compaction both *in vitro* and *in vivo* (Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008). By challenging this chromatin with increasing amounts of MNase, we found that H4K16^{ac} enhances linker DNA accessibility of a chromatin template as short as six nucleosomes (Figure 1A).

Sir3 has been reported to be more susceptible than Sir4 to modifications on histone tails (Carmen *et al*, 2002; Johnson *et al*, 2009). Therefore, we examined first the effect of H4K16^{ac} on the binding of Sir3 to nucleosomal arrays. Increasing amounts of Sir3 were titrated onto unmodified or H4K16^{ac} arrays of nucleosomes. The binding was analysed by gel shift and quantified by scoring the loss of the unbound 6mer. In agreement with previous studies (Carmen *et al*, 2002; Johnson *et al*, 2009), we found that H4K16^{ac} reduces the binding affinity of Sir3 to an *in vitro* reconstituted nucleosomal array by roughly two-fold (Figure 1B). In contrast, the binding affinity of the Sir2-4 heterodimer to chromatin was increased nearly two-fold by the presence of the H4K16^{ac} mark (Figure 1C). Superficially, this appears to contradict the fact that silent chromatin is depleted for this mark, although it is consistent with the notion that H4K16^{ac} is a key substrate of Sir2-4 (see also Johnson *et al* (2009)). Therefore, we decided to perform competition experiments in order to reinforce this observation. The binding of increasing amounts of Sir2-4 to an unmodified Cy5-labelled array was competed with a four-fold excess of either unlabelled unmodified or unlabelled H4K16^{ac} chromatin. Confirming our previous results, H4K16^{ac} chromatin competed roughly two-fold more efficiently for the binding of Sir2-4 compared with unmodified chromatin (Figure 1D).

We previously showed that our recombinant Sir2-4 has efficient histone deacetylase activity in the presence of its co-factor NAD (Cubizolles *et al*, 2006). We reasoned that if Sir2-4 bound H4K16^{ac} with higher affinity because it is a preferred substrate of Sir2, then the complex should have less affinity once H4K16^{ac} had been deacetylated. To test this, we quantified the binding of the Sir2-4 heterodimer to H4K16^{ac} chromatin in the absence or presence of NAD. Confirming our hypothesis, Sir2-4 bound more readily to acetylated chromatin and less readily following deacetylation (Figure 1E). Surprisingly, this shows an enhanced affinity of Sir2-4 for acetylated H4K16, while the opposite is true for Sir3.

Sir2-dependent deacetylation of H4K16^{ac} stabilizes the association of Sir2-3-4 to chromatin

Removal of H4K16^{ac} through the catalytic activity of Sir2 has been reported to be important for silencing (Johnson *et al*, 1990; Suka *et al*, 2001, 2002; Carmen *et al*, 2002; Kimura *et al*, 2002). However, it is not clear whether this is due exclusively

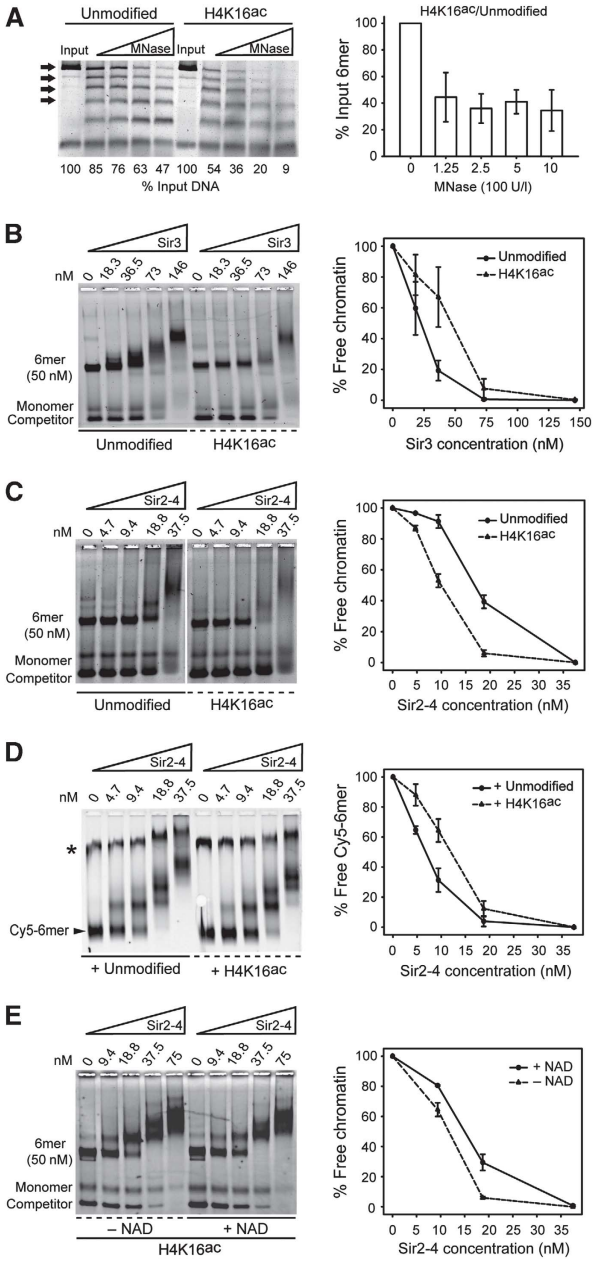

Figure 1 Acetylation of H4K16 decreases the binding affinity of Sir3 but favours the loading of Sir2-4 onto chromatin. (**A**) Equally saturated 6mer of 601 nucleosomes with either unmodified or acetylated H4K16 was digested with increasing amount of MNase, as indicated. After protein digestion, the denatured DNA was separated by electrophoresis and visualized by SYBR® Safe staining. The bands showed by an arrow (6-, 5-, 4- and 3mers) were quantified and normalized to input. The histograms show the ratio between the amount of intact 6-3mers of H4K16^{ac} over unmodified chromatin for the indicated MNase titration point. The Sir3 protein (**B**) or the Sir2-4 heterodimer (**C**) were titrated into a constant amount of unmodified or H4K16^{ac} 6mer of 601 nucleosomes. Samples were separated by native agarose gel electrophoresis and visualized by SYBR® Safe staining. (**D**) The binding of increasing amounts of Sir2-4 to 8 nM of unmodified Cy5-labelled 6mer of nucleosomes (indicated by the arrowhead) was competed with 32 nM of either unlabelled unmodified or unlabelled H4K16^{ac} 6mer of nucleosomes. Cy5 fluorescence was used to monitor the binding of Sir2-4 to the unmodified labelled chromatin. The asterisk indicates a Cy5-labelled contaminant DNA. (**E**) The Sir2-4 heterodimer was titrated into a constant amount of H4K16^{ac} 6mer of 601 nucleosomes. Deacetylation is allowed by the addition of 150 μM NAD where indicated. Samples were separated and visualized as in (**B**, **C**). The images are representative of at least three independent experiments, quantifications show the mean value ± s.e.m. of the % of unbound chromatin compared with the input.

to the generation of unmodified H4K16 or whether it additionally involves a conformational change coupled to *O*-acetyl-ADP-ribose (*O*-AADPR) production (Liou *et al*, 2005; Martino *et al*, 2009). To gain insight into the molecular consequences of H4K16^{ac} deacetylation on the establishment of silencing, we compared the binding of the SIR complex with unmodified or H4K16^{ac} chromatin in the presence or absence of NAD. We first examined the effect of H4K16^{ac} on the binding of the Sir2-3-4 heterotrimer in absence of NAD (Figure 2A) and found that, similar to Sir2-4 (Figure 1C) but in a less pronounced manner, the SIR holocomplex bound slightly better to acetylated chromatin. We then confirmed that our purified Sir2-3-4 complex was able to efficiently deacetylate H4K16^{ac} within chromatin in the presence of NAD (Figure 2E), as shown previously for chemically acetylated histone octamers (Cubizolles *et al*, 2006). In the following experiments, the term 'deacetylated chromatin' will be used whenever H4K16^{ac} marks were actively removed by Sir2 in the presence of NAD, to distinguish it from chromatin assembled from unmodified histones.

We next compared the binding affinity of Sir2-3-4 with unmodified or H4K16^{ac} chromatin in the presence of NAD. We found that the active removal of the H4K16^{ac} mark increased the binding affinity of the SIR complex to chromatin by roughly two-fold (Figure 2B). This effect is not caused by NAD alone as enhanced binding was not observed when H4K16^{ac} chromatin was replaced with unmodified chromatin (Supplementary Figure S2A). Indeed, in absence of Sir2,

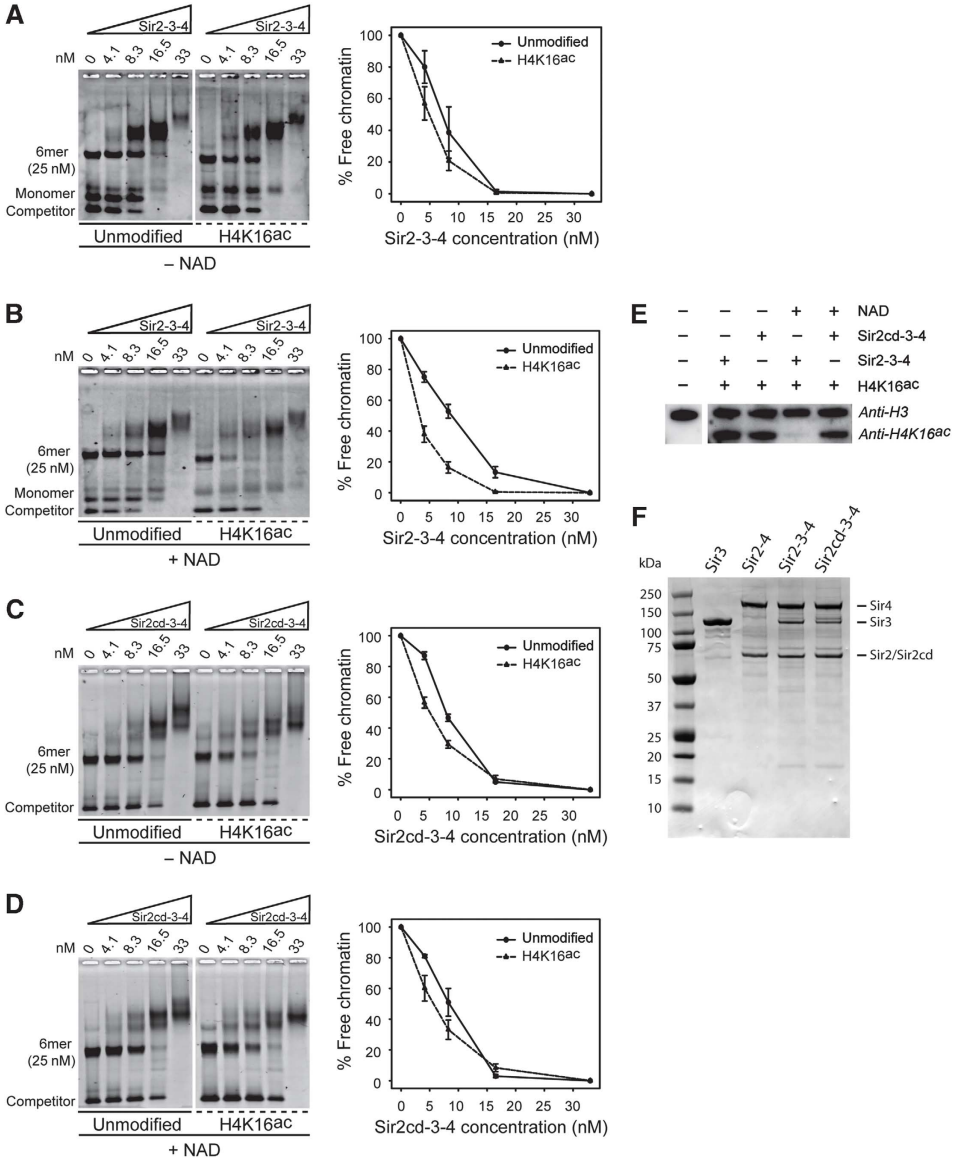

Figure 2 Sir2-dependent deacetylation of H4K16^{ac} stabilizes the association of Sir2-3-4 to chromatin. The Sir2-3-4 complex (**A**, **B**) or the catalytically dead Sir2cd-3-4 mutant (**C**, **D**) was titrated into a constant amount of unmodified or H4K16^{ac} 6mer of 601 nucleosomes. Where indicated, 150 μM NAD was added to the samples. Scatter plot quantifications show the mean value ± s.e.m. of the % of unbound chromatin compared with the input for at least three experiments. (**E**) Reconstituted chromatin fully acetylated on H4K16 was subjected to NAD-dependent deacetylation in presence of a 2.5-fold molar excess of the Sir2-3-4 complex or the Sir2cd-3-4 mutant. The acetylation state was then determined by immunoblotting using acetylation mark-specific antibodies and H3 for loading. (**F**) Two micrograms of the indicated Sir protein were denatured in sample buffer and run on a 4–12% NuPAGE® Novex® Bis-Tris Gel.

NAD does not affect the acetylation state of chromatin (Supplementary Figure S2B). This shows that the binding affinity of the SIR complex for deacetylated chromatin is higher compared with chromatin assembled from unmodified histones.

In order to reinforce this finding, we tested whether the deacetylase activity of Sir2 itself was required for the enhanced binding of acetylated template in the presence of NAD. We generated a catalytic inactive Sir2 (Sir2cd) by introducing the point mutation N345A, which maps to the nucleotide binding motif (Rossman fold). This mutation disrupts Sir2 enzymatic activity *in vitro* and *in vivo* (Imai *et al*, 2000; Armstrong *et al*, 2002). The N345A substitution, however, did not affect the stability of Sir2 or its interaction with Sir3 and Sir4 and we were able to purify the mutated Sir2cd-3-4 from insect cells with the same efficiency as for the Sir2-3-4 complex (Figure 2F). We could furthermore confirm that this mutant did not retain significant deacetylase activity (Figure 2E).

In order to confirm that the Sir2cd-3-4 mutant was still able to recognize its substrate, we monitored the binding of Sir2cd-3-4 to unmodified or H4K16^{ac} chromatin in the absence of NAD. We found that, like Sir2-3-4, Sir2cd-3-4 has a slight preference for H4K16^{ac} chromatin (compare Figure 2A and C). We then monitored the loading of the Sir2cd-3-4 mutant onto unmodified or H4K16^{ac} chromatin in the presence of NAD. Unlike the Sir2-3-4 complex, the catalytic dead Sir2cd-3-4 showed the same slight preference for the H4K16^{ac} chromatin, as it did in the absence of NAD (compare Figure 2C and D). This data reinforce our observation that the deacetylation reaction has a positive role on the loading of the SIR complex onto chromatin (Figure 2B). Given that the deacetylation of H4K16^{ac} chromatin reduced the binding affinity of the Sir2-4 heterodimer (Figure 1E), these results suggest that Sir3 and the deacetylation of H4K16^{ac} by Sir2 jointly promote the binding of the SIR holocomplex to chromatin.

The Sir3 protein and Sir2-dependent deacetylation of H4K16^{ac} are both required to decrease nuclease accessibility of the linker DNA

SIR complex bound chromatin is thought to have a more compact structure *in vivo* as it is less accessible to enzymatic attack (Gottschling, 1992; Loo and Rine, 1994). The SIR complex could compact chromatin in two ways: first by deacetylating H4K16, and second by binding to chromatin. We observed that loading of the SIR complex onto unmodified chromatin greatly reduces the accessibility of the linker DNA to MNase and the restriction enzyme *Ava*I *in vitro*, consistent with a direct role for binding (Supplementary Figure S3A and B; Martino *et al*, 2009). In order to test the impact of H4K16^{ac} deacetylation on compaction *in vitro*, we first incubated H4K16^{ac} chromatin with the Sir2-4 subcomplex in the presence or absence of NAD and then challenged it with increasing amounts of MNase. We found that the presence of NAD did not significantly change the accessibility of the linker DNA (Figure 3A). Since acetylation of chromatin usually results in greater accessibility (Figure 1A), this result is likely a combination of changed accessibility due to removal of H4K16^{ac} and reduced binding affinity of Sir2-4 for deacetylated chromatin (Figure 1E).

We then performed the same experiment but replaced Sir2-4 with the SIR holocomplex. The concentration of Sir2-3-4

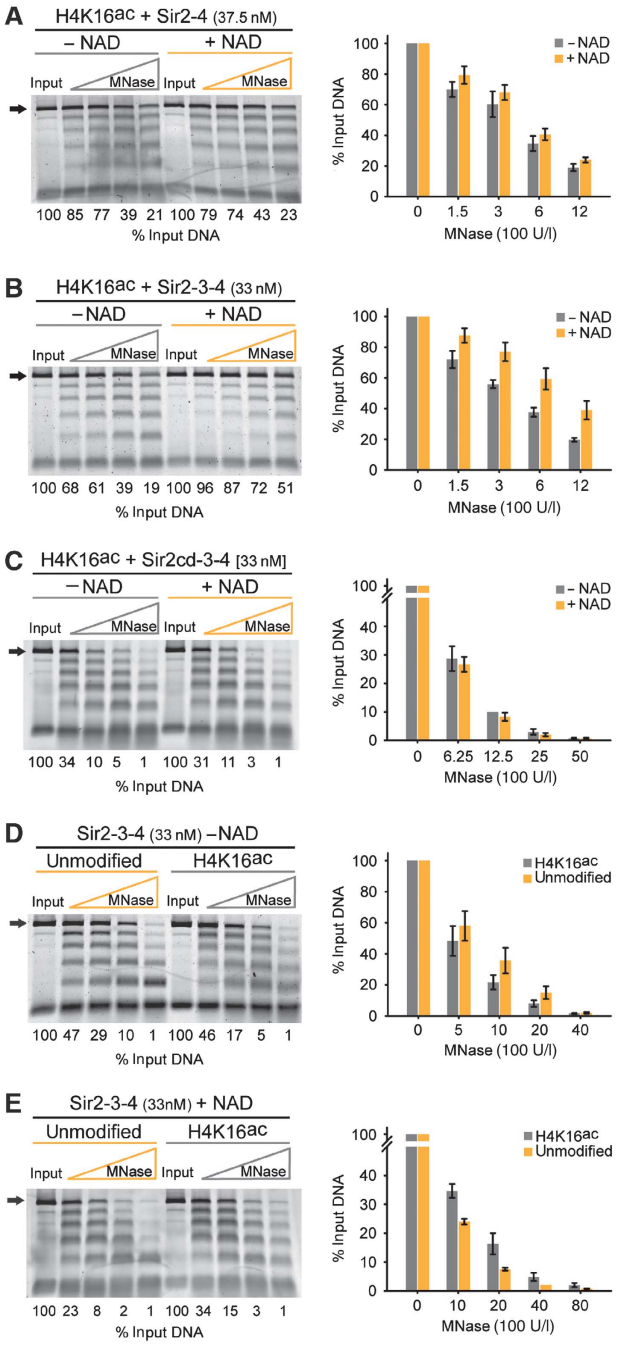

Figure 3 Sir3 is required to translate the Sir2-dependent deacetylation of H4K16^{ac} into a decrease of nuclease accessibility of the linker DNA. Unmodified or H4K16^{ac} 6mer of 601 nucleosomes (50 nM) was incubated with the indicated amount of Sir2-4 (**A**), Sir2-3-4 (**B**, **D** and **E**) or Sir2cd-3-4 (**C**) and was challenged with increasing amounts of MNase. Where indicated, SIR-bound chromatin was supplemented with 150 μM NAD and incubated for 15 min at 30°C before MNase digestion. Deproteinated samples were separated by electrophoresis and the amount of intact 6mer DNA (black arrow) was quantified and normalized to input. Quantification of at least three experiments was used to generate the vertical bar charts, data represent mean value ± s.e.m.

used resulted in a complete upshift of both acetylated and unmodified chromatin in a binding assay, ruling out differential accessibility due to incomplete ligand occupancy. Interestingly, we observed that in the presence of NAD the linker DNA was more protected from MNase than SIR-bound

chromatin in the absence of NAD (Figure 3B). Importantly, the addition of NAD did not change the protection of linker DNA of an array bound by the catalytic inactive Sir2cd-3-4 (Figure 3C). The same analysis in the absence of Sir3 did not increase protection against MNase attack (Figure 3A), arguing that the protective effect of NAD-dependent deacetylation of H4K16^{ac} by Sir2 requires Sir3 (compare Figures 3A and B). Finally, the increased linker DNA protection observed in Figure 3B was not caused by the NAD molecule *per se*, as no NAD-dependent differences were scored for linker DNA accessibility when Sir proteins were bound to unmodified chromatin (Supplementary Figure S3C). These results suggested that the deacetylation of H4K16^{ac} by the SIR complex promotes linker DNA protection.

When comparing unmodified with acetylated chromatin bound by Sir2-3-4 in the absence of NAD, we found that the linker DNA is slightly more protected (Figure 3D), indicating that at least some of the protection observed in Figure 3B is due to loss of H4K16^{ac} *per se*. However, given our previous observation that deacetylated chromatin is bound with higher affinity than unmodified chromatin (Figure 2B), we decided to explore the possibility that the deacetylation reaction itself may also contribute to the increased linker DNA protection observed in Figure 3B. Therefore, we compared the linker DNA accessibility of unmodified and H4K16^{ac} chromatin in the presence of Sir2-3-4 and NAD. We found that deacetylated chromatin is reproducibly more protected from MNase attack compared with chromatin assembled from unmodified histones (Figure 3E). Together, these results show that both Sir2-dependent deacetylation of H4K16^{ac} and Sir3 are required to decrease the nuclease accessibility of linker DNA, which presumably reflects the tighter binding of the SIR holocomplex to chromatin. In addition, there may be a conformational change that enhances linker DNA protection.

H3K56^{ac} loosens Sir protein binding to chromatin, slightly increasing linker DNA accessibility

To ask if our observation for H4K16^{ac} can be generalized to other acetylation marks we tested the effects of H3K56 acetylation, which is found on newly assembled nucleosomes in S phase. Since there are contradictory reports about which enzyme deacetylates H3K56^{ac} (Xu *et al*, 2007; Yang *et al*, 2008b) we first tested if Sir2 can deacetylate H3K56^{ac} as suggested earlier. We incubated H3K56^{ac} chromatin with SIR complex in the presence or absence of NAD. Chromatin homogenously acetylated at H3K56 was obtained by purifying acetylated H3 from *E. coli* using an expanded genetic code strategy (Neumann *et al*, 2008). Probing the histones with H3K56^{ac} antibodies after incubation showed that, unlike for H4K16^{ac}, the level of H3K56 acetylation remained unchanged (Figure 4B). We conclude that H3K56^{ac} is not a substrate of the NAD-dependent deacetylase activity of Sir2. This supports previous work reporting that two Sir2-related enzymes, Hst3 and Hst4, are required for H3K56^{ac} deacetylation *in vivo* (Celic *et al*, 2006; Maas *et al*, 2006; Yang and Kirchmaier, 2006) and suggests that Hst3 and Hst4 are the exclusive deacetylases for this residue.

To address whether acetylation of H3K56 has an effect on Sir protein loading, we compared the binding of the SIR holocomplex with unmodified and H3K56^{ac} chromatin. We found that H3K56^{ac} reduces the affinity of the SIR holocomplex for chromatin by roughly two-fold (Figure 4C). The binding affinity of the Sir2-4 heterodimer was also reduced in presence of the H3K56^{ac} mark (Figure 4D), while the binding affinity of the Sir3 protein alone was mostly unchanged (Figure 4E). To explore whether the slight affinity decrease observed here for the SIR complex could be responsible for the silencing defects seen *in vivo*, we investigated whether the SIR complex efficiently protects linker DNA in chromatin bearing the H3K56^{ac} mark. The acetylation on H3K56 *per se* has been shown to increase transient unwrapping of the DNA from the histone octamer but not to change the higher order structure of a 61mer nucleosomal array (Neumann *et al*, 2009). Consistently we show, by means of an MNase digestion assay, a slight increase in linker DNA accessibility for the chromatin bearing H3K56^{ac} over the unmodified control (Figure 4F). Subsequently, after adding the SIR complex in saturating concentrations (Supplementary Figure S4A), the H3K56^{ac} chromatin continued to show slightly higher linker DNA accessibility as compared with unmodified chromatin (Figure 4G). This is consistent with a previous *in vivo* study indicating that H3K56^{ac} chromatin is more sensitive to DNA methylation by an ectopically expressed bacterial *dam* methylase (Xu *et al*, 2007). Moreover, H3K56 point mutations disrupted silencing at telomeres without affecting Sir protein spreading (Xu *et al*, 2007). We conclude that H3K56^{ac} does not have a role similar to that of H4K16^{ac}, neither in the recruitment of Sir2-4, nor by being a substrate for Sir2.

Methylation of H3K79 by Dot1 neither increases linker DNA accessibility nor reduces Sir2-4 loading

Another mark associated with active chromatin in yeast is methylation of lysine 79 of histone H3 (H3K79). This methylation is exclusively catalysed by Dot1 and is thought to act as a boundary for the inappropriate spreading of the Sir proteins on chromatin (van Leeuwen *et al*, 2002; Frederiks *et al*, 2008; Martino *et al*, 2009; Verzijlbergen *et al*, 2009). Moreover, *in vitro* studies showed that interaction of the Sir3 protein with histone peptides was sensitive to the methylation of H3K79 (Altaf *et al*, 2007; Onishi *et al*, 2007). Previous work from our laboratory showed that we can make use of recombinant Dot1 in order to methylate reconstituted nucleosomal arrays *in vitro* (Martino *et al*, 2009). We have previously shown that even partial methylation of H3K79 decreases the binding affinity of both the SIR complex and the Sir3 protein alone to chromatin (Martino *et al*, 2009). We now provide further evidence that the lowered affinity indeed affects Sir3 binding, since the Sir2-4 heterodimer associates with unmodified and H3K79^{me} chromatin with nearly equal affinity (Figure 5A), while Sir3 clearly prefers unmodified chromatin (Figure 5B).

We then decided to test whether H3K79^{me} also impacts the structure of SIR-bound or SIR-depleted chromatin. To examine the potential impact of H3K79^{me} on linker DNA protection, we challenged *in vitro* methylated chromatin lacking Sir proteins with increasing amounts of MNase. Unlike the case for H3K56^{ac}, the accessibility of the linker DNA in the absence of SIR complex was unaffected by H3K79^{me} (Figure 5C). However, in the presence of substoichiometric amounts of Sir2-3-4 (Supplementary Figure S4B), the accessibility of the linker DNA was higher for H3K79^{me} chromatin than for unmodified chromatin (Figure 5D), consistent with notion that better SIR complex binding enhances linker DNA protection. When we added additional Sir2-3-4 such that

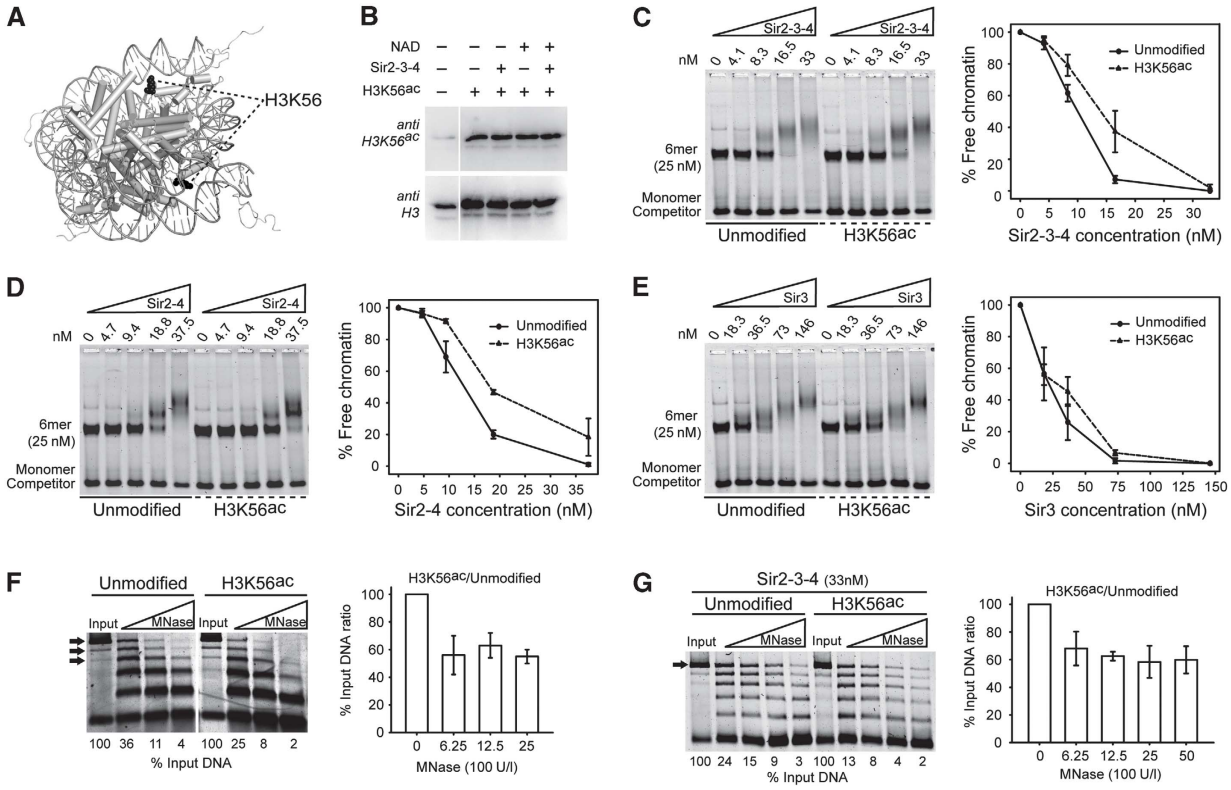

Figure 4 H3K56^{ac} decreases Sir protein binding affinity and slightly increases linker DNA accessibility. (**A**) Cartoon representation of the nucleosome core particle (NCP147; Davey *et al*, 2002) highlighting the position of H3K56 (black) at the entry/exit point of the DNA around the histone octamer. (**B**) Reconstituted chromatin fully acetylated on H3K56 was subjected to NAD-dependent deacetylation in presence of a 2.5-fold molar excess of the SIR complex. The acetylation state was then determined by immunoblotting using acetylation mark-specific antibodies and H3 for loading. The SIR complex (**C**), Sir2-4 heterodimer (**D**) or Sir3 (**E**) were titrated into a constant amount of unmodified or H3K56^{ac} 6mer of 601 nucleosomes. Samples were analysed as in Figure 2. Unmodified or H3K56^{ac} 6mer of nucleosomes were challenged with an increasing amount of MNase in absence (**F**) or presence (**G**) of the SIR complex. The 6mer, 5mer and 4mer bands (**F**) or the band corresponding to the intact 6mer alone (**G**), shown by black arrows, were quantified and normalized to the input. The histograms show the ratio between the amounts of quantified DNA from H4K16^{ac} chromatin over unmodified for the indicated MNase titration point ± s.e.m.

we score an equal degree of binding on both substrates (Supplementary Figure S4C), we observed no difference in accessibility of linker DNA (Figure 5E). This suggests that H3K79^{me} neither changes chromatin structure nor prevents the SIR complex from compacting it, but decreases the affinity of the SIR complex for chromatin. Thus, it antagonizes silencing through a mechanism distinct from H3K56^{ac}.

Discussion

Silent chromatin in *Saccharomyces cerevisiae* is the best studied system of heterochromatic gene silencing, yet we still do not fully understand the molecular mechanisms of its assembly and the role of histone modifications in this process. *In vitro* binding analysis between Sir protein domains and histone peptides have been informative, yet they only reflect a small part of the chromatin template. To examine the molecular basis of SIR-dependent silencing, we have established a fully recombinant system that recapitulates key features of silent chromatin in budding yeast (Martino *et al*, 2009). Here, we extend this system to examine how histone modifications participate in the formation of stable silent and active domains.

We show here that the H4K16^{ac} mark has both a positive and a negative role in SIR binding in a sequential manner (see Figure 6). Importantly, we show that H4K16^{ac} decreases the binding affinity of Sir3, but, in contrast, promotes the association of the Sir2-4 heterodimer to chromatin. Even the binding affinity of the SIR holocomplex is slightly increased by the presence of H4K16^{ac} in the absence of NAD (see also Johnson *et al* (2009)). This result, while initially counter-intuitive, helps elucidate the dual role of H4K16^{ac} in heterochromatin formation. On one hand, H4K16^{ac} prevents the dispersion of its key ligand, Sir3, into euchromatic chromatin. On the other hand, the high affinity of Sir2-4 for H4K16^{ac} may help nucleate silent chromatin, since it is likely in yeast that the targeted nucleosomes are acetylated before SIR complex loading.

In support of this dual role, it was shown that the substitution of H4K16 by not only an acetyl-mimicking residue, but also unacetylatable amino acids, disrupts silencing at telomeres and mating type loci (Johnson *et al*, 1990, 1992; Megee *et al*, 1990; Park and Szostak, 1990; Aparicio *et al*, 1991; Millar *et al*, 2004). Moreover, the deletion of *SAS2*, which encodes the HAT responsible for most H4K16^{ac} in yeast, impaired the repression of reporter genes at certain loci, such as TelVIIL or within the *HML* locus (Reifsnyder *et al*, 1996; Meijsing and Ehrenhofer-Murray, 2001) and led to the spreading of Sir proteins into subtelomeric regions that usually lack SIR-mediated repression (Kimura *et al*, 2002;

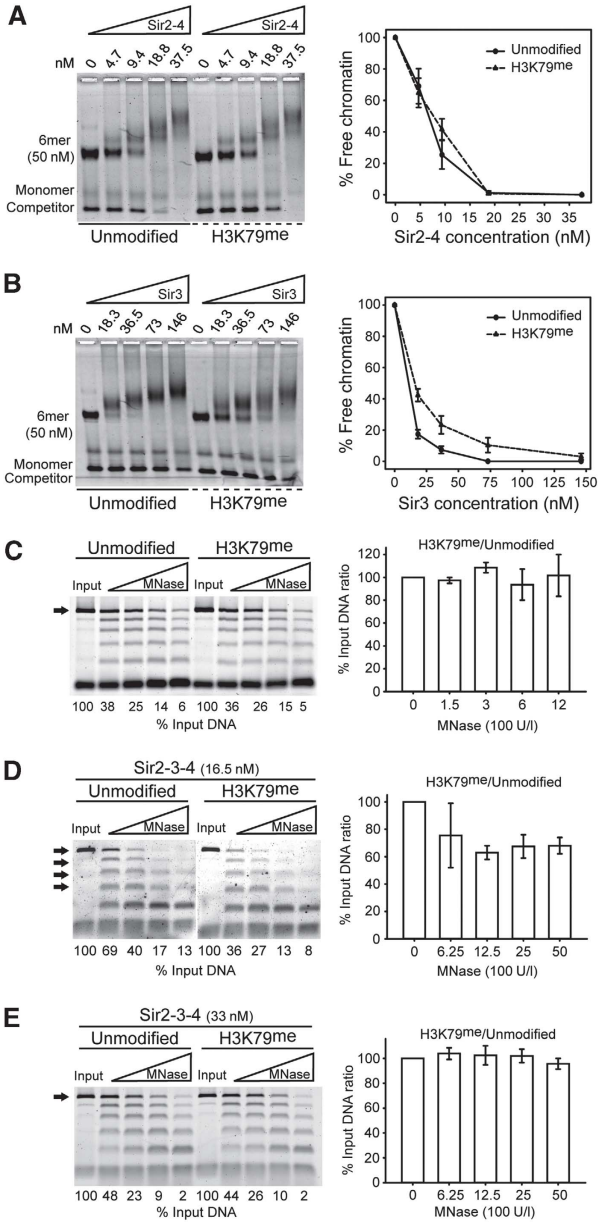

Figure 5 Methylation of H3K79 by Dot1 affects neither linker DNA accessibility nor Sir2-4 loading onto chromatin. The Sir2-4 heterodimer (**A**) or Sir3 (**B**) was titrated into a constant amount of unmodified or H3K79^{me} 6mer of nucleosomes. Samples were analysed as in Figure 2. Unmodified or H3K79^{me} chromatin were challenged with an increasing amount of MNase in absence (**C**) or presence (**D**, **E**) of the indicated amount of the SIR complex. The 6mer DNA band alone (**C**, **E**) or the 3mer to 6mer bands (**D**), shown by black arrows, were quantified and normalized to the input. The histograms show the ratio between the amount of quantified DNA from H3K79^{me} over unmodified chromatin for the indicated MNase titration point.

Suka *et al*, 2002). In contrast, the repression of a reporter at *HMR* was enhanced, probably because this locus has much stronger silencers, which dominate over an indiscriminate spreading of Sirs (Ehrenhofer-Murray *et al*, 1997). Importantly, both the kinetics of Sir3 recruitment to *HMR* and the establishment of silencing at *HML* were slower in cells that lack the H4K16-specific HAT, Sas2 (Katan-Khaykovich and Struhl, 2005; Osborne *et al*, 2009), suggesting a positive role for H4K16 acetylation. Collectively, these results support the model that Sas2-mediated acetylation of H4K16 has more than one role in silent chromatin formation (see also Zou and Bi (2008)). Reporter context appears to determine which role is rate limiting: the recruitment of Sir2-4, or the assembly and propagation of the Sir3-containing holocomplex along nucleosomes.

Sequential assembly of nuclease-resistant SIR-bound chromatin requires H4K16^{ac} deacetylation

In vivo the absence of H4K16^{ac} from silent chromatin suggests that it is removed by Sir2 as soon as the SIR complex is loaded. Moreover, it was shown that in the absence of Sir2 catalytic activity, H4K16^{ac} prevents the formation of silent domains (Yang and Kirchmaier, 2006). On the other hand, as mentioned above, even conservative substitutions at H4K16 decrease silencing efficiency at *HML* and at telomeres (Johnson *et al*, 1990; Meijsing and Ehrenhofer-Murray, 2001; Yang and Kirchmaier, 2006). This supports the notion that not only the recruitment of Sir2-4 by H4K16^{ac}, but the deacetylation reaction itself helps to seed repression (Johnson *et al*, 1992; Imai *et al*, 2000; Millar *et al*, 2004; Liou *et al*, 2005; Yang *et al*, 2008a; Martino *et al*, 2009).

Why is the deacetylation reaction important for silencing? The recapitulation of these steps *in vitro* helped us to address this question. Indeed, by adding NAD to SIR-bound H4K16^{ac} chromatin, we catalysed deacetylation of H4K16 and increased the binding affinity of the SIR holocomplex to chromatin. This shows in a well-defined recombinant system that removal of the single H4K16^{ac} mark by Sir2 increases SIR complex binding. Even more importantly, we found that the linker DNA was better protected from MNase digestion when the SIR complex was assembled on chromatin in the presence of H4K16^{ac} and NAD, as compared with its being loaded onto unmodified chromatin. This protection nicely mimics the DNA shielding observed in SIR-silenced chromatin regions *in vivo* (Gottschling, 1992; Loo and Rine, 1994; Xu *et al*, 2007) and argues that the SIR complex may associate with chromatin in more than one conformation. It was previously proposed that a by-product of Sir2 NAD-dependent deacetylation, *O*-AADPR, might trigger a conformational change of the SIR–chromatin complex to favour repression (Liou *et al*, 2005; Onishi *et al*, 2007; Martino *et al*, 2009).

Importantly, the deacetylation-dependent increase in affinity of the SIR holocomplex for chromatin, and the increase in linker DNA protection, depends crucially on the presence of Sir3. This is consistent with previous results which showed that exogenously added *O*-AADPR enhances the binding of both Sir3 and the SIR holocomplex to chromatin (Martino *et al*, 2009). A further study showed that addition of an excess of acetylated peptides and NAD to SIR–chromatin assemblies generated a structure that appeared more compact by electron microscopy (Johnson *et al*, 2009), perhaps reflecting a conformational change in the SIR complex (Liou *et al*, 2005). Nevertheless, *O*-AADPR is probably neither absolutely required for SIR complex loading nor for silencing, since repression can be achieved in a strain devoid of NAD-dependent deacetylases if an ectopic HDAC is fused to Sir3 (Chou *et al*, 2008) or if Sir3 is overexpressed in a H4K16R background (Yang and Kirchmaier, 2006). Taken together, our data support a scenario in which the sequential loading of Sir2-4

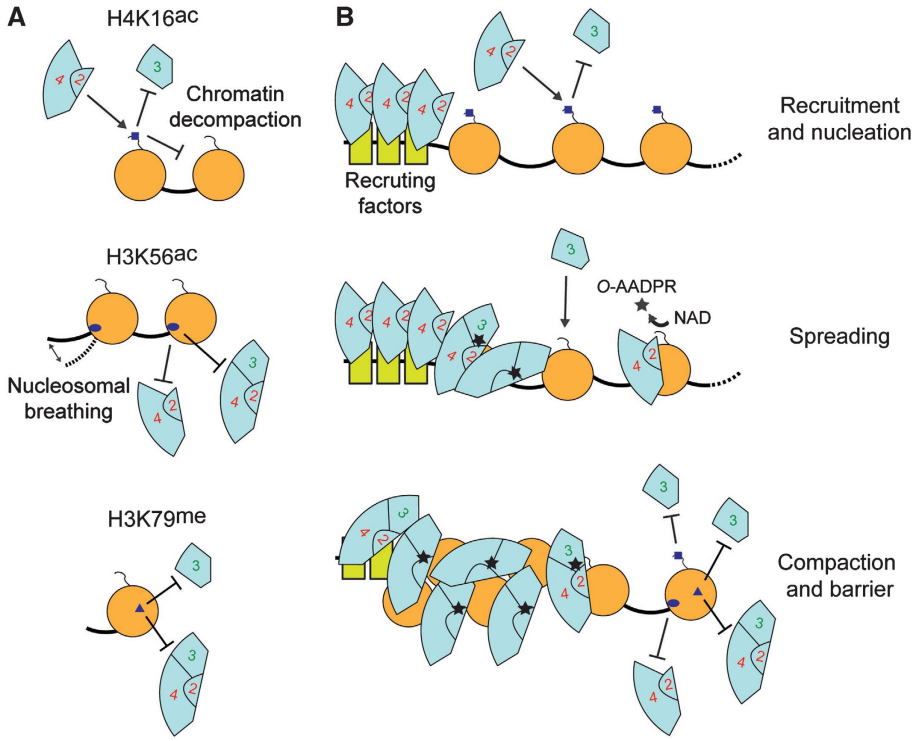

Figure 6 Combinatorial histone modifications distinguish silent and active chromatin regions. (**A**) Outline of the role played by different histone modifications on Sir protein loading and chromatin structure. (**B**) The Sir proteins are recruited onto chromatin by protein–protein interactions and bind tightly unmodified nucleosomes driving gene silencing. Spreading of the SIR complex is promoted by H4K16^{ac} that recruits the Sir2-4 heterodimer yet prevents the ectopic spreading of Sir3 alone. The NAD-dependent deacetylation reaction of H4K16^{ac} by Sir2 generates a high-affinity binding substrate for Sir3 and the synthesis of *O*-AADPR favours the tight association of the SIR complex to unmodified nucleosomes. H3K56^{ac} and H3K79^{me} generate a boundary to the spreading of the SIR complex mainly by reducing the binding affinity of the Sir2-4 heterodimer and the Sir3 protein, respectively. The acetylation of H3K56 and H4K16 also enhance the accessibility of the chromatin fibre, unlike methylation of H3K79.

onto nucleosomes containing H4K16^{ac}, its NAD-dependent deacetylation and the loading of Sir3, sequentially promote a stable assembly that protects linker DNA from exogenous factors (Figure 6).

Boundary formation and reduction of SIR holocomplex affinity by histone modifications

The observation that H4K16^{ac} might provide a boundary for heterochromatin spreading (Kimura *et al*, 2002; Suka *et al*, 2002) seems counterintuitive given the results described above. However, H4K16^{ac} does affect other processes beyond SIR complex association to chromatin, most notably, the recruitment of the histone methyltransferase Dot1 to chromatin (Altaf *et al*, 2007). Given that Sir2-4 preferentially binds chromatin carrying H4K16^{ac}, we propose that Dot1 competes with the recruitment of Sir2-4, and not as proposed earlier, with Sir3 (Altaf *et al*, 2007). On the other hand, the anti-silencing role of the methylation mark itself, H3K79^{me}, is most likely a reflection of reduced interaction between Sir3 and methylated chromatin (Ng *et al*, 2002, 2003; van Leeuwen *et al*, 2002; Altaf *et al*, 2007; Onishi *et al*, 2007; Martino *et al*, 2009). Consistently, we found that nucleosomes bearing H3K79^{me} neither affect the binding of the Sir2-4 heterodimer, nor was there an inherent change in structural properties of H3K79^{me}-containing chromatin. This is consistent with crystallographic analyses which argue that H3K79^{me} does not alter the structure of the nucleosome (Lu *et al*, 2008). Given that no enzyme has been found so far that removes the H3K79^{me} mark, depletion of this mark may depend on histone eviction or on sequential dilution through rounds of DNA replication. Its slow removal renders H3K79^{me} a more stable barrier to the spreading of the SIR complex than H4K16^{ac}, which instead recruits Sir2-4 and promotes the spread of repression (Figure 6).

The third histone mark correlated with active chromatin in yeast is the acetylation of H3 on K56. In contrast with H3K79^{me}, H3K56^{ac} is clearly subject to active deacetylation. Here, we show that Sir2 is unable to remove the H3K56^{ac} mark *in vitro*, which indirectly supports previous work showing that H3K56^{ac} is primarily deacetylated by two Sir2-related enzymes: Hst3 and Hst4 (Celic *et al*, 2006; Maas *et al*, 2006). Indeed, Sir-mediated repression cannot be established in the absence of these two enzymes, although Sir proteins still bind telomeres in an *hst3*Δ*hst4*Δ mutant (Yang *et al*, 2008b). Consistent with our work, this suggests that the H3K56^{ac} mark does not completely block SIR–chromatin interaction.

How then does H3K56^{ac} impair the formation of silent chromatin? Using our *in vitro* system, we found that H3K56^{ac} affects both the affinity with which SIR complexes bind chromatin and the formation of a chromatin structure that is less accessible to MNase attack. The observed drop in affinity of SIR holocomplex for chromatin agrees with an *in vivo* study, which suggested that H3K56^{ac} facilitates Sir protein displacement and RNA polymerase II elongation within heterochromatin regions (Varv *et al*, 2010). Our

observation that H3K56^{ac} increases accessibility of the linker DNA is consistent with an increase in spontaneous (but transient) unwrapping of the DNA from the histone octamer, which may reflect the position of H3K56 at the entry/exit point of the nucleosomal DNA (Figure 4A; Neumann *et al*, 2009). It is striking that even SIR-saturated arrays showed increased linker DNA accessibility in the presence of H3K56^{ac}, indicating that SIR binding cannot overcome the effect of H3K56^{ac} on nucleosomal structure. Although it is unclear why this modification reduces SIR complex binding, this and the increased linker DNA exposure are likely to account for the anti-silencing effect of the H3K56^{ac} mark.

To conclude, we propose that the euchromatic mark H4K16^{ac} is required for the formation of both active and silent chromatin. The process of creating stable silent and active states is not an one-step event, but requires positive feedback loops. H4K16^{ac} may be the starting point for silent domains, which are reinforced by the Sir2 deacetylation reaction and possibly the generation of *O*-AADPR, and active domains, where it promotes H3K79 methylation. These interdependent pathways are conserved throughout evolution and mathematical modelling clearly shows that such networks are required to establish a stable binary switch (Dodd *et al*, 2007; Mukhopadhyay *et al*, 2010). Here, we have demonstrated that H4K16^{ac} is actively implicated in the establishment of yeast silent chromatin, being the first histone mark shown to recruit Sir proteins to chromatin.

Materials and methods

SIR purification and chromatin reconstitution

In vitro reconstitution of SIR-bound chromatin was carried out essentially as described (Cubizolles *et al*, 2006; Martino *et al*, 2009). Briefly, the Sir proteins were expressed in sf21 insect cells with baculoviruses generated using BD BaculoGold^{TM}, BD-Biosciences. Co-infection was used to produce the Sir2-3-4 complex, the catalytic dead Sir2cd-3-4 and the Sir2-4 heterodimer, a single infection was used to produce the Sir3 protein alone (Cubizolles *et al*, 2006). Recombinant *X. laevis* histones were use to reconstitute histone octamers as described previously (Luger *et al*, 1997). Chromatin was assembled *in vitro* by adding increasing amounts of purified histone octamer to a constant amount of DNA arrays containing six 601-Widom positioning elements separated by 20 bp of linker DNA, referred as 601-167-6mer (Lowary and Widom, 1998). An unspecific DNA sequence of 147 bp (referred as 'competitor' on the figures) was added to the mix in order to bind the excess of histone octamers subsequent to the saturation of the 601-167-6mer (Huynh *et al*, 2005). Cy5-labelled 601-167-6mer was generated by filling the 5′ overhang-ends of an *Eco*RI site with Klenow enzyme (NEB, accordingly to manufacturer's instruction), using d-CTP-Cy5 (GE Healthcare). The free nucleotides were then separated from the Cy5-labelled array using small Bio-spin columns (Bio-Rad). DNA and histones were mixed in 40 μl of buffer A (10 mM TEA pH 7.4 and 1 mM EDTA) and 2 M NaCl on ice, and chromatin was reconstituted by step dialysis in buffer A containing 1.2, 1, 0.8 or 0.6 M NaCl for 2 h at 4°C and in buffer A overnight (Lee and Narlikar, 2001). The 601-167-6mer was routinely prepared at a final nucleosomal concentration of 10^{-6} M. Increasing amounts of Sir proteins were added to the 601-167-6mer diluted to 5×10^{-8} M or 2.5×10^{-8} M in 10 mM TEA pH 8, 25 mM NaCl, 0.05% Tween-20 on ice and after 10 min incubation the samples were fixed with 0.0025% glutaraldehyde for 10 min on ice. The fixation yields slightly sharper bands but the results are very similar without fixation. When chromatin deacetylation was coupled to Sir protein loading, SIR-bound chromatin was incubated with or without 150 μM NAD for 15 min at 30°C before incubation on ice for a 10 min fixation as above. The samples were routinely run at 80 V for 90 min at 4°C in a 0.7% agarose gel 0.2 × TB: 18 mM Tris, 18 mM Boric acid. The gel was soaked for 20 min in 1 × SYBR® Safe and the DNA was visualized in a Typhoon 9400 scanner.

Preparation of the histone modifications

Methylation of H3K79 was carried out on reconstituted chromatin as described before (Martino *et al*, 2009). Briefly, 0.8 pmol of recombinant Dot1 was incubated with 8 pmol of reconstituted 601-167-6mer in 25 mM Tris pH 7.9, 20 mM NaCl, 0.4 mM EDTA, with or without 160 pmol of S-adenosylmethionine (SAM) at 30°C for 30 min, then 160 pmol of SAM was added and the reaction was continued for 30 min. Mass spectrometry analysis showed that H3K79 is mono-, di- and, to a lesser extent, tri-methylated on at least 50% of the available K79 residues (Frederiks *et al*, 2008; Martino *et al*, 2009). The chromatin was then stored at 4°C.

Homogeneous acetylated histone H3 at the lysine 56 was obtained using an aminoacyl-tRNA synthetase and $tRNA_{CUA}$ pair created by directed evolution in *E. coli* (Neumann *et al*, 2008). An unmodified control was prepared in parallel. Histone octamers were assembled as described previously (Luger *et al*, 1997) and kept at 4°C before chromatin reconstitution.

Full acetylation of H4K16 was obtained by NCL as described previously (Shogren-Knaak *et al*, 2006). Briefly, the H4 N-terminal peptide containing residues 1–22 and acetylated lysine at position 16 was synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based solid-phase synthesis and activated at the C-terminus by thioesterification. Subsequently, the globular *X. laevis* H4Δ1-22,R23C was ligated to the activated H4 peptide and the ligation product was purified as described previously (Shogren-Knaak *et al*, 2006). Identity and purity of the histones were verified by SDS–PAGE as well as ESI-MS (Supplementary Figure S1). Histone octamers were assembled as described previously (Luger *et al*, 1997) and kept at 4°C before chromatin reconstitution.

MNase digestion assay

MNase digestion was carried out in 20 μl of 10 mM TEA pH 8, 1.5 mM $CaCl_2$, 25 mM NaCl, 0.05% Tween-20. In all, 1 pmol of 601-167-6mer was digested with increasing amounts of MNase, as detailed in the figures, for 12 min on ice. The digestion was stopped by adding 10 mM EGTA, proteins were removed by proteinase K digestion for 15 min at 30°C and the samples were run at 65 V for 60 min in a 1.2% agarose gel 1 × TBE: 90 mM Tris, 90 mM Boric acid, 2 mM EDTA. Digestion of SIR-bound chromatin was performed on 601-167-6mer pre-incubated with the indicated amount of Sir proteins for 10 min on ice. MNase digestion of deacetylated chromatin was performed on 601-167-6mer incubated with 0.66 pmol of Sir2-3-4, Sir2cd-3-4 or Sir2-4 for 15 min at 30°C with or without 150 μM NAD and recovered on ice. Concerning the Sir2-3-4 complex, similar results were obtained by incubating the nucleosomal array with 0.66 pmol of Sir2-4 at first and adding 0.66 pmol of Sir3 before the recovery on ice. In order to strengthen our observations, different batches of modified and unmodified chromatins were compared.

Deacetylation reaction

Deacetylation of 2 pmol of reconstituted chromatin was performed in 30 μl of 25 mM Tris pH 8, 50 mM NaCl in presence of 5 pmol of the Sir2-3-4 complex, the Sir2cd-3-4 mutant or Sir2-4 and 150 μM NAD for 30 min at 30°C and stopped by addition of 4 × Laemmli buffer. Similar results were obtained in 25 mM Tris pH 8, 137 mM NaCl, 2.7 mM KCl and 1 mM $MgCl_2$. The acetylation state was determined by immunoblotting using acetylation mark-specific antibodies (anti-H3K56^{ac} Upstate #07-677, anti-H4K16^{ac} Serotec AHP417) and H3 for loading (anti-H3 Abcam ab1791-100).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: MO, SK and SMG designed the experiments and interpreted results. MO performed the experiments. SK and FM contributed reagents. SS and WF contributed the H4K16^{ac} histone octamers. SMH and JC contributed the H3K56^{ac} histone octamers. MO, SK and SMG wrote the manuscript. SMG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

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