Formation of an SIR-Nucleosome Filament *in vitro* and Its Modulation by O-Acetyl-ADP-Ribose

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It is not possible to clearly visualize how chromatin condenses to heterochromatin *in vivo*. However, in an *in vitro* system for *Saccharomyces cerevisiae,* the requirements for heterochromatin filament formation mirror those found *in vivo*. Here we report that the nucleosomes and the Sir2, Sir3 and Sir4 proteins, which are required for *in vitro* filament assembly, are also components of these filaments, confirming that the filaments are SIR-nucleosome filaments. We show the individual localization patterns of the Sir proteins on this SIR-nucleosome filament, and demonstrate that the metabolite, AAR, plays a specific and essential role in promoting the formation of this SIR-nucleosome filament.

Main

In eukaryotic cells, nuclear DNA is packaged with histones and other proteins into chromatin. The epigenetic modification of histones, including by acetylation, methylation, phosphorylation, sumoylation, and ubiquitination, modulates their affinity for DNA, and for histone-associated proteins (1) and can lead to the formation of silent heterochromatin. This plays important roles

not only in the regulation of gene expression but also in the maintenance of chromosome stability. The silent heterochromatin of *Saccharomyces cerevisiae* offers an excellent system for studies on epigenetics.

In vivo genetics and *in vitro* biochemical studies in *Saccharomyces cerevisiae*, determined that the silent information regulator (Sir) proteins, Sir2, Sir3 and Sir4, are required to establish and maintain silent heterochromatin domains at the telomeres and the two mating type loci (2-5). The Sir proteins form the SIR complex, capable of nucleosome binding (6-9), and which brings together two types of biochemical activities, critical for silent heterochromatin assembly. Sir2 is a nicotinamide adenine dinucleotide (NAD) dependent histone deacetylase (10-11). The deacetylase activity of Sir2 has been shown to be required for the hypoacetylation of histone proteins H3 and H4 (12), for Sir2 and Sir3 binding to the telomeric and mating type loci (13-16), and for Sir3 and Sir4 binding to histone tail regions (1). Sir2 couples this deacetylation to NAD hydrolysis and generates the metabolite, *O*-acetyl-ADP-ribose (OAADPR, or AAR) (12, 17-18). This deacetylation of histone amino terminal tails is followed by their association with Sir3 and Sir4, which leads to iterative recruitment cycles of Sir proteins along the adjacent chromatin fiber to form extended silent heterochromatin regions (13-15).

Sir2 and Sir4 are co-purified as a heterodimeric complex, which contains varying amounts of Sir3 (13, 19). Purified Sir3 exists as dimers and higher order oligomers (20). The synthesis of AAR during the deacetylation promotes the association of multiple copies of Sir3 with Sir2/Sir4 and induces a dramatic structural rearrangement of the SIR complex (20). Thus both the deacetylation event and the AAR itself might contribute to SIR complex assembly *in vitro,* with both being required for the formation of silent heterochromatin *in vivo*.

Models for the formation of silent heterochromatin based on the spreading of SIR complexes along the chromatin fiber have been proposed (13-15), however, the mechanisms and regulation of heterochromatin formation remain to be investigated using well-defined *in vitro and in vivo* systems. It has been demonstrated *in vitro* that purified Sir proteins can associate with purified yeast nucleosomes to form filament structures (21). Although these filaments display requirements that closely mirror those observed for the formation of silent chromatin *in vivo*, their exact components remain to be clarified.

We verified by electron microscopy that purified Sir2/Sir4, Sir3, nucleosomes and NAD, were able to form filamentous structures *in vitro* and determined more detail on their structure and composition. These filaments appeared to be non-rigid structures, 15-20 nm in diameter and over 100 nm in

length. This compares with a 30-nm diameter for a condensed heterochromatin fiber and 10-nm for beads-on-a-string nucleosomal array filaments (review in 22), raising the possibility that these *in vitro* filaments might be incompletely formed pre-heterochromatin filaments, particularly as they were larger and longer than those expected in single SIR complexes and single nucleosome arrays (Fig 1a-f). When any one of the Sir proteins, nucleosomes or NAD was absent from the *in vitro* assembly system, no filaments were detected (Fig 1g-j). This indicates that all these components are necessary for *in vitro* filament assembly and suggests that the filaments were SIR-nucleosome filaments. We were interested to determine the filament components to verify that they were indeed SIR-nucleosome filaments, and to study the assembly mechanism.

We used two approaches to address the first issue. Briefly, to determine the presence of Sir proteins and nucleosomes in the filaments, assembly reactions were centrifuged at speeds sufficient to pellet the filaments but not individual reaction components. Aliquots of the resuspended pellet and supernatant fractions were analyzed by Western blot. As shown in Figure 2a, Sir2, Sir3 and Sir4 were all detected in the pellet fraction as was histone H3, when the filament assembly conditions included the presence of NAD.

However, when NAD was absent, filamentous structures were not seen and under the same centrifugation conditions, Sir2, Sir3, Sir4 and histone H3 could not be detected in the pellet fraction. Thus all the Sir proteins and the histone H3 protein are components of these filamentous structures, and an assembled filament is an SIR-nucleosome filament.

Using electron microscopy and immuno-gold labeled antibodies specific to each of the Sir proteins, all the Sir proteins could be detected on the filaments and displayed interesting and discontinuous localization patterns (Fig 2b-d). The major localization of Sir2 was at a terminal region of the filament with light distribution on the rest of the fiber, whereas Sir3 was not detected at a filament end, and Sir4 only rarely localized there. The length of the distribution of Sir3 was similar to that of Sir4 but longer than that of Sir2. When we used either a histone H3 or a histone H4 antibody, we unfortunately failed to detect the immuno-gold signal on the filament. It is therefore possible that the recognized epitopes were not exposed, either because the nucleosomes were tightly packaged, and entirely surrounded by associated Sir proteins, or recognition sites had been covered (or interfered with) by other associated molecules. Meanwhile, we also noticed that some filaments showed a helical shape (Fig 1b, 2c, 3b-c) and that both Sir3 and Sir4 signals seemed to also

show a helical distribution (Fig 2c-d). Whether these characteristics are because the Sir proteins preferentially surround the nucleosomes helically or are due to a feature of the filament itself still remains to be investigated.

That the Sir2 and Sir4 distributions barely co-localize is not expected given that they co-purify from the filament fraction as heterodimers. This may indicate that the epitopes of one or both are hidden or altered when within the complex. That only small amounts (even none) of monomers are purified from the complex leads us to hypothesize that at the end of the fibers, the epitope on Sir4 is hidden while that on Sir2 is exposed. The possibility of co-localization of Sir3 with Sir4 on the fiber might suggest that on the binding of Sir3 to the complex, substantial conformational changes may then expose the Sir4 recognition site and conceal or alter that of Sir2. However, we cannot rule out the possibility that Sir4 may also associate with nucleosomes in the absence of bound Sir2, and the similarity of the Sir4 and Sir3 localization patterns raises the possibility of Sir3-Sir4 interaction.

Purified Sir3 has been shown to self-polymerize to form oligomers which possess a higher affinity for interaction with the Sir2/Sir4 complex and nucleosomes than monomeric Sir3. Although purified Sir2/Sir4 and Sir3 are able to associate with histone proteins without NAD or AAR present, AAR is

able to induce the stoichiometric change and structural rearrangement of SIR complexes (20). AAR is therefore known to play an important role in the regulation of SIR complex assembly. We were interested to show whether this AAR-dependent process is important in the mechanism of SIR-nucleosome filament formation. We used an enzymatically inactive Sir2 mutant, Sir2-H364Y, to address this issue. In this mutant, the mutation does not affect the ability of the protein to assemble into a SIR complex, nor the regulation of SIR complex assembly by exogenously added AAR (20).

From Figure 3a, in contrast to SIR-nucleosome filaments from reactions containing wild type Sir2 (Fig 1b), no long filamentous structures formed in assembly reactions containing Sir2-H364Y in the presence or absence of NAD. Interestingly, there were some particles with a larger and longer shape than single SIR complexes or nucleosomes, and similar particles were also present in the assembly reactions containing wild type Sir2 in the absence of NAD (Fig 1j). We were not able to distinguish whether these particles consisted of multiple Sir proteins and/or multiple nucleosomes. However, we determined that Sir2 mediated NAD-dependent deacetylation with AAR generation was responsible for the formation of SIR-nucleosome filaments in our assembly reactions.

We next asked whether AAR could regulate the formation of SIR-nucleosome filaments. As shown in Figure 3b, purified AAR added to the assembly reaction containing Sir2-H364Y promoted the formation of SIR-nucleosome filaments. The same result was found in assembly reactions containing Sir2 in the presence of AAR but absence of NAD (Fig 3c). We conclude that AAR, a product of NAD-dependent deacetylation, acts as a modulator to regulate the formation of SIR-nucleosome filaments. Consistent with this we have also demonstrated that AAR is associated with yeast heterochromatin regions (data not shown).

Put together, we propose that Sir2/Sir4 binds the nucleosome. Sir2 then carries out histone deacetylation, generating AAR. AAR induces the structural rearrangement of the SIR complex, promotes polySir3-SIR complex formation and increases the affinity of this polySir3-SIR complex for nucleosomes. These polySir3-SIR complexes then spread along nucleosomes to create an extended SIR-nucleosome filament (Fig 3d).

These results suggest that a small number of proteins and molecules may be sufficient to mediate the formation of a minimal set of yeast pre-heterochromatin. And, at least in our *in vitro* system, these results highlight the importance of the metabolite AAR, indicating it is not merely a

non-functional by-product of the histone deacetylation reaction but an essential component of pre-heterochromatin formation in the yeast, *Saccharomyces cerevisiae*.

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Figure legends

Fig 1. *In vitro* assembly of SIR-Nucleosome filaments. (a) Purified proteins, Sir3, Sir2/Sir4, Sir2H364Y/Sir4 and Nucleosomes, were separated by SDS-PAGE and visualized by Coomassie Blue staining. Protein standard markers were labeled, shown to the left of gels. Nucl represents the native yeast nucleosome purified by H2A-TAP. (b) Electron micrograph shows the negatively stained i*n vitro* assembly reaction of SIR-Nucleosome filament, containing Sir2/Sir4, Sir3, nucleosome and NAD. (c-f) Electron micrographs show negative staining for Sir2/Sir4, Sir2H364Y/Sir4, Sir3 and nucleosomes, respectively, as indicated. (g-j) Electron micrographs show negative staining for the *in vitro* assembly reactions without Sir2/Sir4, Sir3, nucleosomes and NAD, respectively, as indicated. Bar represents 100 nm.

Fig 2. Components of SIR-Nucleosome filaments. (a) Western blotting of the pellet (ppt) and supernatant fractions (sup) from centrifuged *in vitro* assembly

reactions (Tot), containing Sir2/Sir4, Sir3 and nucleosomes in the absence (left panel) or presence (right panel) of NAD, were detected by Sir4, Sir3, Sir2 and histone H3 antibodies, respectively, as indicated. (b-d) Electron micrographs showing i*n vitro* assembled SIR-Nucleosome filaments, immuno-gold labeled with Sir2, Sir3 and Sir4 antibodies, respectively. Bar represents 100 nm.

Fig 3. Modulation of SIR-Nucleosome filament formation by AAR. (a) Electron micrographs showing the i*n vitro* assembly reactions containing Sir2H364Y/Sir4, Sir3, nucleosomes and NAD. (b) Electron micrographs showing the i*n vitro* assembly reaction containing Sir2H364Y/Sir4, Sir3, nucleosomes and AAR. (c) Electron micrograph showing the i*n vitro* assembly reaction containing Sir2/Sir4, Sir3, nucleosome and AAR. Bar represents 100 nm. (d) Model illustrating the formation of SIR-Nucleosome pre-heterochromatin filaments. The SIR complex is recruited to nucleosomal array chromatin, and NAD-dependent deacetylation of histone tails by Sir2 induces AAR-mediated stoichiometric changes and a structural rearrangement of SIR complexes. These SIR complexes then spread along the nucleosomal array chromatin to create an extended SIR-nucleosome pre-heterochromatin filament.

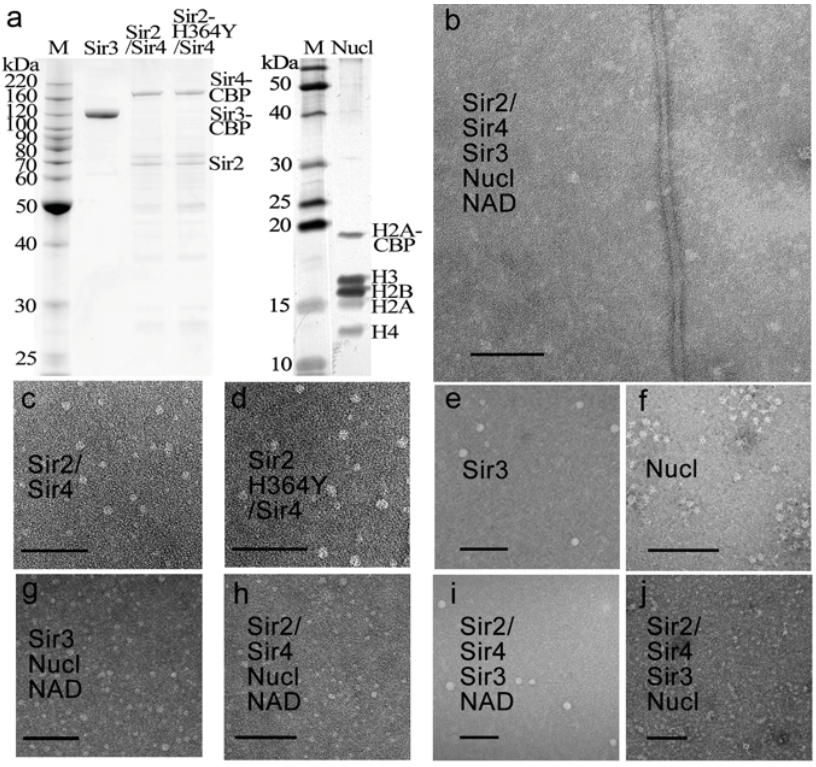

Figure 1_Wu et al

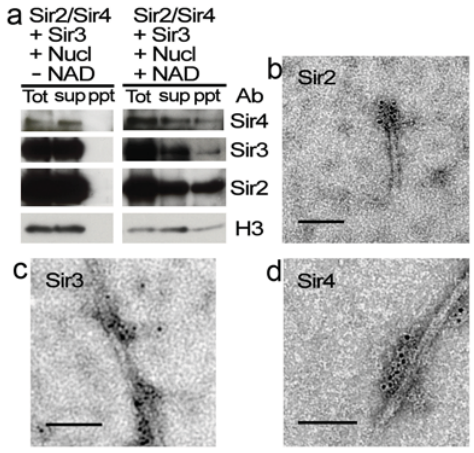

Figure 2_Wu et al

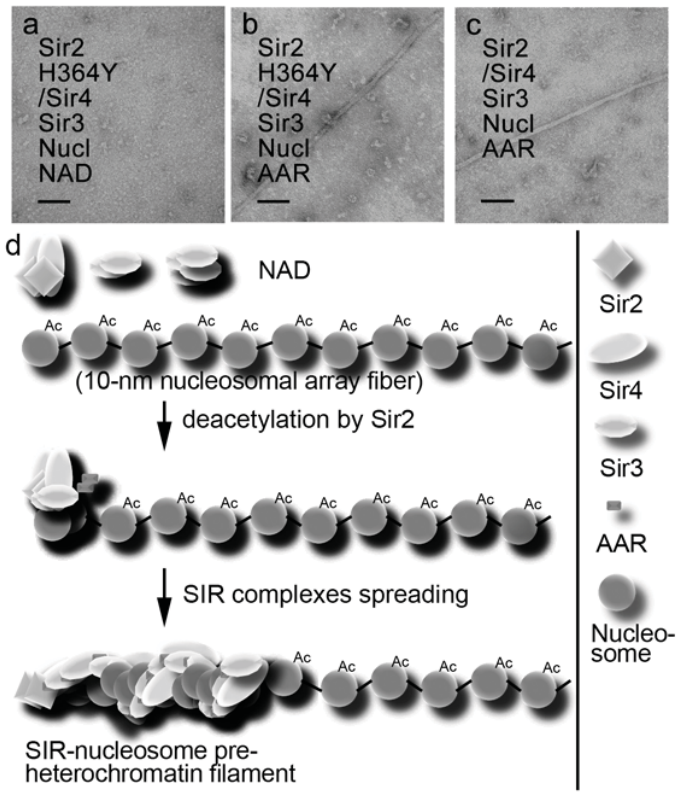

Figure 3_Wu et al