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Determinants of Sir2-Mediated, Silent Chromatin Cohesion

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Cohesin associates with distinct sites on chromosomes to mediate sister chromatid cohesion. Single cohesin complexes are thought to bind by encircling both sister chromatids in a topological embrace. Transcriptionally repressed chromosomal domains in the yeast *Saccharomyces cerevisiae* represent specialized sites of cohesion where cohesin binds silent chromatin in a Sir2-dependent fashion. In this study, we investigated the molecular basis for Sir2-mediated cohesion. We identified a cluster of charged surface residues of Sir2, collectively termed the EKDK motif, that are required for cohesin function. In addition, we demonstrated that Esc8, a Sir2-interacting factor, is also required for silent chromatin cohesion. Esc8 was previously shown to associate with Isw1, the enzymatic core of ISW1 chromatin remodelers, to form a variant of the ISW1a chromatin remodeling complex. When *ESC8* was deleted or the EKDK motif was mutated, cohesin binding at silenced chromatin domains persisted but cohesion of the domains was abolished. The data are not consistent with cohesin embracing both sister chromatids within silent chromatin domains. Transcriptional silencing remains largely intact in strains lacking *ESC8* or bearing EKDK mutations, indicating that silencing and cohesion are separable functions of Sir2 and silent chromatin.

Cohesin is best known for its role as the molecular glue that holds sister chromatids together. The ring-shaped complex associates with chromatin in a topological manner with DNA passing through the central void (1, 2). Substantial evidence supports a model in which single cohesin complexes embrace both sister chromatids. Such a double-embrace model, however, cannot account for additional evidence that suggests that the complex mediates cohesion at some locations without encircling both chromatids (3).

Other nuclear processes utilize the ability of cohesin to hold unlinked or distant DNAs together. The complex binds double-stranded DNA breaks to assist in repair using DNA homology from the sister chromatid (4, 5). The complex also binds in and around genes to influence their transcription and provide insulation from neighboring chromosomal domains (6). In the yeast *Saccharomyces cerevisiae*, the complex binds chromosome arms at sites between genes that are oriented toward one another (7, 8). Convergent transcription is thought to slide the topologically bound complexes toward one another to accumulate between gene pairs.

In budding yeast, cohesin is guided to additional sites by the chromatin-bound, histone deacetylases Sir2 and Hst1 (9, 10). Both enzymes belong to an evolutionarily conserved family of NAD-dependent, protein deacetylases known as sirtuins (11, 12). There are five sirtuins in yeast (Sir2 and Hst1 to Hst4) and seven in humans (SirT1 to SirT7). Both yeast Sir2 and yeast Hst1 repress the transcription of the genes to which they bind, albeit through the assembly of radically different chromatin structures. In humans, deacetylation of histones and other proteins by sirtuins regulates aging, as well as pathologies related to human health and disease. In this study, we focus primarily on Sir2 to understand how the yeast sirtuins participate in sister chromatid cohesion.

Sir2 represses genes at the *HM* mating-type loci and telomeres through the formation of an extended heterochromatin-like structure known as silent chromatin (13). The protein associates with additional factors, Sir3 and Sir4, to form a complex that is recruited to nucleation sites on chromatin known as silencers. Sir2 deacetylates neighboring nucleosomes to create additional recruitment sites for Sir3 and Sir4, both of which possess an affinity for deacetylated histone tails. Through cycles of histone deacetylation and histone binding, the Sir complex spreads several kilobases from silencers to yield large domains of silent chromatin. Sir2 is also recruited to the rRNA gene cluster (known as the rDNA), where it represses RNA polymerase II transcription by a mechanism that is less well understood.

Cohesin binds to silenced loci, including the well-characterized *HMR* locus, in a manner that requires silent chromatin assembly (3). At *HMR*, binding of cohesin was shown to mediate cohesin-dependent cohesion of the silenced sister chromatids. A tRNA gene adjacent to *HMR* acts as a genomic loading site for cohesin complexes that ultimately accumulate at *HMR* (14). Evidence that Sir2 was critical for the cohesin function within silent chromatin domains came from experiments that reconstituted cohesion by tethering Sir2 directly to DNA (9). In this context, a nonenzymatic, 63-amino-acid fragment of the Sir2 C terminus was sufficient for cohesin-dependent cohesion. We refer to this fragment as the cohesion-proficient domain of Sir2. How the domain interacts with cohesin is not known.

In this study, we identified two determinants of Sir2-mediated cohesion of silent chromatin: a cluster of charged residues on the surface of Sir2 and the Sir2-interacting factor Esc8, which is a subunit of a presumed ISW1a chromatin-remodeling complex. By mutating each of these factors, we show that robust silencing and cohesion are separable functions of silenced chromosomal domains. Interestingly, neither Esc8 nor the Sir2 surface is re-

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quired for cohesin binding. Instead, they are required for cohesin to function in promoting cohesion of silent chromatin domains.

MATERIALS AND METHODS

Strains and plasmids. All plasmids used in this study are listed in Table S1 in the supplemental material. The oligonucleotides used in plasmid or strain construction are listed in Table S2 in the supplemental material. Plasmid pYFC12 was constructed with two overlapping fragments bearing the 3′ end of *SIR2*, the *AKAA* mutation, and the *SIR2* 3′ untranslated region, which were cloned into EcoRI-digested pRS423 by PCR-mediated plasmid gap repair in yeast. The 2μ region of the plasmid was then removed by digestion with AfeI and religation to yield pYFC13. Plasmids pYFC29, pYFC30, and pYFC31 were constructed similarly. All modifications within open reading frames were confirmed by sequencing.

All strains used in this study are listed in Table S3 in the supplemental material. Complete open reading frame deletions were generated by PCR-mediated gene replacement and confirmed by PCR. Strains with mutations in *SIR2* were constructed by transformation with StuI-digested plasmids (pYFC13, pYFC29, pYFC30, and pYFC31), which generated tandem alleles of *sir2*: the first contained a full-length *sir2* mutant, and the second lacked the promoter and 5′ end of the gene. Additional strains were derived from crosses and confirmed by genetic selection: YFC8 is a segregant of MC52 × CSW84, YFC101 is a segregant of YCF8 × CSW116, and YFC109 is a segregant of CRC83 × YFC8.

Cohesion assays. Assays for cohesion of DNA circles were performed as described previously for the native *HMR* locus and the locus with lexA binding sites (3, 9). Briefly, cells were grown for 8 h in either yeast extract-peptone-dextrose-adenine (YPDA) or synthetic dextrose complete (SC) dropout medium (to select for plasmids) before inoculation of similar media containing 2% raffinose for overnight growth. On the following morning, the cultures were diluted back to an optical density at 600 nm of roughly 0.1 in yeast extract-peptone-adenine containing raffinose. When the cultures reached mid-log phase, nocodazole was added to arrest the cells in M phase (final concentration [C_f], 10 μg/ml; stock concentration, 1 mg/ml in dimethyl sulfoxide [Sigma]). Three hours later, galactose was added to a final concentration of 2% to induce DNA circle formation. After 2 h, cells were harvested and fixed with paraformaldehyde (C_f, 4%). In each case, data from at least three independent trials were pooled because they satisfied χ^2 tests of the homogeneity of proportions. Error bars represent the standard errors of proportions. Each reported value was compared to that for an appropriate control by a χ^2 test and judged to be significant using a 95% confidence interval.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as described in reference 9, with the following exceptions. Cultures were arrested in M phase and either cross-linked immediately to evaluate chromosomal loci or cross-linked 2 h after addition of galactose to evaluate DNA circles. Chromatin was sheared with a Bioruptor UCD-200 bath sonicator (Diagenode) in ice water using 20 cycles, each consisting of 15 s at high power followed by a 15-s rest. Anti-TAP antibody (Open Biosystems) and protein A Dynabeads (Invitrogen) were used for immunoprecipitation. DNA was eluted from the beads at 65°C for 10 min in 100 μl of buffer containing 1% SDS, 50 mM Tris-HCl, pH 7.5, and 10 mM EDTA. Cross-links were reversed by adding an equal volume of 10% Chelex 100 resin (Bio-Rad) and incubating at 95°C for 10 min. Quantitative PCRs were performed with a Rotor-Gene Q real-time PCR system (Qiagen) using the primers listed in Table S2 in the supplemental material. Pairwise Student's *t* tests relative to an appropriate control in each figure were performed to assess the statistical significance of at least three independent trials.

Silencing assays. (i) RT-PCR assay for expression of *HMR* a*1* gene. RNA extraction and reverse transcription-PCR (RT-PCR) were performed as described in reference 15 using the oligonucleotides listed in Table S2 in the supplemental material.

(ii) Spotting assays for *HML*, rDNA, and telomeric silencing. Strains were grown overnight to saturation in YPDA medium and spotted in 10-fold serial dilutions on appropriate indicator plates.

(iii) Sectoring assay for *HMR* silencing. For strains without plasmids, 300 to 500 cells from saturated cultures were spread on yeast extract-peptone-dextrose (YPD) plates. After 2 to 3 days, the plates were transferred to 4°C to enhance the red pigmentation. For strains carrying plasmids, cultures were grown in SC medium lacking Ura supplemented with extra adenine (C_f, 720 mg/liter) overnight at 30°C before they were spread cells on SC medium plates lacking Ura that contained 5 mg/liter of adenine.

Immunoblotting. Cell pellets were suspended in lysis buffer (100 mM Tris, pH 8.0, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and agitated with glass beads in a Mini-BeadBeater-16 cell disrupter (BioSpec) at 4°C in a cold room for five cycles, each of which was 1 min long. Protein concentrations were determined by the Bradford method (catalog number 500-0006; Bio-Rad). After SDS-PAGE, protein transfer and immunoblotting were performed as described previously (16). Blots were incubated with anti-PGK1 antibody at a 1:5,000 dilution (catalog number ab-113687; Abcam) and either anti-lexA antibody at a 1:1,000 dilution (catalog number 06-719; EMD Millipore) or anti-Sir2 antibody at a 1:5,000 dilution (catalog number sc-6666; Santa Cruz Biotechnology). Membranes were washed and then incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated (HPC) anti-rabbit immunoglobulin antibodies (catalog number W401B; Promega) and either HPC anti-mouse immunoglobulin antibodies (catalog number W402B, Promega) or HPC anti-goat immunoglobulin antibodies (catalog number sc2033; Santa Cruz Biotechnology). Immunoblots were developed with an enhanced chemiluminescence system (catalog number 32106, Thermo Scientific) according to the manufacturer's specifications.

Yeast two-hybrid screening. Bait strains L40 (wild type [wt]) and YFC35 (*sir2*Δ) carrying plasmid pCSW55 (lexA-Sir2$^{243–562}$-H364Y/TRP1, where Sir2$^{243–562}$-H364Y indicates Sir2 from amino acids 243 to 562 with the H364Y mutation) were transformed with a *LEU2* library of genomic fragments fused to GAL4$_{AD}$ (17). Positive interactors were first selected on SC medium lacking Trp, Leu, and His and containing 3-aminotriazole (0 to 20 mM) and/or nicotinamide (NAM; 0 to 2.5 mM). Positive candidates were then screened with a secondary *lacZ*, filter lift assay (16). All positive candidates were retransformed into a screening strain and tested again on SC medium lacking Trp, Leu, and His and containing 3-aminotriazole.

RESULTS

Full-length Hst1 mediates cohesion. To help define regions of Sir2 that mediate cohesion, we sought to make comparisons between the protein sequences of Sir2 and Hst1. Previous work had shown that a small C-terminal fragment of Hst1 was sufficient for cohesion when tethered to DNA (9). To validate that full-length Hst1 also mediates cohesion, we used genetic manipulation to direct the protein to a locus where cohesion can be measured easily. Ordinarily, Hst1 is recruited by Sum1 to target promoters where it represses transcription via localized histone deacetylation (18–20). The well-characterized *SUM1-1* mutant gene encodes a protein that gains the ability to bind silencers and spread in an *HST1*-dependent manner, even in the absence of Sir proteins (21–23). In this context, histone deacetylation and silencing of distal genes require the enzymatic activity of Hst1. We introduced the *SUM1-1* mutation into a strain where the cohesion of *HMR* can be measured with fluorescence microscopy. In this strain, the locus is tagged nearby with a lac operator array that binds green fluorescent protein (GFP)-tagged lacI (GFP-lacI). A pair of target sites for the R site-specific recombinase flanks the construct. Induction of the recombinase in M phase-arrested cells generates a pair of GFP-

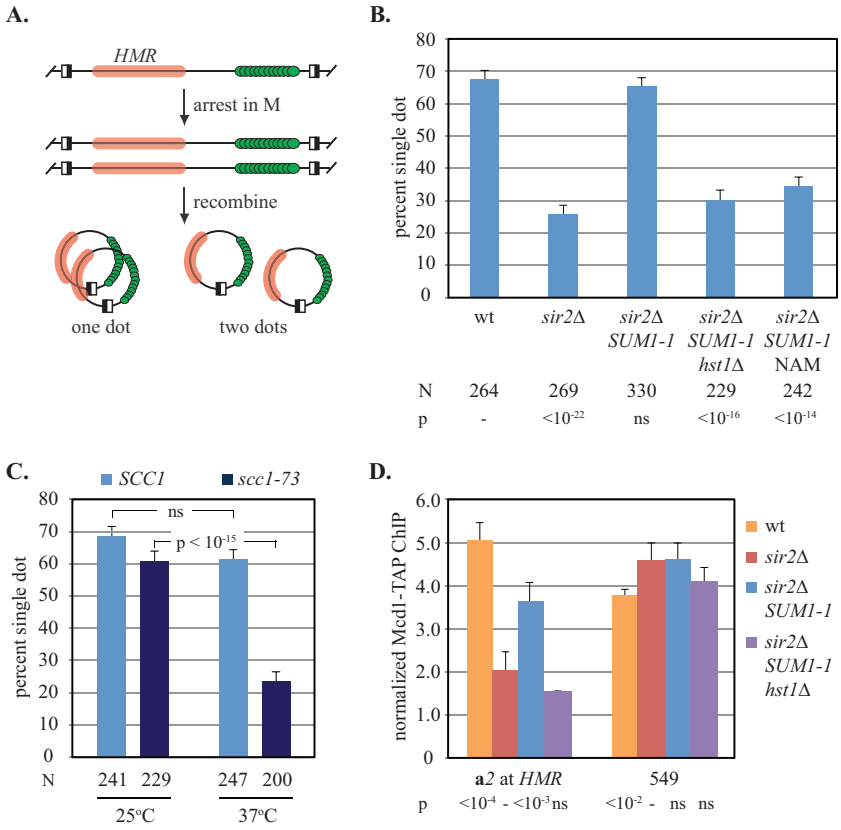

FIG 1 Full-length *HST1* mediates cohesion at *HMR* in a *SUM1-1* background. (A) Flow chart of assay. Silent chromatin, GFP-lacI, and recombinase sites are depicted with pink bars, green circles, and half-filled boxes, respectively. (B) Cohesion by full-length Hst1. Strains CSW10 (wt), CSW84 (*sir2*Δ), YFC8 (*sir2*Δ *SUM1-1*), and YFC97 (*sir2*Δ *SUM1-1 hst1*Δ) were used. NAM, nicotinamide; N, number of cells examined; p, *P* values obtained by pairwise χ^2 tests relative to the results for a benchmark strain (−); ns, not significant. (C) Cohesion by *HST1* requires cohesin. Strains YFC8 and YFC109 (*sir2*Δ *SUM1-1 scc1-73*) were arrested in M phase, and DNA ring formation was induced at 25°C before the cultures were split and half was shifted to 37°C for 2 h. (D) *HST1* is required for cohesin binding at *HMR* in a *SUM1-1* background. ChIP of Mcd1-TAP was performed in strains CSW116 (wt), YFC9 (*sir2*Δ), YFC101 (*sir2*Δ *SUM1-1*), and YFC103 (*sir2*Δ *SUM1-1 hst1*Δ). Binding at the both the **a***2* gene of *HMR* and the position 549 positive-control site was measured relative to binding at position 534, a negative-control site. *P* values obtained by Student's *t* tests relative to the results for a benchmark strain (−) are reported under the other strains.

tagged *HMR* DNA circles that yield one fluorescent dot if they cohere and two dots if they do not (Fig. 1A) (3).

In the parental strain with wild-type sirtuins and *SUM1*, cohesion of the *HMR* circles occurred in 67% of the M-phase cells (Fig. 1B). In the absence of *SIR2*, the frequency of cohesion dropped to 26%. Importantly, introduction of the *SUM1-1* mutation restored cohesion, but this was abolished either by simultaneous omission of *HST1* or by addition of nicotinamide (NAM), an inhibitor of sirtuin enzymatic activity. Cohesion in the *SUM1-1* strain was also abolished by introduction of a temperature-sensitive allele of *MCD1 (SCC1)*, which encodes a core subunit of cohesin (Fig. 1C). This result indicates that cohesion of *HMR* by *HST1* in the *SUM1-1* mutant also requires cohesin. Binding of cohesin at *HMR* was analyzed by chromatin immunoprecipitation (ChIP) of a TAP-tagged *MCD1* allele with an anti-TAP antibody in M-phase-arrested cells. All measurements were made relative to the measurement for a negative-control site and compared to the measurement for a positive-control site (positions 534 kb and 549 kb on chromosome V, respectively). In Fig. 1D, cohesin bound the chromosomal *HMR* locus in the parental strain, but that binding was lost when *SIR2* was deleted (9). Significantly, binding of cohesin was partly restored when the *SUM1-1* allele was introduced, but this binding was abolished when *HST1* was deleted. At the positive-control site (labeled 549 in Fig. 1D), introduction of *SUM1-1* or deletion of *HST1* did not influence cohesin binding. Collectively, these results show that full-length Hst1, like Sir2, generates cohesion of silenced sister chromatids by recruiting cohesin.

A Sir2 surface required for heterochromatic cohesion. Using amino acid conservation between Hst1 and Sir2 homologs in *Saccharomyces sensu stricto* species as one guiding criterion, we sought mutations within the cohesion-proficient domain of Sir2 that disrupt cohesion (Fig. 2A). Our attention was drawn to a cluster of charged amino acids, each mapping to a compact, solvent-exposed surface of a Sir2 crystal structure, defined by an antiparallel beta strand and adjoining hairpin loop (Fig. 2B). The glutamic and aspartic acids at positions 545 and 547, respectively, are absolutely conserved. The lysine at position 548 is fairly conserved and is replaced by isoleucine only when a second isoleucine is at position 546. Here we describe the consequences of simultaneously mutating the charged residues at E545, D547, and K548 to alanines. Other surface residues meeting our criteria were tested but did not produce effects as strong as those described below. Here,

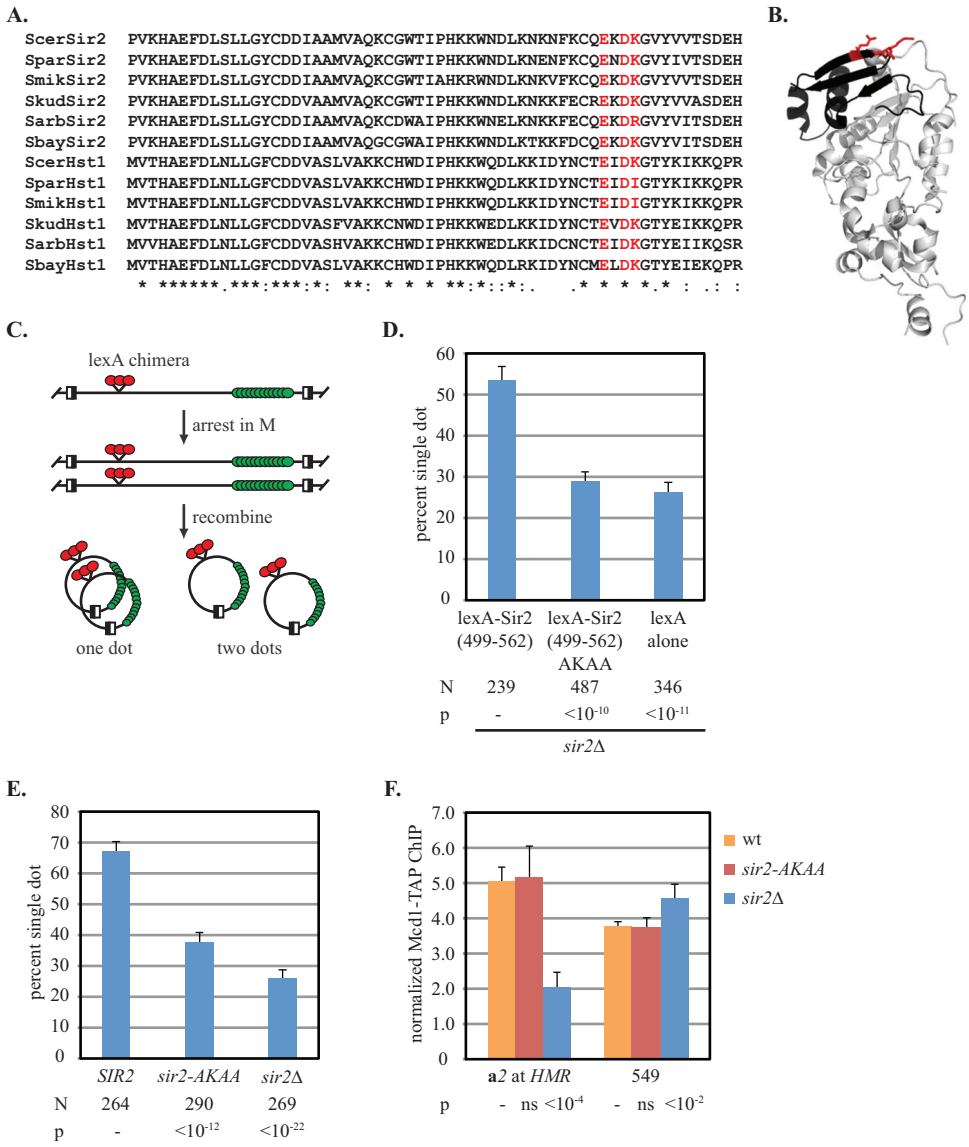

FIG 2 The EKDK motif is required for *SIR2*-mediated cohesion. (A) Alignment of *S. cerevisiae* Sir2 (residues 499 to 558) and Hst1 (residues 440 to 449) to homologs in *Saccharomyces sensu stricto* species, with conservation noted according to Clustal Omega criteria (61): asterisks, identical residues; colons, conservative substitutions; periods, semiconservative substitutions. Charged residues that were mutated are highlighted in red. Scer, *Saccharomyces cerevisiae*; Spar, *Saccharomyces paradoxus*; Smik, *Saccharomyces mikatae*; Skud, *Saccharomyces kudriavzevii* ; Sarb, *Saccharomyces arboricola*; Sbay, *Saccharomyces bayanus*. (B) Crystal structure of Sir2 residues 220 to 556. Sir2 crystallizes as a dimer of nearly identical subunits, but only one is shown (PDB accession number 2HJH, deposited by T. Ellenberger). The cohesion-proficient domain is shown in black, and the mutated residues of the EKDK motif are shown in red. (C) Flow chart of tethering assay. (D) The *sir2-AKAA* mutation abolishes cohesion by tethered Sir2. Strain CSW42 (*sir2*Δ) expressing lexA-Sir2$^{499-562}$ (pYFC1), lexA-Sir2$^{499-562}$-AKAA (pYFC10), and lexA alone (pBTM116H) was evaluated. (E) The *sir2-AKAA* mutation abolishes the cohesion of *HMR*. Strains CSW10 (wt), YFC1 (*sir2-AKAA*), and CSW84 (*sir2*Δ) were evaluated. (F) Cohesin recruitment by full-length Sir2-AKAA. ChIP of Mcd1-TAP was performed with strains CSW116 (wt), YFC7 (*sir2-AKAA*), and YFC9 (*sir2*Δ). *P* values obtained by Student's *t* test are reported relative to the results for the wild-type strain.

we refer to the charged cluster motif of Sir2 as EKDK and the mutant *sir2* allele with the designation *AKAA* (*sir2-AKAA*).

The role of the EKDK motif in Sir2-mediated cohesion was measured first with a protein-targeting assay (9). In this assay, a protein fragment of interest is tethered via lexA to GFP-tagged DNA circles formed by recombination in M-phase-arrested cells (Fig. 2C). To this end, lexA was fused to the 63-amino-acid cohesion-proficient domain of Sir2 (residues 499 to 562 [Sir2$^{499-562}$]). The domain is not sufficient to nucleate silencing when tethered directly to DNA (see Fig. S1 in the supplemental material). Cohesion of the DNA circles occurred in 54% of the cells expressing lexA-Sir2$^{499-562}$, as reported previously (9). When lexA-Sir2$^{499-562}$-AKAA was expressed, cohesion occurred in only 30% of the cells (Fig. 2D). By comparison, expression of lexA alone yielded cohesion in only 25% of the cells. The negative impact of the *AKAA* mutation on cohesion was not due to differential protein expression. Immunoblotting with antibodies against lexA showed that both chimeras were expressed equally (see Fig. S2A in the supplemental material). These data show that the EKDK motif mediates cohesion by Sir2.

To test whether the Sir2 EKDK cluster is required for cohesion in a silent chromatin context, *HMR* DNA circles were examined in a strain bearing the *AKAA* mutation within full-length *SIR2*. Immunoblotting with antibodies against Sir2 showed that the mutant was expressed at normal levels, as were all other full-length *sir2* alleles used in this study (see Fig. S2B in the supplemental material). As first shown in Fig. 1B, *HMR* circles cohered in 67% of the parental strain, whereas cohesion dropped to 26% in a *sir2*Δ negative control (Fig. 2E). The *sir2-AKAA* allele yielded cohesion in only 37% of the cells. We conclude that the EKDK motif of Sir2 is important for cohesion of native silent chromatin. That cohesion was not entirely eliminated by the *AKAA* mutation in full-length Sir2 as it was with the tethered cohesion-proficient domain indicates either that the mutation is not fully penetrant or that other features of silent chromatin, perhaps even other domains of Sir2, contribute to cohesion at *HMR*.

Cohesin recruitment by the Sir2 EKDK motif. ChIP was used to measure the impact of the *AKAA* mutation on cohesin binding at the chromosomal *HMR* locus, as described in the legend to Fig. 1D. Unexpectedly, Mcd1-TAP bound as well at *HMR* in the *sir2-AKAA* mutant as it did in the wild-type strain (Fig. 2F). Similar results were obtained when ChIP was performed on the *HMR* DNA circles used in the cohesion assay (see Fig. S3 in the supplemental material). These data indicate that cohesion loss in the *AKAA* mutant, as indicated in Fig. 2E, is not due to the loss of cohesin binding. If the EKDK motif is the sole cohesin recruitment surface of Sir2, then mutation to AKAA may result in loading of nonproductive cohesin complexes. Alternatively, additional features of silent chromatin may participate in cohesin recruitment, but those surfaces cannot utilize cohesin without participation of the EKDK motif. Either way, it should be noted that inactivation of Sir2 with an inhibitor during M phase also caused a loss of cohesion without a loss of cohesin binding (3).

Cohesion defects in *sir2-AKAA* strains are not caused by defects in silencing. A simple explanation for the loss of cohesion in strains bearing the *AKAA* mutation in full-length Sir2 is that the alteration interferes with proper silent chromatin assembly. Therefore, RT-PCR was used to determine whether the **a***1* gene at *HMR* remained silent when the locus was excised from the chromosome and circularized in the cohesion assay. mRNA from the nonsilent *KCC4* locus was used as an internal control. Figure 3A shows that **a***1* transcripts were not detectable either in a wild-type strain or in the *sir2-AKAA* mutant. In contrast, **a***1* transcripts were easily measured in a strain lacking *SIR2*. These results indicate that the *sir2-AKAA* mutation does not impair silencing of *HMR*.

Silencing of *HMR* is particularly robust, which might mask subtle defects in Sir2-mediated transcriptional repression. Therefore, phenotypic assays were used to measure the impact of the *AKAA* mutation on silencing at other locations where Sir2 acts. At the *HML* mating-type locus, silencing was measured with an *ADE2* reporter gene, which is required for adenine prototrophy (24). Saturated cell cultures were spotted in 10-fold serial dilutions on indicator plates with SC medium lacking Ade. Figure 3B shows that neither the wild-type strain nor the *sir2-AKAA* strain grew in the absence of adenine, whereas the *sir2*-null strain grew unhindered. These results show that the *sir2-AKAA* mutation has no substantial impact on silencing at *HML*.

Silencing at telomeres and the rDNA was measured with *URA3* reporter genes inserted either at the terminus of a chromosome VII truncation or within the intergenic regions of the rRNA gene repeat unit (*IGS1* and *IGS2*). Tenfold serial dilutions of saturated cultures were spotted on SC medium plates containing 5-fluoroorotic acid (5-FOA), a toxic metabolite of the *URA3* gene product (25). In each case, the wild-type strain and the *sir2-AKAA* mutant grew equally well, whereas the *sir2*-null strain did not grow at all (Fig. 3C and D). These results indicate that silencing at the rDNA and telomeres is not affected by the *sir2-AKAA* mutation. If it is presumed that the *AKAA* mutation causes a loss of cohesion at all of these loci as it does at *HMR*, the results indicate that robust transcriptional silencing does not require cohesion of silent chromatin.

Silencing and cohesion are separable functions of Sir2. Collectively, the silencing assays described in the preceding section indicate that mutation of the Sir2 EKDK motif does not cause a gross alteration of silent chromatin structure. To evaluate silencing at *HMR* with greater sensitivity, we turned to an assay that measures persistence of the silent state during colony formation (26). The assay utilizes an attenuated *HMR* locus with a partially disabled silencer (*hmr*Δ*a*) and an *ADE2* reporter gene that suppresses accumulation of a red pigment on medium with low levels of adenine. Lineages of cells that have switched from the silent to the nonsilent state produce white sectors in an otherwise red background, whereas lineages of cells that restore silencing yield red sectors in a white background. We binned colonies into four categories based on visual inspection: (i) fully red, (ii) mostly red, (iii) mostly white, and (iv) fully white. Figure 4A shows that the *hmr*Δ*a*::*ADE2* reporter was fully silenced (completely red) in 72% of the colonies of a wild-type strain, whereas no red pigment was observed in a *sir2*Δ strain. Significantly, only 1% of the colonies were fully silenced in the *AKAA* mutant. Thus, *sir2-AKAA* shifts the silencing equilibrium toward the derepressed state in this sensitized assay.

The silencing defects highlighted by the sectoring assay raised the possibility that the loss of cohesion was due to subtle differences in silencing rather than a cohesin-specific defect introduced by the *AKAA* mutation. In a recent cocrystal of Sir2 and Sir4 fragments, the last residue of the EKDK motif (K548) was found to be precariously close to a Sir2 cleft occupied by Sir4 (27). Binding of Sir4 displaces the K548 residue from the cleft (see Fig. S4 in the supplemental material). Thus, it was conceivable that the *AKAA* mutation destabilizes the silent chromatin structure by perturbing the Sir2-Sir4 interaction. To disentangle the roles of the EKDK motif in silencing and silent chromatin cohesion, we attempted to suppress the silencing defect of the *AKAA* mutant by providing additional Sir4. The protein is limiting for silencing, and even subtle increases in the *SIR4* gene dosage can improve transcriptional repression (26, 28–30). In the case of the sectoring assay, extra *SIR4* provided by a low-copy-number centromere plasmid in a wild-type strain increased the fraction of completely red colonies from 65% to nearly 100% (Fig. 4B). Significantly, the additional *SIR4* also restored silencing in a *sir2-AKAA* mutant, increasing the number of completely red colonies from 1% to 86%. These results show that roughly doubling the level of *SIR4* suppresses the silencing defect caused by *sir2-AKAA*.

We next asked whether suppression of the *AKAA* silencing defect also suppresses the cohesion defect. To address this question, we evaluated cohesion of *HMR* DNA circles in strains bearing extra *SIR4* on a low-copy-number centromere plasmid. Irrespective of the *SIR2* allele tested (*SIR2*, *sir2*Δ, or *sir2-AKAA*), the additional *SIR4* did not improve cohesion (Fig. 4C). These results

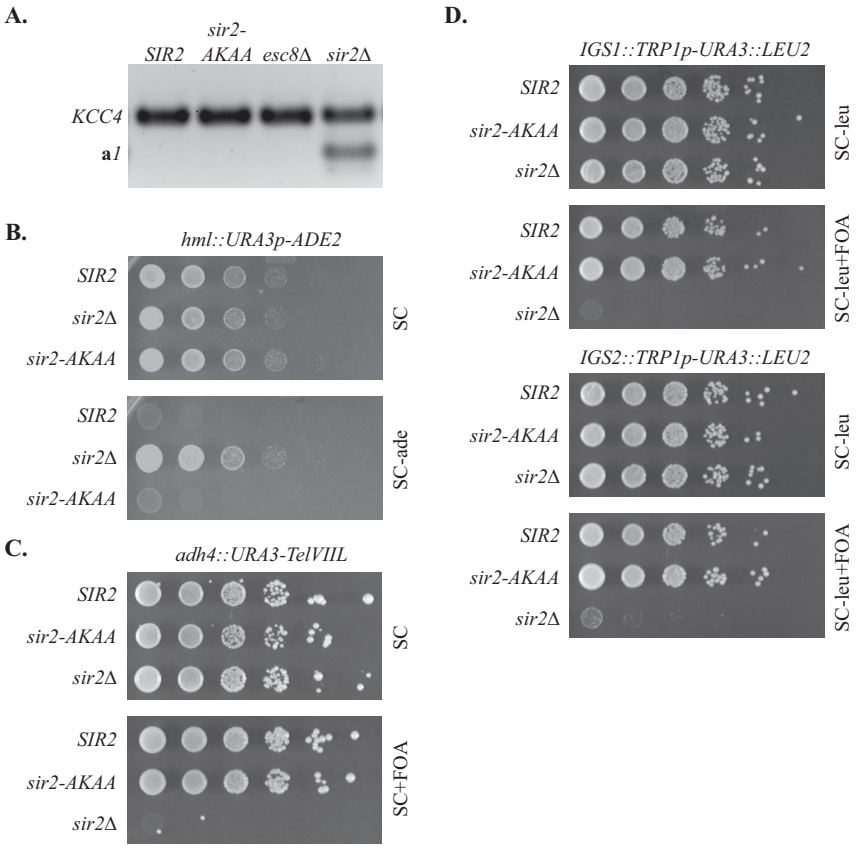

FIG 3 Persistence of transcriptional silencing in strains with *sir2-AKAA*. (A) Analysis of *HMR* **a***1* by RT-PCR. RNA extracts were prepared from strains YFC150 (wt), YFC152 (*sir2-AKAA*), YFC153 (*esc8*Δ), and YFC151 (*sir2*Δ) that had been grown according to the cohesion assay protocol. (B) Silencing of *URA3p-ADE2* reporter at *HML*. Strains THC74 (wt), YFC24 (*sir2-AKAA*), and CCC3 (*sir2*Δ) were spotted in 10-fold serial dilutions on SC medium lacking Ade (SC-ade) to measure silencing and on SC medium as a loading control. (C) Silencing of *URA3* at a telomere created at *ADH4* by truncation of the chromosome VII left arm (*TelVIIL*). Strains YDS631 (wt), YFC12 (*sir2-AKAA*), and CCC1 (*sir2*Δ) were spotted on SC medium plus 5-FOA (SC+FOA) to measure silencing and on SC medium as a loading control. (D) Silencing of *URA3* within a *TRP1p-URA3*::*LEU2* cassette at the *IGS1* and *IGS2* sites of an rRNA gene repeat. Strains DMY2804 (wt), YFC13 (*sir2-AKAA*), and DMY2835 (*sir2*Δ) with the reporter at *IGS1* and strains DMY2800 (wt), YFC3 (*sir2-AKAA*), and DMY2831 (*sir2*Δ) with the reporter at *IGS2* were spotted on SC medium lacking Leu plus 5-FOA (SC-leu+FOA) to measure silencing and SC medium lacking Leu (SC-leu) as a loading control.

demonstrate that cohesion defects imposed by *sir2-AKAA* persist even when silencing is restored to full capacity. We conclude that silencing and cohesion are separable functions of *SIR2* and that the *sir2-AKAA* mutation affects the cohesion of silent chromatin directly.

To analyze the EKDK motif in finer detail, individual residues of Sir2 were mutated. Replacement of E545 with alanine yielded the *sir2-AKDK* mutant, replacement of D547 with alanine yielded the *sir2-EKAK* mutant, and replacement of both amino acids with alanine yielded the *sir2-AKAK* mutant. Importantly, the *sir2-AKDK* mutant altered the EKDK residue that lies furthest from Sir4 in the cocrystal (see Fig. S4 in the supplemental material). Figure 4D shows that each of the three new mutants disrupted the cohesion of silent chromatin to the same extent as the original *AKAA* mutation. Additionally, each of the new mutants yielded a derepression phenotype that was intermediate between that of the wild type and that of the original *sir2-AKAA* mutant in the sensitive *hmr*Δ*a*::*ADE2* sectoring assay (Fig. 4E). Collectively, the results obtained with the new mutants show that cohesion defects do not scale linearly with silencing defects. These results reinforce the notion that mutations of the EKDK domain do not disrupt silent chromatin cohesion by interfering with Sir4.

Esc8: a Sir2-interacting partner required for silent chromatin cohesion. Traditional two-hybrid screening was performed to identify Sir2-interacting factors responsible for Sir2-mediated cohesion. A bait protein containing the Sir2 enzymatic core and the adjacent cohesion-proficient domain (collectively spanning amino acids 243 to 562) recovered a number of previously identified Sir2-interacting factors from a genomic library. Our studies focused on one factor that was isolated, Esc8, which was first identified in a screen for proteins that restore silencing when tethered to a mutated silencer (31). Esc8 has been shown to associate directly with Sir2 in glutathione *S*-transferase pulldown experiments. Our two-hybrid studies showed that the C-terminal domain of Esc8 (amino acids 640 to 714) contains a Sir2 interac-

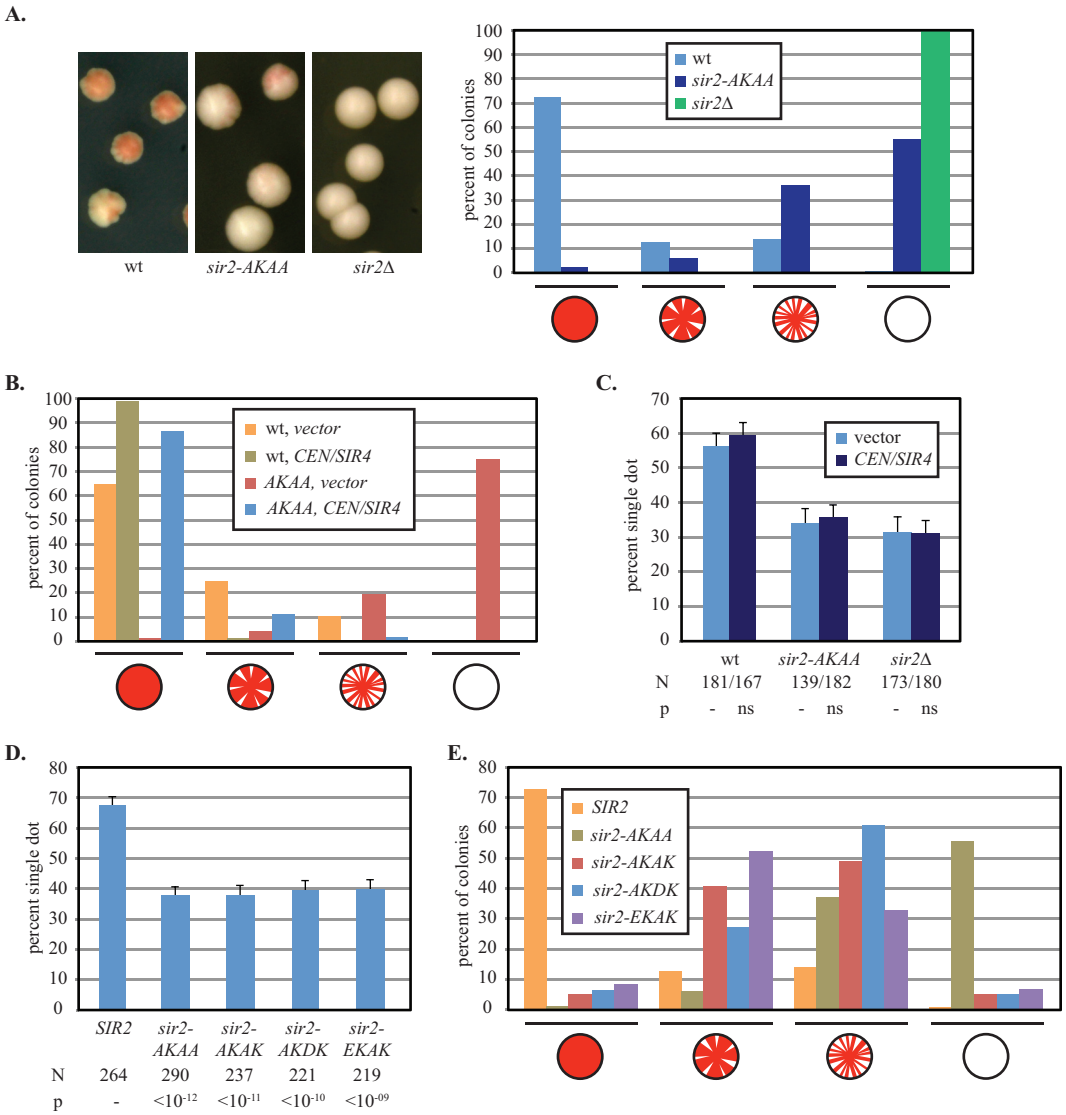

FIG 4 Separation of silencing and cohesion functions of Sir2 with the *AKAA* mutation. (A) Variegated silencing of an attenuated *HMR* locus by the *sir2-AKAA* mutation. Strains GCY317 (wt), YFC44 (*sir2-AKAA*), and YFC116 (*sir2*Δ) bearing the *hmr*Δ*a*::*ADE2* allele were used. (B) Suppression of silencing defects in the strains described for panel A by a centromeric *SIR4* expression plasmid (pJR368). Empty vector (pRS416) was used as a control. At least 300 cells for each strain-plasmid combination were scored in at least three independent trials. (C) Extra *SIR4* does not suppress the *sir2-AKAA* cohesion defect at *HMR*. *HMR* DNA circles were evaluated in strains CSW10 (wt), YFC1 (*sir2-AKAA*), and CSW84 (*sir2*Δ) bearing either a centromeric *SIR4* expression plasmid (pJR368) or empty vector (pRS416). (D) Point mutations within the Sir2 EKDK motif disrupt cohesion of *HMR* DNA circles. Strains CSW10 (wt), YFC1 (*sir2-AKAA*), YFC137 (*sir2-AKAK*), YFC138 (*sir2-AKDK*), and YFC139 (*sir2-EKAK*) were evaluated. (E) Point mutations of the Sir2 EKDK motif impact silencing minimally. Silencing of the *hmr*Δ*a*::*ADE2* reporter was evaluated in strains GCY317 (wt), YFC44 (*sir2-AKAA*), YFC140 (*sir2-AKAK*), YFC141 (*sir2-AKDK*), and YFC142 (*sir2-EKAK*).

tion domain. Additionally, Esc8 and Sir2 interacted when the *AKAA* mutation was present in Sir2, suggesting that the EKDK motif is not required for the association of the two proteins (see Fig. S5 in the supplemental material).

Esc8 is a paralog of Ioc3, a protein that associates with the Isw1 ATPase to form ISW1a, a chromatin-remodeling complex (32). Homology modeling with a crystal structure of ISW1a suggests that Esc8 retains the residues necessary for Isw1 binding (33). Indeed, Esc8 and Isw1 interact in proteome-scale studies (34). Thus, we infer that Esc8 associates with Isw1 *in vivo* to form a variant of the ISW1a chromatin-remodeling complex.

Cohesion of *HMR* DNA circles was measured in strains lacking *ESC8*, *IOC3*, or *ISW1*. Figure 5A shows that cohesion of the circles was disrupted in the *esc8* and *isw1* mutants and to a less severe extent in the *ioc3* mutant. Isw1 associates with Ioc2 and Ioc4 to form a distinct chromatin-remodeling complex named ISW1b. Deletion of *IOC4* affected *HMR* cohesion to a similar extent as deletion of *IOC3* (Fig. 5A). These results suggest that the ISW1 chromatin-remodeling complexes facilitate the cohesion of *HMR* but that ISW1a complexes containing Esc8 play the most prominent role.

Cohesion by tethered lexA-Sir2$^{499-562}$ was also measured in strains lacking subunits of the Isw1 remodeling complexes. Whereas cohesion by the chimeric protein was abolished in a strain lacking *ISW1*, deletion of *ESC8* alone or in combination with *IOC3* bore no effect (Fig. 5B). Deletion of *IOC4* also had no

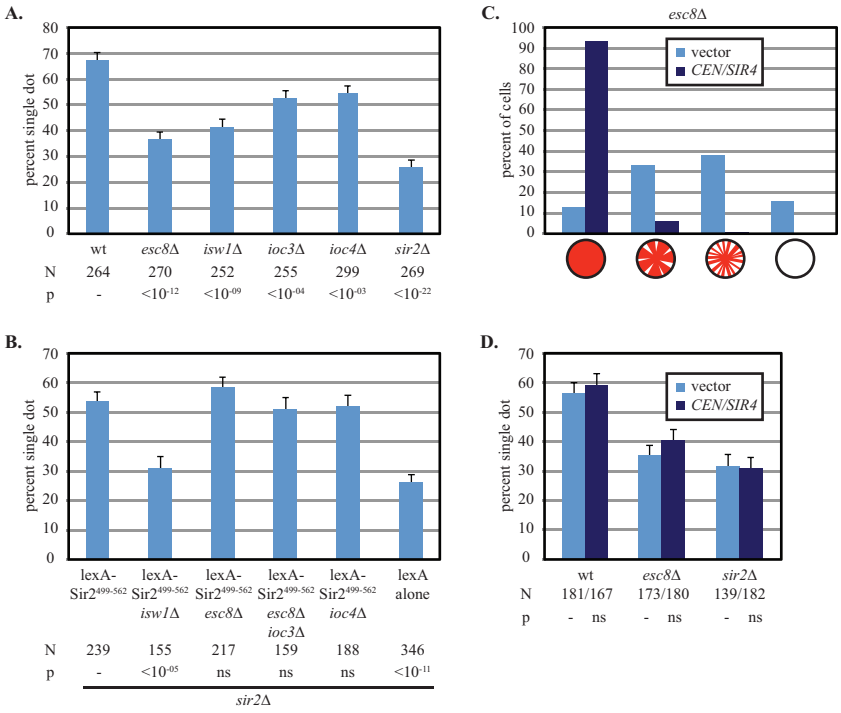

FIG 5 *ESC8* in silent chromatin cohesion and transcriptional silencing. (A) Cohesion of *HMR* requires *ESC8*. *HMR* DNA circles were produced in strains CSW10 (wt), YFC18 (*esc8*Δ), YFC133 (*isw1*Δ), YFC130 (*ioc3*Δ), YFC144 (*ioc4*Δ), and CSW84 (*sir2*Δ). (B) Cohesion by tethered Sir2 does not require *ESC8* or the *IOC* genes. DNA circles bearing the lexA binding sites were produced in strains CSW42 (*sir2*Δ), YFC135 (*sir2*Δ *isw1*Δ), YFC21 (*sir2*Δ *esc8*Δ), YFC128 (*sir2*Δ *ioc3*Δ *esc8*Δ), and YFC145 (*sir2*Δ *ioc4*Δ) expressing lexA-Sir2$^{499-562}$ (pYFC1). Strain CSW42 expressing lexA alone (pBTM116H) was included as a negative control. (C) Silencing defects in the absence of *ESC8* are suppressed by extra *SIR4*. Assays were performed with strains GCY317 (wt) and GCY310 (*esc8*Δ) bearing either a centromeric *SIR4* expression vector (pJR368) or empty vector (pRS416). (D) Extra *SIR4* does not suppress cohesion defects caused by the loss of *ESC8*. *HMR* DNA circles were produced in strains CSW10 (wt), CSW84 (*sir2*Δ), and YFC18 (*esc8*Δ) bearing either an empty vector (pRS416) or a centromeric *SIR4* expression vector (pJR368).

impact. Similar results were obtained with lexA-Sir2$^{78-562}$, which nucleates silent chromatin domains when tethered to DNA (see Fig. S1 and S6 in the supplemental material). Why do the two assays for cohesion, one using native chromatin and the other using tethered proteins, display different dependencies on *ESC8*? We suspect that the distinction lies in the *cis*-acting silencers of the loci under study. The native silent chromatin assay includes the *E* and *I* silencers that normally flank *HMR*, whereas the tethering assay replaces both silencers with a single highly engineered construct that contains lexA sites (35). Apparently, the replacement of bona fide silencers with sites for artificial Sir2 recruitment interferes with the specification for the ISW1a chromatin remodeling complex. The results suggest that Isw1 acts without Ioc proteins on the synthetic construct, as was found for Isw1 in transcriptional silencing at the rDNA (36).

Disentangling of the roles of *ESC8* in silencing and sister chromatid cohesion. Previous characterization of ISW1 complexes in transcriptional silencing demonstrated that deletion of *ESC8* yielded phenotypes less severe than those yielded by deletion of *ISW1* (31, 37). In support, the RT-PCR assay in Fig. 3A shows that the gene was not required for silencing of *HMR* DNA circles. Deletion of *ESC8*, however, did produce silencing defects when the sensitive *hmr*Δ*a*::*ADE2* reporter was used, as confirmed in Fig. 5C (compare the result to that for the wild type in Fig. 4B). Given the minimal but measurable role of Esc8 in silencing, we focused on *esc8* mutants for the remainder of the study.

If an *esc8* mutant compromises silencing even in subtle ways, then the diminution of silencing could be responsible for the loss of cohesion. To investigate this possibility, we tested whether extra *SIR4* could suppress the silencing defect of an *esc8* mutant. Figure 5C shows that a centromeric plasmid bearing *SIR4* restored silencing to normal levels. Importantly, restoration of silencing by extra *SIR4* did not suppress the cohesion defect in the absence of *ESC8* (Fig. 5D). This result suggests that ISW1a complexes containing Esc8 affect the cohesion of silent chromatin, in addition to the role that the complexes play in transcriptional silencing.

ChIP was used to test whether *ESC8* is required for cohesin binding at silenced loci. Figure 6A shows that the association of the Mcd1 cohesin subunit with *HMR* was not diminished by the loss of *ESC8*. Similar results were obtained when ChIP was performed on the *HMR* DNA circles used in the cohesion assay (see Fig. S3 in the supplemental material). As with the *AKAA* mutation, these data indicate that *ESC8* is required for the formation of productive cohesin complexes at silent chromatin but not recruitment of the complex to the locus.

To test whether *ESC8* is a global cohesion regulator, cohesion at other loci was tested. Centromeric cohesion was evaluated with a centromeric plasmid bearing tet operators in a strain expressing tetR-GFP (38). Figure 6B shows that the plasmid exhibited a high level of cohesion (84%) in wild-type cells arrested in M phase. Cohesion was not diminished by deletion of *ESC8*. As a positive

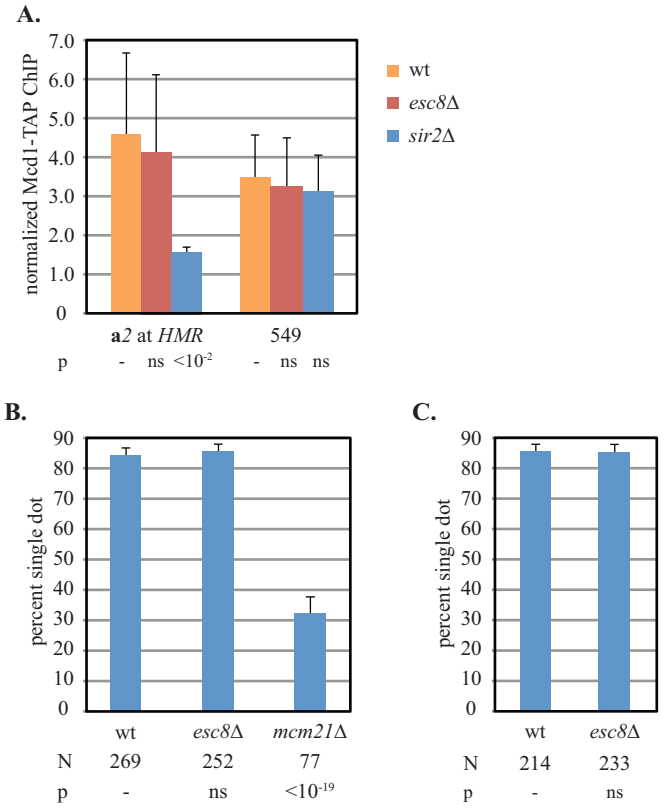

FIG 6 Loss of *ESC8* disrupts the cohesion of silent chromatin selectively without causing a loss of cohesin. (A) Binding of cohesin at *HMR* does not require *ESC8*. ChIP of Mcd1-TAP was measured in strains CSW116 (wt), YFC9 (*sir2*Δ), and YFC95 (*esc8*Δ). (B) *ESC8* is not required for pericentric cohesion. Cohesion of plasmid pPCM14tet0 was measured in nocodazole-arrested strains pMY127 (wt), YFC96 (*esc8*Δ), and YFC108 (*mcm21*Δ). (C) *ESC8* is not required for cohesion of the right arm of chromosome III. The cohesion of the *HMR* locus in strains CSW10 (wt) and YFC18 (*esc8*Δ) was evaluated immediately after arrest in the M phase without forming DNA circles.

control, *MCM21*, a gene required for pericentromeric cohesion, was deleted (39). Accordingly, cohesion of the centromeric plasmid was abolished. These results indicate that *ESC8* is not required for pericentromeric cohesion. Cohesion of euchromatic sites distal to pericentromeric domains was tested in two ways. First, we evaluated chromosome arm cohesion using the strain described above with a GFP-tagged *HMR* locus. In the absence of the galactose-induced recombinase, the GFP tag reports on cohesion of the right arm of chromosome III. In cells arrested in M phase, chromosome III arm cohesion persists in the absence of *ESC8*, even if the local cohesion of *HMR* is compromised (Fig. 6C). As a second measure of the role of *ESC8* in cohesion, we examined cohesion of the euchromatic gene *URA3*. Concurrent studies in our lab have shown that *URA3* is sufficient for cohesion when the gene is present in DNA circles (J. S. Campor, M. S. Borrie, and M. R. Gartenberg, unpublished data). Deletion of *ESC8* did not disrupt *URA3*-mediated cohesion either (data not shown). Together, these results suggest that the ISW1a complex containing Esc8 is not a general cohesion factor but, rather, one that acts specifically on a subset of chromosomal domains that include Sir2-mediated silent chromatin.

DISCUSSION

Sirtuin-mediated sister chromatid cohesion. This study extends our understanding of the specialized form of sister chromatid cohesion that occurs at Sir2-related, silent chromatin domains. To help identify features of Sir2 that are important for cohesion, we first evaluated the cohesive properties of another sirtuin, Hst1. We showed that full-length Hst1, like Sir2, promoted cohesion when the protein was redirected to form a repressive chromatin structure at *HMR*. By extrapolation, we propose that both Sir2 and Hst1 similarly promote cohesion at other locations in the genome where the two proteins bind (10). Since the sirtuins are not required for all sister chromatid cohesion, the sirtuin-bound locations constitute a distinct class of chromosome arm cohesion sites.

A surface motif of Sir2 that supports sister chromatid cohesion. By mapping the cohesion-proficient domains of the sirtuins to a Sir2 crystal structure, we identified a surface motif necessary for Sir2-mediated cohesion. Mutating conserved residues in the EKDK motif abolished *HMR* cohesion with virtually no impact on silencing at any of the loci where Sir2 acts. Silencing defects at *HMR* were detected only with a highly sensitive colony formation assay, and the defects were easily suppressed, as described below. These results indicate that silencing and cohesion are separable functions of Sir2 and that cohesion of silent chromatin is not an obligatory prerequisite for transcriptional silencing.

The subtle silencing defects associated with mutations in EKDK were reversed by mild increases in Sir4, a factor whose quantity is limiting for silencing (30). Thus, if cohesion promotes silencing in any way, it might be to increase the local concentration of Sir4. According to this argument, the emergent silent chromatin of each sister chromatid recruits dynamically equilibrating Sir4 proteins, which are then enriched locally for both sister chromatids that are held together by cohesion. This so-called Circe effect has also been invoked to explain the benefits to silencing of clustered silent chromatin domains at the nuclear periphery (40).

Previous reports documented a role for cohesin as an inhibitor of silent chromatin in assays for the establishment and expansion of silent chromatin domains (41–43). In those cases, the chromatin association of cohesin was modulated by experimental means. Here, cohesin function is disrupted by mutations that do not disrupt cohesin binding. Thus, the negative regulation of silent chromatin by cohesin in the previous studies may arise from the presence of the cohesin complex and not from the cohesion of sister chromatids that the complex imparts.

We propose that Sir2 associates directly with cohesin or an intermediary adaptor protein and that the EKDK motif of Sir2 controls the function of that factor. It is unlikely that the EKDK motif is the sole cohesin recruitment surface because cohesin still accumulates at *HMR* when the EKDK domain is mutated. Thus, we propose that a second cohesin recruitment site exists within Sir2 or some other component of silent chromatin.

Esc8 and the ISW1a chromatin-remodeling complex in silent chromatin cohesion. Our attempts to find Sir2-interacting factors that mediate cohesion did not yield cohesin or other intuitive adaptor proteins. Direct tests for interactions between Sir2 and the Smc1 and Scc3 cohesin subunits by coimmunoprecipitation yielded negative results (data not shown). We did, however, find that Esc8, an understudied Sir2-interacting factor, facilitates silent chromatin cohesion. Esc8 associates with the Isw1 ATPase and is thought to form an alternative ISW1a chromatin-remodeling

complex. In general, members of the ISWI family create regularly spaced nucleosome arrays, maintain chromatin organization across transcribed regions, and negatively affect transcription (32, 44, 45). ISW1a, in particular, has been shown to oppose chromatin opening at promoters by repositioning nucleosomes near the nucleosome-depleted regions at the ends of genes (46–48). Certain transcription factors, like Rap1 and Abf1, create nucleosome-depleted regions that recruit ISW1 complexes for their subsequent roles in adjacent chromatin domains (49). Both Rap1 and Abf1 bind the nucleosome-free silencers that flank the *HM* loci (50, 51). If *HM* silencers recruit ISW1 complexes, then it seems likely that Sir2 at silencers favors recruitment of ISW1a complexes bearing Esc8. Recruitment of Esc8 by Sir2 may explain why ISW1a complexes bearing Esc8 are not required for cohesion at the other genomic locations tested.

We propose that ISW1a complexes bearing Esc8 promote the cohesion of silenced sister chromatids by remodeling nucleosomes within silent chromatin domains. While the specific changes in chromatin structure are unknown, they must be subtle. Silencing remained essentially intact in the absence of *ESC8*, and the micrococcal nuclease digestion patterns of silent chromatin were essentially unchanged in the absence of *ISW1* (37). Nevertheless, the stability of silent chromatin, as measured by a DNA supercoiling assay, was diminished. It is possible that ISW1a complexes bearing Esc8 provide a remodeling activity that counteracts the disruption of silent chromatin by other remodelers, such as RSC (48).

Previous work has identified roles for chromatin remodeling in sister chromatid cohesion, primarily at the step of cohesin loading. In humans, cohesin loading requires ISW1, a homolog of the yeast ISW1 remodeling complexes (52). In yeast, cohesin loading requires the evolutionarily conserved Scc2/Scc4 complex and RSC chromatin remodeler, which together create nucleosome-free regions (53). Indeed, much of the cohesin at *HMR* arrives via an adjacent, nucleosome-depleted tRNA gene, *tT(AGU)C*, that binds Scc2/Scc4 and RSC (14, 54, 55). At *HMR*, Esc8 is required for the function of cohesin but not loading of the complex. Thus, our data imply that there are roles for chromatin remodeling in cohesion beyond cohesin loading, at least within silent chromatin domains.

The cohesin embrace at silent chromatin domains. Our initial analysis of silent chromatin cohesion identified a topological component of cohesin binding by using a small-molecule inhibitor of Sir2: cohesin complexes were lost from *HMR* if the inhibitor was added before DNA circularization, but the complexes were trapped at the locus if the inhibitor was added after circularization (3). Importantly, the trapped cohesin complexes on DNA circles could no longer mediate cohesion. In the double-embrace model, a loss of cohesion arises from opening of the cohesin ring, which should release cohesin from DNA. Thus, an alternative model was proposed in which single cohesin complexes bind one sister primarily through topological engagement but the second sister through silent chromatin-specific interactions (Fig. 7). In this study, mutation of the EKDK motif or Esc8 eliminated silent chromatin cohesion without a loss of cohesin binding. In the model shown in Fig. 7, we propose that these perturbations disrupt one of the two linkages that underpin cohesion of silent chromatin domains. At present, it is not known whether the residual binding of cohesin in either of these mutants is topologically based or not. A recent study proposed that cohesin complexes bind single chromatids stably and then mediate cohesion through cohesin-cohesin interactions (56). Whether such interactions also operate at silent chromatin domains has not yet been tested.

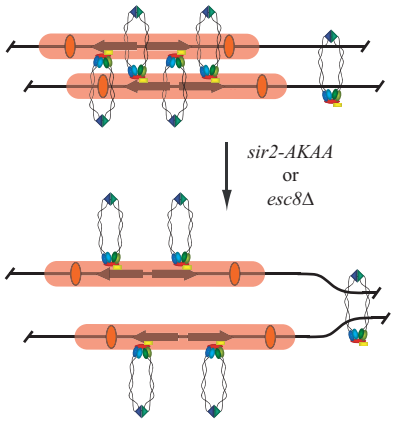

FIG 7 A model for cohesin binding at *HMR* and the impact of mutation of the Sir2 EKDK domain or loss of Esc8. Silent chromatin, silencers, and cohesin complexes are represented by pink bars, orange ovals, and multicolored rings, respectively. On the basis of evidence from earlier work (3), cohesin embraces one chromatid topologically and associates with silent chromatin of the second chromatid directly or through an adaptor protein. The *sir2-AKAA* mutation or deletion of *ESC8* breaks one of the two linkages between cohesin and the chromatids, leaving cohesin bound but unable to perform cohesion. It is unknown whether the residual cohesin binds chromatin topologically.

While our studies have been limited to silent chromatin and silencing-related factors, it should be noted that numerous studies have also created situations where cohesin bound without generating cohesion (see reference 57 and references therein). For example, conditional depletion of cohesin accessory protein Pds5 caused the dissolution of cohesion genome-wide without a loss of cohesin from the chromatids (58–60). That cohesin can release one but not both sisters under these conditions, like the case with Sir2 inactivation described above, may suggest that the mode of cohesin binding at silent chromatin domains is at least thematically related to the mode of cohesin binding elsewhere in the genome.

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