Supplemental Data
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Reconstitution of Heterochromatin-Dependent Transcriptional Gene Silencing

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Proteins and DNA Templates

S. cerevisiae histones were expressed in *E. coli* using codon-optimized constructs (gift of T. Richmond), purified, and reconstituted into octamers as described (Dyer et al., 2004; Vary et al., 2004) (Figures S1A and S1B). Point mutations were made using the Quik-Change method (Stratagene). *S. cerevisiae* GST-Nap1 was expressed in *E. coli* from pGEX-6P-1, affinity purified, treated with Precision Protease (GE Healthcare) to remove the GST, and further purified by HiTrap-Q column (GE Healthcare) chromatography. Isw1a complex was obtained from an IOC3-TAP strain as described (Vary et al., 2003). Sir3-FLAG and HA-Sir2/TAP-Sir4 were purified as previously described (Buchberger et al., 2008; Liou et al., 2005). Protein concentrations were determined by Coomassie blue staining of SDS-polyacrylamide gels using BSA as a standard. The DNA template used in many experiments was a PCR fragment containing the HMR locus (Ch. III, 292598-295673), three LexA binding sites upstream, and 79 bp downstream linker. PCR was performed with a biotinylated downstream oligo, and the PCR product was purified using a PCR Purification kit (QIAGEN).

Chromatin Assembly

Chromatin assembly reactions were performed as described previously (Vary et al., 2004) with modifications. A typical reaction contained 1.7 μg (15 pmol) histone octamer, 3.4 μg (50 pmol

monomer) Nap1, 5-10 ng (22.5-45 fmol) Isw1a, and 1 μg (1.5 nmol, bp) DNA in 110 μl 10 mM HEPES (pH 7.5), 10% glycerol, 10 mM KCl, 1.5 mM EGTA, 2.5 mM β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, and an ATP-regeneration system of 30 mM Creatine phosphate, 3 mM ATP, 4.1 mM $MgCl_2$, and 6.4 μg/ml creatine kinase (see Figure S1D). Reactions were incubated for 5 hr at 30°C. All reactions using free chromatin were performed in siliconized microcentrifuge tubes blocked with 2 mg/ml BSA and 0.1% NP-40. To assess chromatinization, samples were supplemented with 3 mM $CaCl_2$ and digested with micrococcal nuclease for 10 min at RT, deproteinated, purified by phenol/chloroform and ethanol precipitation, separated on a 1.3% agarose-0.5X TBE gel, and stained with ethidium bromide.

Subsequent conjugation of biotinylated chromatin to Dynabeads M-280 (Invitrogen/Dynal) was performed at RT for 1 hr with rotation using 370 μg of beads per μg of DNA in a final buffer containing 30 mM HEPES (pH 7.5), 150 mM NaCl, 4% PEG 8000, 10% glycerol, 0.02 % NP-40, 2.5 mM β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT and 0.5X ATP-regeneration system. Bead-conjugated chromatin was washed four times with six times the volume conjugation buffer without PEG 8000 and ATP regeneration system. Protein was stripped from the beads with 2 M NaCl, and DNA was quantified either by boiling beads in 0.1 % SDS or by restriction enzyme digestion and gel quantification.

To generate an acetylated nucleosome template, bead-conjugated chromatin was incubated with an equal amount of Piccolo HAT complex v7 (a gift from B. Hnatkovich and S. Tan; see Selleck et al., 2005 for details), μg Piccolo: μg DNA, in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5% glycerol, 5 mM DTT, 1 mM PMSF for 1 hr at 30ºC. Beads were washed once in the same buffer plus an additional 500 mM KCl and resuspended in appropriate reaction buffer and stored on ice for no more than 6 hr.

Chromatin Association Assays

Sir protein association with magnetic bead-conjugated chromatin was assayed by coprecipitation by magnetic concentration. Reactions were performed in 50 mM HEPES (pH 7.5), 150 mM KCl, 1 mM Magnesium Acetate, 0.1 mg/ml BSA, 10 % glycerol, 0.02 % NP-40, 0.3 mM EGTA, 2.5 mM β-glycerophosphate, 0.1 mM PMSF and 1 mM DTT. Sir3 (1.05 pmol) and the Sir2/Sir4 (Sir2/4) subcomplex (340 fmol) were preincubated on ice in experiments where the SIR complex was used. Sir proteins were mixed with bead-conjugated chromatin (60 ng DNA, ~480 fmol nucleosomes, assuming 18 nucleosomes per ~3.4 kb DNA fragment) in 20 μl. Reactions were incubated at RT with rotation for 10 min followed by magnetic concentration, one wash in 200 μl reaction buffer, magnetic concentration, and resuspension in SDS-PAGE loading buffer. Samples were incubated at 95ºC for 1 min, beads were separated on a magnetic concentrator, and samples were run on an 11% SDS-PAGE gel. Gels were cut width-wise at the 40 kDa prestained protein marker; the upper portion was transferred onto nitrocellulose and the lower portion was transferred onto Immobilon-P^{SQ} (Millipore) membrane and subjected to standard western analysis as described previously (Onishi et al., 2007).

Deacetylation reactions were performed as above with acetylated chromatin in the presence of 0.2 mM NAD. Reactions were incubated for 1 hr at 30ºC with rotation then at 4ºC for 1 hr. For deacetylation with ^{3}H-acetyl-chromatin, reactions were performed in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5% glycerol, 0.05 mg/ml BSA, 5 mM DTT, 1 mM PMSF and washed twice with 50 μl reaction buffer with 500 mM NaCl. Beads were counted for ^{3}H by liquid scintillation.

Mononucleosome Binding Assays

Mononucleosome reconstitution was performed as described previously with salt dialysis (Luger et al., 1999). The DNA template used was a gel-purified 218 bp PCR fragment containing the “601”

nucleosome-positioning sequence (Li and Widom, 2004) labeled with ^{32}P-phosphate at the 5´ ends. Histone octamer was added to DNA in approximately a 1:1 molar ratio in 10 mM Tris-HCl (pH 7.6), 2 M NaCl, 1 mM EDTA, 0.5 mg/ml BSA, 0.05% NP-40, 5 mM β-mercaptoethanol. Salt dialysis was performed at 4ºC for approximately 20 hr from 2 M NaCl buffer to 50 mM NaCl and a final dialysis step for 1 hr at 50 mM NaCl. Samples were incubated at 55ºC overnight before storage on ice up to 4 weeks.

Mononucleosome binding assays were performed by incubating reconstituted mononucleosome with increasing amounts of Sir3 protein in Lo Buffer (10 mM Tris-HCl [pH7.6], 1 mM EDTA, 50 mM NaCl, 0.05% NP40, 5 mM 2-mercaptoethanol) with 0.5 mg/ml BSA. The binding reaction was incubated at 30ºC for 1 hr. Glycerol was added in the reactions to reach final concentration of 5%, and reactions were stored on ice before gel shift analysis. Samples were separated on a 5% polyacrylamide-0.25X TBE gel at 100V at 4ºC for 1.5 hr. The gel was prerun for 1 hr at 50V, at which time the running buffer was replaced with fresh 0.25X TBE. Gels were dried and analyzed by storage phosphor screen and Quantity One software (Bio-Rad). The amount of Sir3-nucleosome complex was determined by the difference in free nucleosome for each sample. The data were fit to determine an apparent K_d using a simple model ($A + B \rightarrow AB$, where A refers to the mononucleosome, B refers to a Sir3 dimer) analyzed by the Kaleidograph software (Synergy Software).

Restriction Enzyme Protection

Chromatin was assembled with the biotinylated HMR PCR product produced in a PCR reaction with α-^{32}P-labeled dCTP. The chromatin was conjugated to magnetic beads and acetylated as above. Bead-conjugated acetylated wild-type or H4K16A mutant chromatin (100 ng DNA, ~800 fmol nucleosomes) was incubated with an increasing amount of the SIR complex (Sir3 and Sir2/4 pre-

incubated at a ratio of 3.3:1 and titrated as 170, 340, and 510 fmol Sir2/4) in 50 µl 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 0.02% NP-40, and 0.2 mM PMSF at RT for 45 min with rotation. Subsequently, reactions were initiated with 250 U BglII restriction enzyme (NEB) and incubated at 31ºC with rotation for 20 hr. A 25 µl aliquot was removed and quenched with 3 µl 0.5 M EDTA, the beads were separated by magnetic concentrator, and the supernatant was removed and mixed with 25 µl 1% SDS. Quenched reactions were divided in two: 20% was counted by liquid scintillation and 80% was digested with Proteinase K; the DNA was extracted with phenol-chloroform, precipitated with ethanol, and run on a 1% agarose-0.5X TBE gel. The released DNA fragment was visualized by storage phosphor screen.

Electron Microscopy

For samples prepared for EM analysis, the HMR fragment used in this study was generated by PCR with one primer modified at the 5´ end with desthiobiotin-TEG (W.M. Keck Oligo Facility, Yale University). This modified form of biotin can be eluted in a solution of free biotin (Hirsch et al., 2002). Chromatin was acetylated as above, and bead-bound chromatin was washed and stored in 20 mM HEPES (pH 8.0), 5 mM NaCl, 0.5 mM EDTA (HES). A solution of 0.1 M biotin was prepared by dropwise addition of 1 N NaOH until solubilized and immediately diluted to 20 mM in HES buffer. Chromatin was eluted from beads by incubation in HES with 20 mM biotin with agitation for 15 min (see scheme in Figure S4A). Eluted chromatin DNA was quantitated by gel electrophoresis. Reactions were performed in 15 µl containing 100 ng chromatin DNA (~800 fmol nucleosomes), SIR complex (630 fmol Sir2/4, 2.2 pmol Sir3) dialyzed in HES, and 0.2 mM NAD, when present. Samples were incubated for 1 hr at 30ºC and stored on ice before dialysis into HES containing 0.1 % glutaraldehyde at 4ºC for 4 hr, followed by 2 hr in 10 mM HEPES (pH 8.0), 5 mM NaCl, 0.5 mM EDTA, and storage at 4ºC.

Preparation of samples for electron microscopy was essentially as described (Nikitina et al., 2007) and 16 bit images recorded with a Tecnai 12 electron microscope (FEI Corporation) using a 2048 × 2048 CCD camera and tilted beam darkfield optics with pixel size of 0.9 nm. Images were processed by applying a mild low-pass filter to remove large scale illumination gradients and adjusting brightness and contrast for optimal clarity. Forty images for each sample were recorded and used to quantify chromatin particle diameter, based on the smallest circle that can encompass the entire array, as described previously (Francis et al., 2004). Multiple experiments were performed and representative images and histograms are presented (Figures 5E, 5F, and S4).

RNA Polymerase III Transcription Assays

A whole-cell yeast extract was prepared as described previously (Schultz, 1999) using the protease-deficient strain SF10 (Hoppe et al., 2002). Transcription assays were performed in a final volume of 25 μl 20 mM HEPES (pH 7.5), 70 mM KCl, 60 mM Potassium glutamate, 10% glycerol, 1 mM β-glycerophosphate, 10 mM Magnesium acetate, 3.5 mM EGTA, 6 μM $ZnSO_4$, 1 mM DTT, 0.08 mM PMSF, 0.25 mM ATP/GTP/CTP, 10 μM UTP, 0.25 U Protector RNase Inhibitor (Roche), 4 μCi α-^{32}P-UTP, and an ATP-regeneration system as above. Either 90 ng of naked biotinylated-HMR PCR product or a 90 ng (DNA) aliquot of a chromatin assembly reaction (~720 fmol nucleosomes) as described above was used as template and preincubated with the SIR complex (950 fmol Sir2/4 and 3.5 pmol Sir3), when present, for 1 hr at RT. The NTPs were added on ice, and the reaction was initiated with 2 μl (80 μg) of whole-cell extract. Reactions were quenched after 10 min for reactions in Figures 5B and 5C or 1 hr for Figure 5A with 25 μl 10 mM Tris-HCl (pH 8.0), 40 mM EDTA, and 1% SDS. Quenched reactions were deproteinated, phenol-chloroform extracted, ethanol precipitated, and resuspended in 95% formamide. Samples were heated to 65ºC for 5 min and products were

separated on a 5% acrylamide-TBE-urea gel, dried, and analyzed by storage phosphor screen as above.

Pol II Transcription

Strain BY4741 (Brachmann et al., 1998) was grown in 4 liters of YPD medium at 30°C to an optical density at 600 nm of 3–4. Cells were harvested by centrifugation at 5000× *g* for 12 min, suspended in 140 ml of 50 mM of Tris-HCl (pH 7.5, 30 mM DTT) and shaken slowly for 15 min at 30°C. The cells were harvested, suspended in 50 ml of YPD, 1 M sorbitol, and digested with 15 mg of Zymolyase 100T (Seikagaku Corp.) at 30°C until ~80% spheroplast formation (45 min). Digestion was stopped by addition of 100 ml of YPD-1 M sorbitol, and the spheroplasts were harvested. The spheroplasts were suspended in 250 ml of YPD-1 M sorbitol and incubated at 30°C with slow shaking for 30 min and recovered. After washing the spheroplasts with cold 1 M sorbitol, they were resuspended in 100 ml Buffer A (18% Ficoll 400, 10 mM Tris-HCl, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 3 mM DTT, 1X protease inhibitors). The spheroplasts were lysed with a motor-driven homogenizer, and cell debris and unlysed spheroplasts were removed by five centrifugations of the supernatant at 3000× *g* for 5 min. Nuclei were harvested by centrifugation at 25,000× *g* for 30 min and suspended in 10 ml of buffer B (100 mM Tris-acetate [pH 7.9], 50 mM potassium acetate, 10 mM magnesium sulfate, 20% glycerol, 3 mM DTT, 2 mM EDTA, lX protease inhibitors). Nuclei were lysed by dropwise addition of ammonium sulfate to a final concentration of 0.5 M with gentle stirring at 4°C. The suspension was mixed at 4°C for an additional 30 min and centrifuged in a Beckman type 71 rotor at 37,000 rpm for 1.5 hr at 4°C. The supernatant was adjusted to 75% saturation with ammonium sulfate by slow addition of solid ammonium sulfate. The ammonium sulfate precipitate was recovered by centrifugation at 10,500× *g* for 20 min, and the pellet was suspended in 4 ml of buffer C (20 mM

HEPES [pH 7.6], 10 mM MgSO4, 1 mM EGTA, 20% glycerol, 3 mM DTT, l× protease inhibitors). Finally, the suspension was dialyzed three times against 500 ml of the same buffer supplemented with 75 mM ammonium sulfate. Aliquots of the extract were flash frozen on dry ice. Assays for RNA Polymerase II activity were performed in 25 mM HEPES (pH 7.5), 6 mM magnesium acetate, 2.5 mM EGTA, 95 mM potassium acetate, 20 mM ammonium sulfate, 2 mM DTT, 1.7 mM β-mercaptoethanol, 0.01% NP-40, 0.34 U/μl Protector RNase inhibitor, 3.4 mM phosphocreatine, 0.034 U/μl creatine kinase in a final volume of 30 μl as described previously (Keogh et al., 2002). Chromatin assembly was performed with plasmid pUC18-G5cyc1^{G-} bearing five Gal4 binding sites upstream of a CYC1 promoter-driven G-less cassette with two predicted start sites producing transcripts of ~250 and 277 nt (see Sawadogo and Roeder, 1985; Woontner et al., 1991 for details on use of G-less templates). An additional larger product observed was likely a result of read-through transcription of the G-less region. When present, 50 ng of Gal4-VP16 fusion protein was incubated with the assembly reaction (100 ng chromatinized DNA, ~800 fmol nucleosomes) for 30 min at 30ºC and treatment with acetyltransferase was as described above. SIR complex (950 fmol Sir2/4 and 3.5 pmol Sir3) was preincubated with the chromatin template for 1 hr at RT and then placed on ice. A 10X mixture of magnesium acetate, RNase inhibitor, phosphocreatine, and creatine kinase was added. The resulting mixture was preincubated with 8 μl (320 μg) nuclear extract for 5 min, then NTPs were added to a final concentration of 500 μM ATP, GTP, and CTP; 10 μM UTP; and 5 μCi α-^{32}P-UTP. The reaction was incubated at RT for 20 min and quenched with 200 μl 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, and 100 U/ml RNase T_1. Quenched reactions were incubated for 25 min at 25ºC, then treated with SDS and Proteinase K for 30 min at 37ºC. The RNA was extracted with phenol-chloroform and ethanol precipitation in ammonium acetate. Products were separated on a 6% polyacrylamide-TBE-urea gel, which was dried and analyzed by storage phosphor screen.

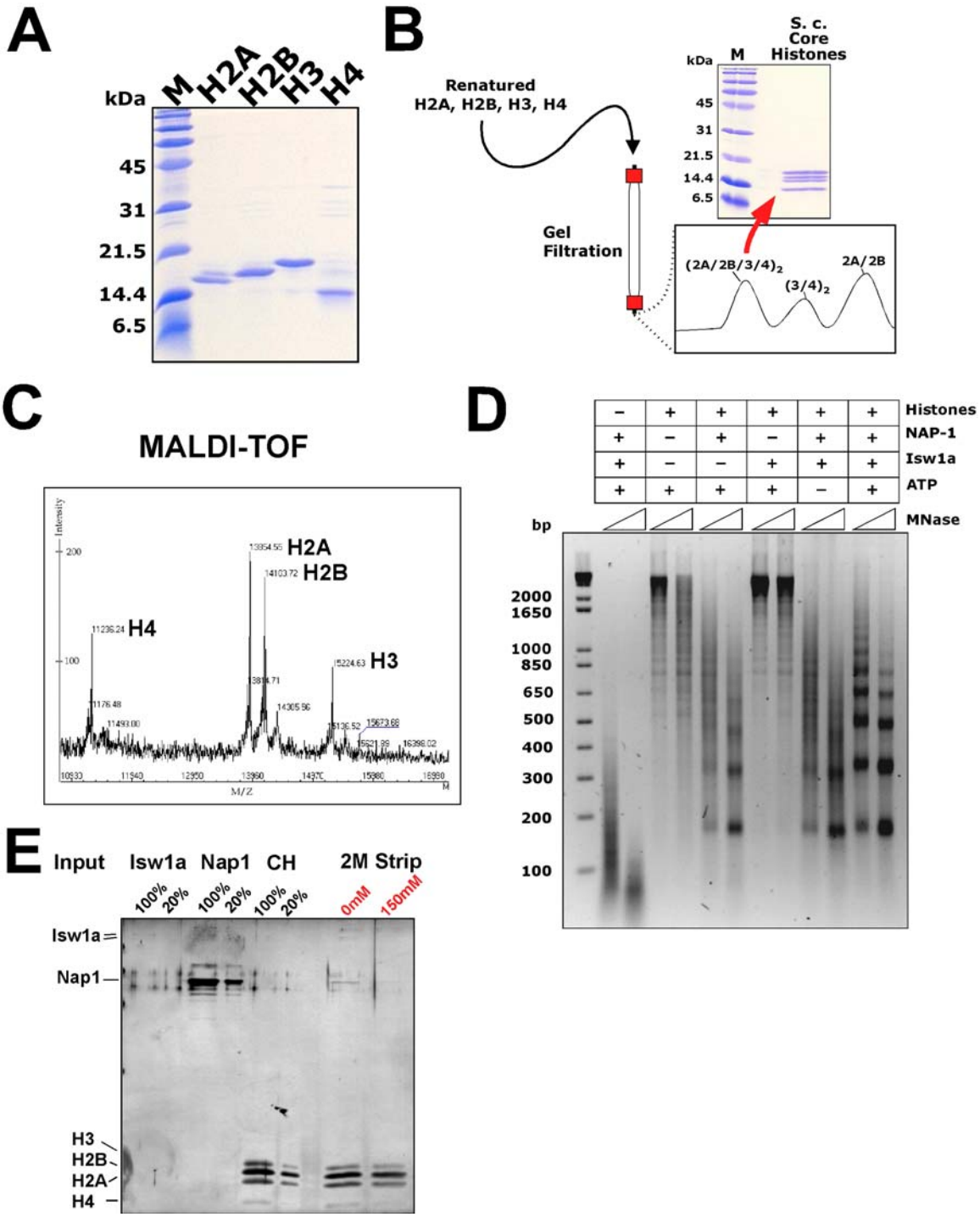


Figure S1.
(A) Purified yeast histone preparations.
(B) Scheme for histone octamer reconstitution by renaturation and gel filtration chromatography in 2M NaCl.
(C) MALD-TOF mass spectrometric analysis of the histone octamer preparation. Major peaks correspond to the molecular weights of the four core histones lacking the first methionine residue.
(D) Dropout assay for chromatin assembly. Assembly products were subjected to limited micrococcal nuclease (MNase) digestion as described in Experimental Procedures.
(E) Bead-conjugated chromatin was stripped with 2 M NaCl and the supernatant separated by SDS-PAGE. Chromatin assembly reaction inputs are shown on the left: The Isw1a complex (Isw1a), Nap1 histone chaperone (Nap1), and yeast recombinant core histones (CH). Bead washes with 0 mM or 150 mM NaCl were performed.

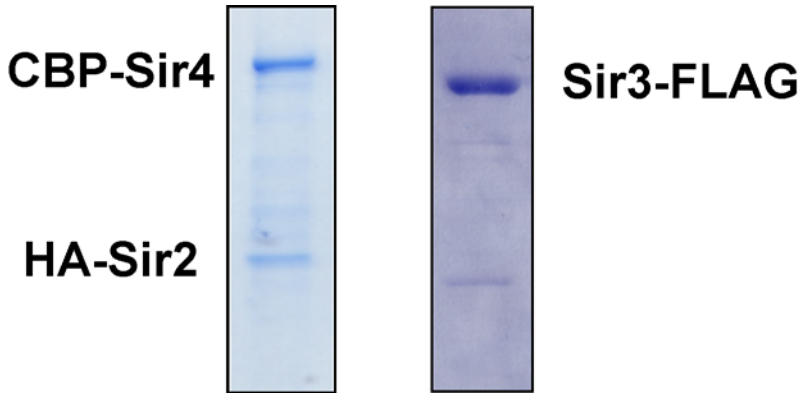


Figure S2. **Affinity Purifications of HA-Sir2/TAP-Sir4 and Sir3-FLAG**

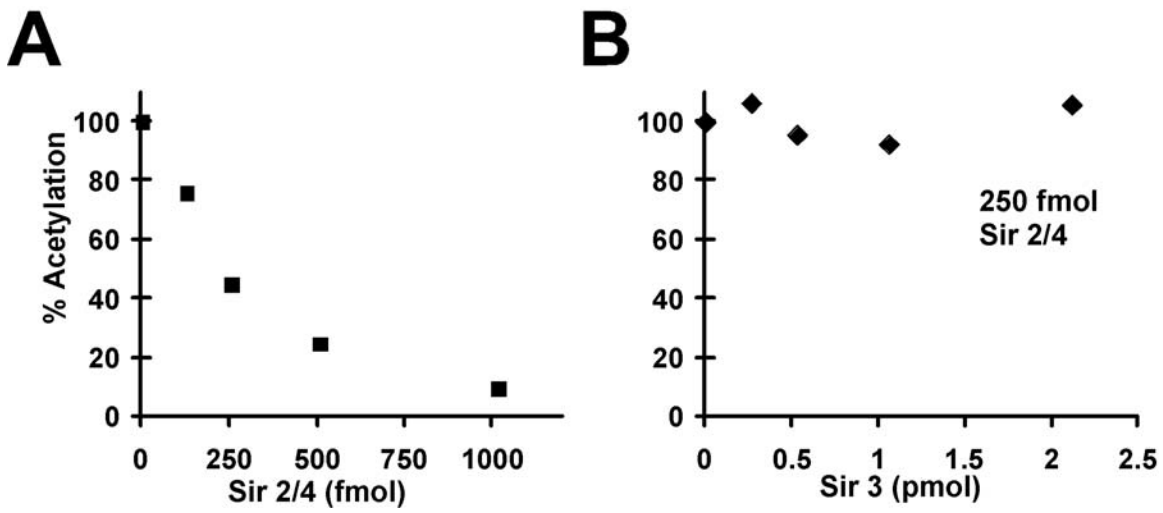


Figure S3.
(A) Titration of the Sir2/4 subcomplex into a reaction containing ^{3}H-acetylated chromatin and NAD as in Figure 3E.
(B) Titration of Sir3 into a similar reaction as in (A) with a constant amount of Sir2/4 subcomplex, 250 fmol, in all points.

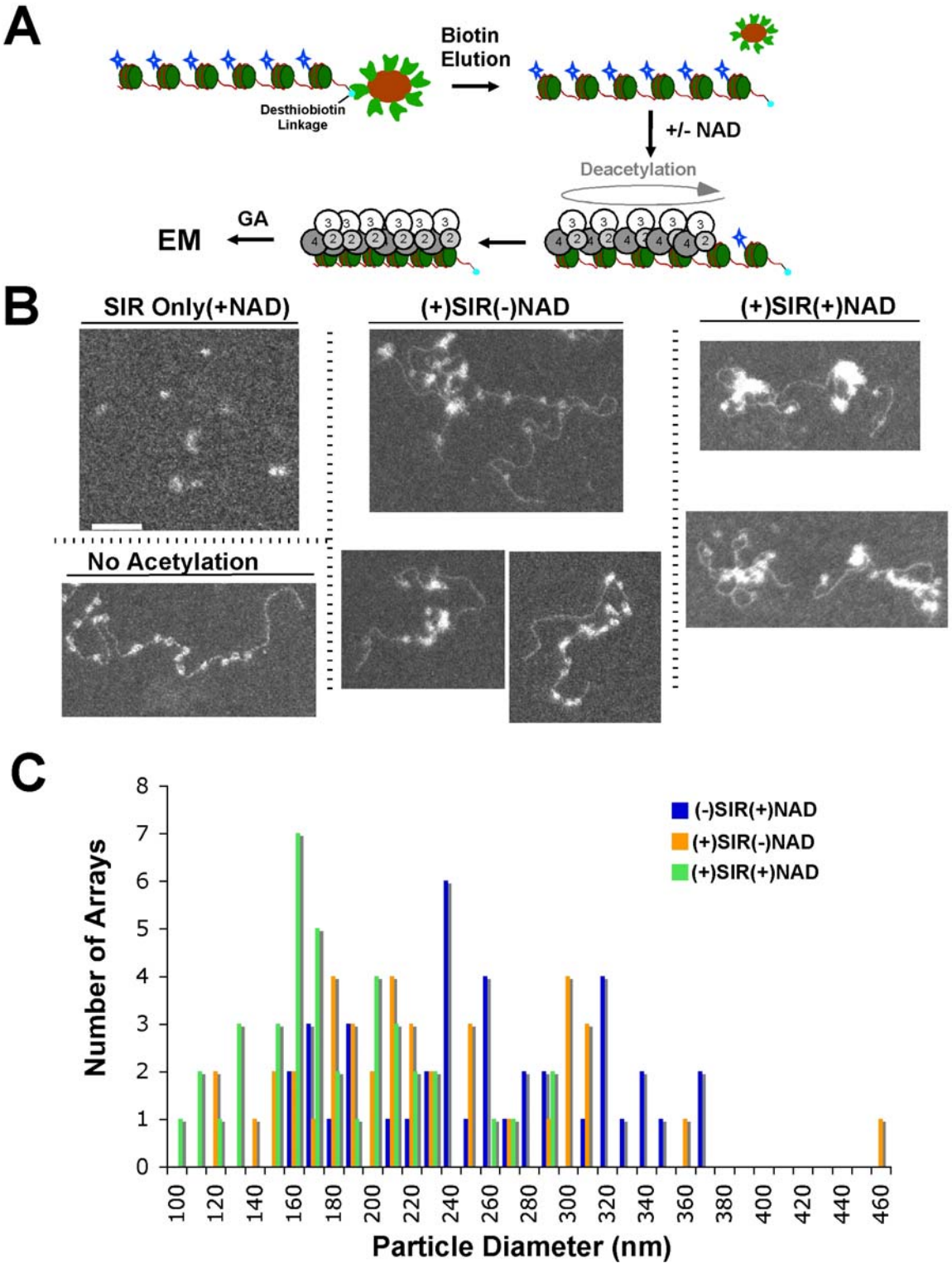


Figure S4.
(A) Scheme for preparing chromatin for samples analyzed by EM.
(B) Further representative samples under indicated conditions, including sample with SIR complex in the absence of chromatin and chromatin without acetylation.
(C) Full histogram of all three samples shown in Figures 5E and 5F.

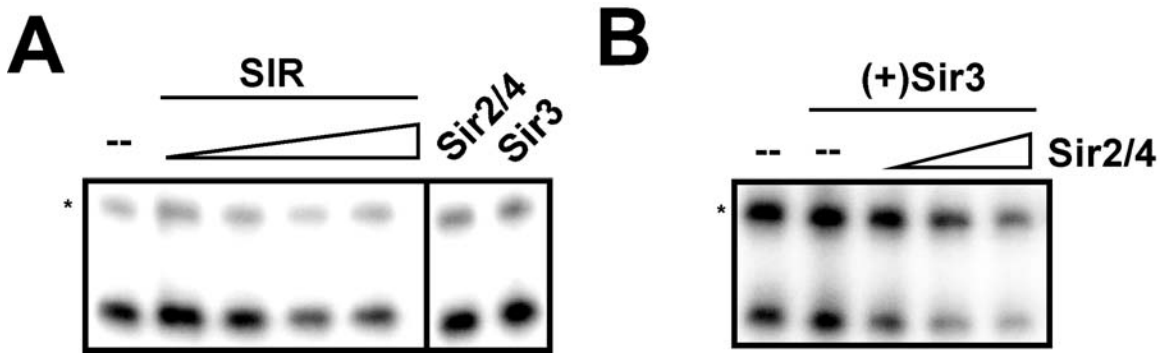


Figure S5.
(A) Titration of the SIR complex into Pol III transcription assay as described in Experimental Procedures.
(B) Titration of Sir2/4 into a Pol III transcription assay containing a constant amount of Sir3.

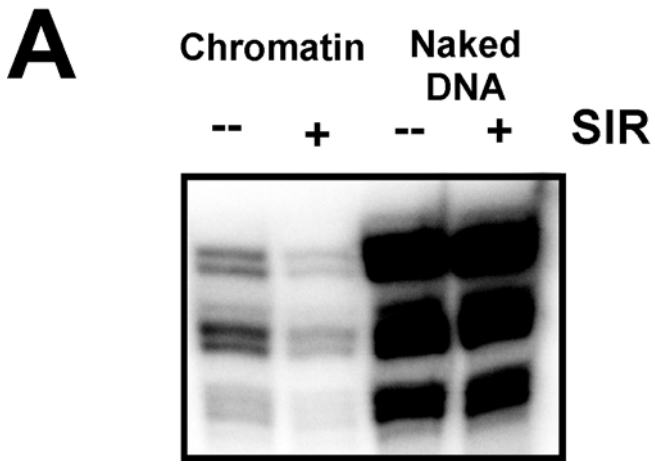
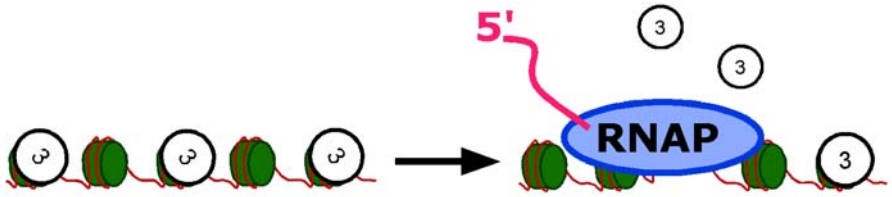
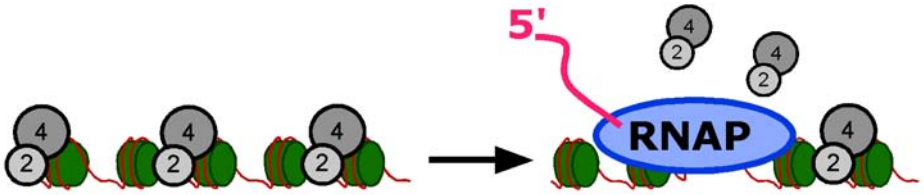


Figure S6.
(A) Activator-dependent Pol II transcription reactions as in Figure 7C. The activator-bound chromatin template was pre-incubated with or without SIR complex with chromatinized or naked DNA template. All reactions contain excess pBluescript plasmid DNA as a competitor.

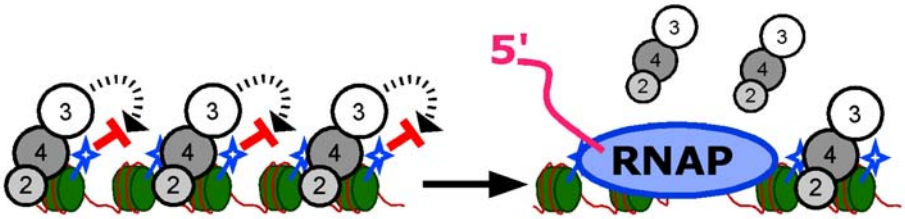
A. Sir3 binds to unmodified chromatin but cannot silence transcription



B. Sir2/4 binds to unmodified chromatin but cannot silence transcription



C. The SIR complex binds to acetylated chromatin but cannot silence transcription



D. The SIR complex deacetylates chromatin in the presence of NAD and silences transcription

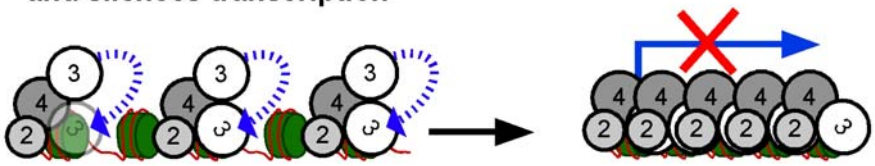


Figure S7. Expanded Model of Requirements for Transcriptional Silencing by the SIR Complex
(A and B) The individual components of the SIR complex bind to unmodified chromatin, but cannot silence transcription.
(C) Acetylated chromatin allows Sir2/4 binding, but prevents direct association of Sir3 with nucleosomes. This loosely associated SIR complex is permissive to transcription.
(D) NAD-dependent deacetylation generates a high-affinity substrate for Sir3 lockdown onto chromatin and promotes an altered SIR-chromatin complex that is repressive to transcription.

Table S1. Rates of Cleavage of Bead-Bound Templates by BglII as Described in Figures 5B-5D and Experimental Procedures

	Naked DNA	WT Chrom.	WT + SIR	H4K16A	H4K16A + SIR
%Digested/hr	>200	2.75	0.41	1.11	1.09

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