Cohesin recruits the Esco1 acetyltransferase genome wide to repress transcription and promote cohesion in somatic cells

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The cohesin complex links DNA molecules and plays key roles in the organization, expression, repair, and segregation of eukaryotic genomes. In vertebrates the Esco1 and Esco2 acetyltransferases both modify cohesin's Smc3 subunit to establish sister chromatid cohesion during S phase, but differ in their N-terminal domains and expression during development and across the cell cycle. Here we show that Esco1 and Esco2 also differ dramatically in their interaction with chromatin, as Esco1 is recruited by cohesin to over 11,000 sites, whereas Esco2 is infrequently enriched at REST/NRSF target genes. Esco1's colocalization with cohesin occurs throughout the cell cycle and depends on two short motifs (the A-box and B-box) present in and unique to all Esco1 orthologs. Deleting either motif led to the derepression of Esco1-proximal genes and functional uncoupling of cohesion from Smc3 acetylation. In contrast, other mutations that preserved Esco1's recruitment separated its roles in cohesion establishment and gene silencing. We conclude that Esco1 uses cohesin as both a substrate and a scaffold for coordinating multiple chromatin-based transactions in somatic cells.

mitosis | gene expression | sister chromatids | chromosomes | acetylation

SMC (structural maintenance of chromosome) proteins are an ancient group of ATP-dependent DNA tethers that support virtually all aspects of chromosome metabolism (1–4). The most well known member of this family, cohesin, forms a ring-shaped complex and is thought to bind and connect chromatin fibers topologically (5, 6). Through the formation of *cis*-acting loops, cohesin divides the genome into discrete domains and regulates gene expression by inhibiting or promoting enhancer-promoter communication (7, 8). Similarly, cohesin's *trans*-catenation of the products of DNA replication (termed sister chromatid cohesion) enables error-free DNA repair via homologous recombination (9), as well as chromosome biorientation and accurate segregation in mitosis (4, 5). Underscoring the importance of these roles at the organismal level, germ-line mutations in cohesin or its regulators result in a spectrum of related developmental syndromes known as "cohesinopathies" (10), whereas somatic alterations in cohesin are frequent in human tumors and regarded as drivers of malignancy (11).

Cohesion between sister DNAs is established during S phase and depends on the acetylation of two residues (K105 and K106 in humans) on Smc3′s N-terminal ATPase domain (12–14), as well as other factors (including the PCNA sliding clamp, the RFC^{Ctf18} clamp loader, the DNA polymerase α-primase scaffold Ctf4, the ChlR1 helicase, and the Timeless-Tipin complex) with important roles in DNA replication (15). Acetylation neutralizes the anti-cohesive activity of Wapl and Pds5 (16-19), which may activate a DNA exit gate at the Smc3-Rad21 interface (20–23). Neutralization of Wapl-Pds5 not only builds cohesion during S phase but also promotes fork progression and restart (24, 25), and in higher organisms is supported by an additional Wapl antagonist called sororin (26, 27).

Smc3 acetylation is catalyzed by cohesin acetyltransferases (CoATs) related to yeast Eco1 (12–14, 27–29). Although the catalytic domains of CoATs are well conserved, their noncatalytic N-termini have diverged extensively between and within taxa (30–32). For instance, although Esco1 and Esco2 both contribute to Smc3 acetylation and cohesion in vertebrates (27, 30, 33), Esco2 binds chromatin via N-terminal motifs that are missing from Esco1 or other CoATs (28, 29). As Esco2 alone is maternally expressed and both necessary and sufficient for cohesion in early embryonic divisions (27–29), why Esco1 becomes important in somatic cells (14, 30) remains to be clarified.

By analyzing the genomewide distribution of Esco1 and Esco2 in human cells, we discovered that Esco1 is poised at thousands of sites occupied by cohesin and CTCF, which mediate long-range chromatin interactions and regulate transcription globally (7, 8, 34). In contrast, Esco2 was targeted to a much smaller group of genes under the control of REST/NRSF, which represses transcription of neuron-specific genes in other tissues (35, 36). Like cohesin, Esco1 was recruited to its binding sites in G1 phase and maintained thereafter, suggesting a potential role in gene expression. Consistently, depleting Esco1 or Esco2 derepressed genes bound by these CoATs and cohesin. We also identified two isoform-specific motifs in Esco1 that dictate its recruitment by cohesin but not overall binding to chromatin or CoAT activity. Strikingly, these motifs were required not only to repress transcription, but also to establish cohesion during DNA replication. In contrast, removal of a different motif produced a version of Esco1 that supported cohesion

Significance

The cohesin complex holds sister chromatids together from their duplication in S phase to their separation in anaphase. Cohesin is also involved in other aspects of chromosome structure and function, including the regulation of gene expression. To connect sister chromatids, cohesin must be modified by conserved acetyltransferases, known as Esco1 and Esco2 in humans. We investigated how Esco1 and Esco2 interact with chromosomes. Surprisingly, cohesin recruits Esco1 to numerous sites throughout the genome, while Esco2 is rarely seen at these sites. Esco1's colocalization with cohesin requires two short regions that have been conserved throughout its evolution. Deleting these regions prevents Esco1 from establishing cohesion, and from silencing genes near its binding sites, a newly identified function of this factor.

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Data deposition: ChIP-Seq data have been deposited in the NCBI Sequence Read Archive (accession no. SRP061269) Microarray data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE71420).

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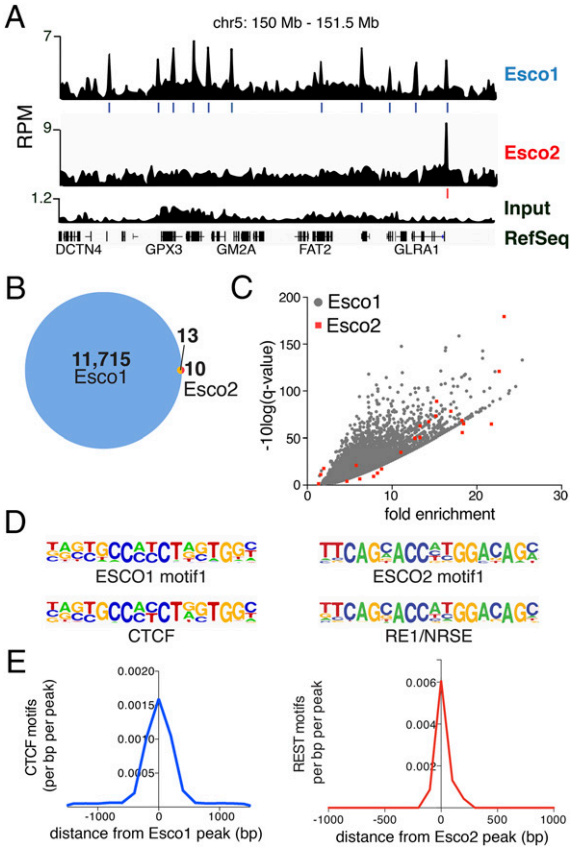

Fig. 1. Human cohesin acetyltransferases (CoATs) differ significantly in their sites of interaction with chromatin. (*A*) Sequence coverage of Esco1 and Esco2 over a 1.5-Mb region of chromosome 5. Bars below coverage tracks indicate peaks called in both biological replicates. Input used for FLAG-Esco1 ChIP is shown. RPM, reads per million. (*B*) Scaled Venn diagram comparing replicate-consensus peaks for Esco1 and Esco2 (see also Fig. S1). (*C*) Scatter plot comparing enrichment and confidence metrics for Esco1 and Esco2 consensus peaks. (*D*) Esco1 and Esco2 binding sites are enriched for CTCF and REST/NRSF transcription factor motifs. (*E*) Plots of CTCF and REST motif density around Esco1 and Esco2 peaks.

establishment but not gene silencing, demonstrating that the two activities are separable. Together our results reveal how vertebrate CoATs have evolved to control multiple aspects of chromosome metabolism in somatic cells.

Results

To facilitate the analysis and comparison of Esco1 and Esco2's binding to chromatin, we generated HeLa cell lines expressing FLAG-tagged and RNAi-resistant versions of each CoAT. Both transgenes restored cohesion in cells depleted of endogenous Esco1 or Esco2, validating their functionality (Fig. S1 *A* and *B* and below). Chromatin immunoprecipitation with FLAG-specific antibodies and next-generation sequencing (ChIP-Seq) identified over 11,000 sites of Esco1 binding, whereas Esco2 was reproducibly bound at just 23 sites (Fig. 1 *A*–*C* and Fig. S1*C*). Specific CoAT enrichment was confirmed in quantitative ChIP-PCR assays performed with or without FLAG peptide as a competitor (Fig. S1 *D* and *E*). To identify genomic features associated with Esco1 and Esco2 binding, 300-bp regions around each site were used for de novo motif discovery and pattern searches, revealing significant overrepresentation of consensus motifs for CTCF (39.5% of Esco1 sites versus 1.3% background; $P = 1 \times 10^{-4760}$) and REST (79% of Esco2 sites versus 0.01% background; $P = 1 \times 10^{-40}$) (Fig. 1 *D* and *E*). For Esco1 our *in silico* analysis underestimates the overlap with CTCF, as 20% of CTCF-bound sites do not match this consensus (37).

CTCF recruits cohesin to a large fraction of its binding sites, where the two proteins form intrachromosomal loops that dictate nuclear architecture and transcriptional activity (7, 34). To correlate Esco1 binding with the presence and acetylation status of cohesin, we performed additional ChIP-Seq experiments with antibodies to Rad21 to detect total cohesin or a new monoclonal that recognizes Smc3 acetylated on K105 (Fig. S2). The latter reagent was developed because a previous report with a different antibody suggested that only 10% of cohesin-binding sites are acetylated (38). In contrast, we detected K105 acetylation at 81% of Rad21 sites and 70% of Esco1 sites (Fig. 2 *A* and *B*) and maximum Esco1 enrichment at Rad21 peaks (Fig. 2*C*), suggesting that cohesin may be both a substrate of and a scaffold for Esco1. Consistent with this hypothesis, Esco1 recruitment was reduced after Rad21 knockdown by RNAi (Fig. 2 *D* and *E*). When immunoprecipitated from native chromatin, Esco1 copurified with cohesin and Pds5 in a benzonase-sensitive manner (Fig. 2*F*), indicating that the interactions responsible for their colocalization are nucleic acid-dependent.

Esco1 was positioned at its binding sites throughout the cell cycle (Fig. 3 *A* and *B*) and supported site-specific Smc3 acetylation in G1 phase, when Esco1 but not Esco2 (30) is expressed (Fig. 3*C*). The constitutive nature of this regulation suggested a potential role for Esco1 in controlling gene expression. To test this idea we compared the transcriptional profiles of Esco1- and Rad21-depleted cells by microarray hybridization (Fig. 3*D* and Fig. S3 *A*-*D*). Both conditions resulted in distinct but overlapping patterns of increased expression, with 34–51% of Esco1-regulated genes scoring as Rad21-regulated at various thresholds (Table 1). Esco1-dependent regulation was further confirmed using quantitative RT-PCR assays (Fig. 3*E*). Among 239 genes strongly regulated by Esco1, 86 (36%) were within 5 kb of an Esco1-binding site (Fig. 3*F*), and 118 (49%) were within 20 kb, in line with previously reported distance correlations for cohesin-regulated genes (39).

In addition to its enrichment at CTCF sites, Esco1 was found at over half of REST sites bound by Esco2 (Fig. 1*B* and Fig. S3*F*). As REST is known to silence its target genes by recruiting corepressors (35), we asked whether Esco1 or Esco2 are important for this outcome. Depleting either CoAT led to two- to fivefold increases in transcript levels (Fig. S3 *G* and *H*), an effect comparable in scale to the loss of REST or its known corepressors (40, 41), whereas database searches identified 9 other Esco1-repressed genes as annotated REST targets. Our findings are consistent with the reported interaction between Esco2 and the CoREST complex in nuclear extracts (42) and suggest that mammalian CoATs collaborate with cohesin and sequence-specific transcription factors to silence gene expression.

To understand how Esco1 is targeted to cohesin-occupied chromatin, we traced its evolution in vertebrates (Fig. S4*A*). In addition to the acetyltransferase domain, zinc finger, and PCNA-binding motifs found at the C-termini of all CoATs, two short regions of high sequence identity were identified at the N terminus, neither of which was present in Esco2 or other CoATs (Fig. 4*A* and Fig. S4 *B* and *C*). Due to their enrichment for acidic and basic residues, we refer to these regions as the A-box and B-box respectively. Flag-tagged and RNAi-resistant mutants with deletions in the A-box (amino acids 298–322; Esco1$^{\Delta A}$), B-box (383-400; Esco1$^{\Delta B}$), or a nearby sequence found only in tetrapods (332–336; Esco1$^{\Delta T}$; Fig. S4*D*) were stably expressed in HeLa cells and analyzed by chromatin fractionation and quantitative ChIP-qPCR. Previous studies demonstrated that the N termini of Esco1 and Esco2 mediate their nuclear localization and deposition onto chromatin (26, 28, 30), which were preserved in all three mutants (Fig. 4*B*). However, deleting either the A-box or the B-box abolished Esco1's recruitment to cohesin

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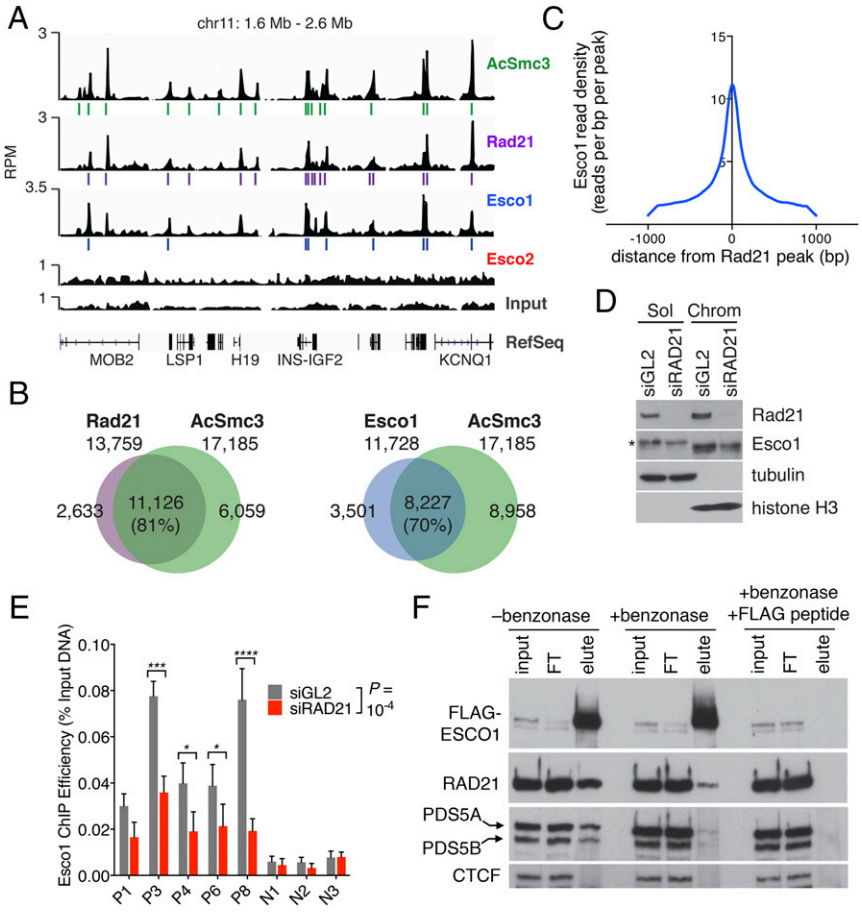

Fig. 2. Esco1 is recruited to its binding sites by cohesin. (*A*) Sequence coverage of K105-acetylated Smc3 (AcSmc3), Rad21, Esco1, and Esco2 over a 1-Mb region of chromosome 11. Input used for AcSmc3 ChIP is shown. (*B*) Venn diagram comparing replicate-consensus peaks for AcSmc3, Rad21, and Esco1. (*C*) Plot of Esco1 read density around Rad21 consensus peaks. (*D*) FLAG-Esco1 cells were transfected with control (siGL2) or Rad21-specific siRNAs, then synchronized in S phase with thymidine. Soluble and chromatin fractions were prepared (24), resolved by SDS/PAGE, and blotted with the indicated antibodies. (*E*) Cells in *D* were subjected to FLAG ChIP and quantitative real-time PCR (for primers, see Table S1). Error bars indicate SEMs from triplicate measurements in three independent experiments. Familywise and individual *P* values were computed with two-way ANOVA and Holm–Sidak multiple comparison tests (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$). (*F*) Native chromatin from FLAG-Esco1 cells was digested with benzonase or left intact, then immunoprecipitated with FLAG antibodies in the presence or absence of competitor peptide. Input, flowthrough, and bead-eluted fractions were resolved by SDS/PAGE and blotted as shown.

sites, whereas Esco1$^{\Delta T}$ retained site-specific binding (Fig. 4*C*). We then analyzed the effect of each mutant on gene expression. Strikingly, all three mutants induced partial derepression of Esco1 target genes in cells that still expressed the endogenous CoAT (Fig. 4*D*). This dominant-negative effect is analogous to "squelching" by transcriptional activators (43, 44) and suggests that these mutants competitively titrate or displace a limiting corepressor from Esco1 target genes. Consistent with this interpretation, all three mutants elicited more severe transcriptional defects when combined with Esco1 knockdown, with one target gene (*CLDN11*) induced up to 50-fold (Fig. 4*E*). We were unable to test the acetyltransferase-dead point mutant of Esco1 (G768D) in this assay, due to difficulties in achieving its stable expression. However, a C-terminal truncation encompassing this domain (Esco1$^{\Delta C}$, 1–579) could be stably expressed and was found to increase gene expression in a dominant-negative manner (Fig. S5). Collectively these results indicate that the transcriptional repression of Esco1 target genes depends on the CoAT's recruitment to cohesin-bound chromatin sites and localized acetylation of cohesin and/or other substrates.

Finally, we asked whether Esco1's recruitment by cohesin is important for cohesion establishment, and if so, whether it promotes Smc3 acetylation or acts in parallel with the latter, a possibility suggested by recent work in other model systems (26, 28, 45). Because cohesin binds and tethers sister chromatids through chromatin regions lacking CTCF (39), we used quantitative immunoblotting to follow its acetylation globally. Consistent with their additive impact on cohesion (30, 33), knockdown of either Esco1 or Esco2 reduced Smc3 acetylation by 50% (Fig. 5*A* and Fig. S2*A*). In the case of Esco1, this decrease could be suppressed by all forms of the enzyme (Fig. 5*A*), indicating that Esco1 retains CoAT activity even without its continuous colocalization with cohesin. Nonetheless, this unscaffolded mode of Smc3 acetylation was functionally inadequate, as Esco1$^{\Delta A}$ and Esco1$^{\Delta B}$ failed to restore cohesion, unlike Esco1^{WT} and Esco1$^{\Delta T}$ (Fig. 5 *B* and *C*). Because Esco1$^{\Delta T}$ is equally defective in transcriptional repression (Fig. 4*E*), our data demonstrate that Esco1-dependent gene regulation is not a prerequisite for cohesion establishment. Nevertheless, both functions depend on Esco1's sustained recruitment by cohesin, which we propose coordinates acetylation with other structural changes in cohesin that lead to the tethering of chromatin fibers in *cis* or *trans* configurations (Fig. 5*D*).

Discussion

Although first studied as a mediator of sister chromatid cohesion in postreplicative cells (4–6), cohesin is now known to regulate chromosome architecture and gene expression in a broader and largely orthogonal manner (7–9). Nevertheless, both the cohesive and noncohesive roles of this complex are thought to involve its topological binding and tethering of chromatin fibers (11). As mutations in cohesin or its binding partners are linked to birth defects and cancer (10, 11), understanding how these factors interact with and regulate chromosomes has both fundamental and medical relevance.

In vertebrates, cohesion establishment depends on two separate CoATs with divergent N-terminal extensions and contrasting temporal regulation (30, 33). We were intrigued by the fact that Esco1 is absent from unfertilized eggs and early embryos (26, 28) yet constitutively expressed in somatic cells (30). As the latter are also transcriptionally active, these observations suggested that Esco1 may have evolved an additional role in gene

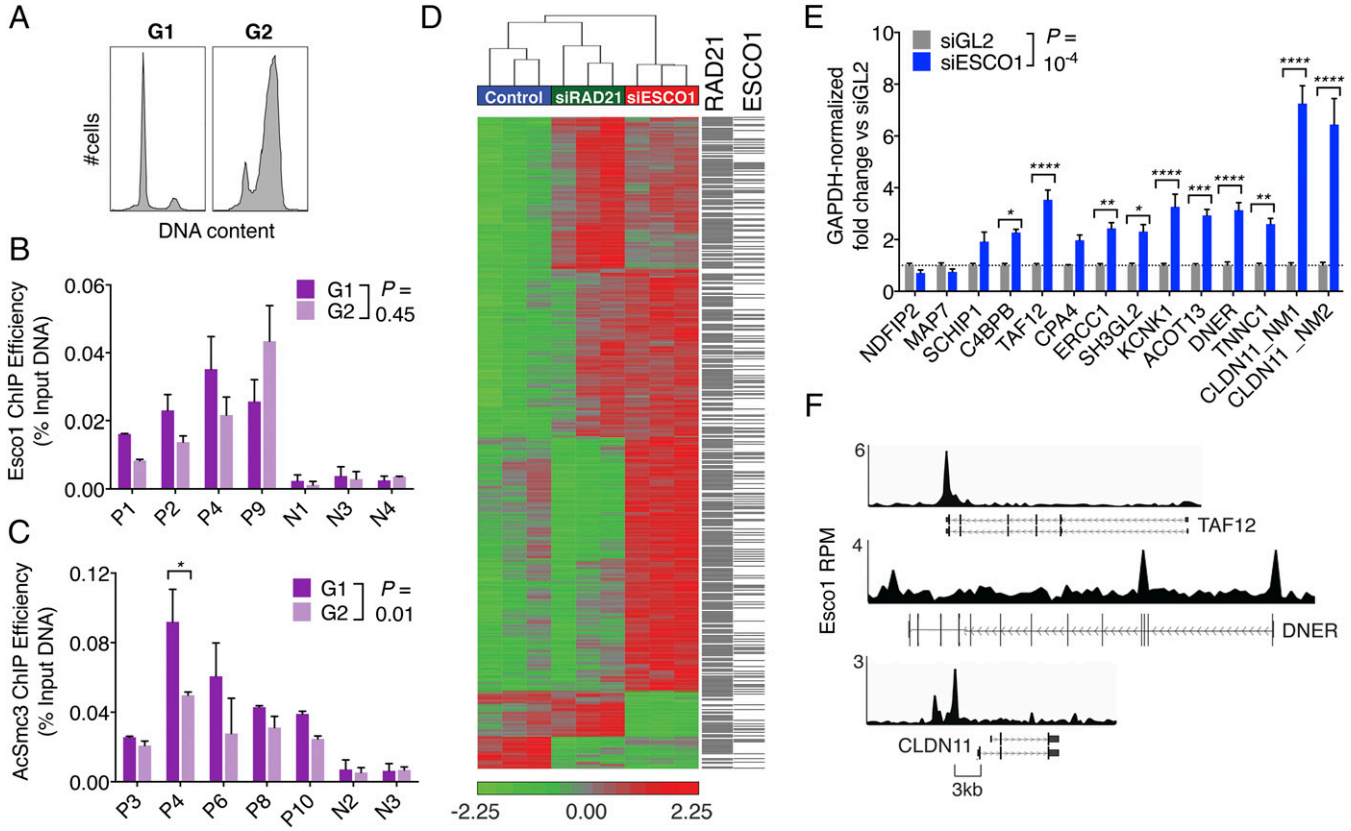

Fig. 3. Esco1 colocalizes with cohesin throughout the cell cycle and down-regulates expression of neighboring genes. (*A*) FLAG-Esco1 cells were synchronized using a double-thymidine block and collected 5 h (G2 phase) or 13 h (G1 phase) after release. DNA content was determined by propidium iodide staining and flow cytometry. (*B* and *C*) Cells in *A* were subjected to FLAG ChIP (*B*) or AcSmc3 ChIP (*C*). Error bars reflect SEM of triplicate measurements from two independent experiments. *P* values were computed using two-way ANOVA and Holm–Sidak multiple comparison tests. (*D*) Heatmap of genes differentially expressed after Esco1 or Rad21 depletion. A set of 548 Esco1-regulated genes (FDR ≤ 0.05; see Table 1) was used for hierarchical clustering of control, Esco1-, and Rad21-depleted HeLa cells (three biological replicates per condition). Scale displays standardized probe intensities. The final two columns indicate proximity (≤5 kb) to Rad21 or Esco1 binding sites. (*E*) Validation of Esco1-regulated gene expression. Transcript levels in control and Esco1-depleted HeLa cells were determined by reverse transcription and quantitative PCR (RT-qPCR). Primer sequences are given in Table S2. Fold change is reported relative to the siGL2 control, using *GAPDH* as an internal reference. Error bars indicate SEMs from triplicate measurements in two experiments. *P* values were computed using two-way ANOVA and Holm–Sidak multiple comparison tests. (*F*) Esco1 ChIP-Seq reads at three genes (*TAF12*, *DNER*, and *CLDN11*) validated by RT-qPCR.

regulation. Our findings support this hypothesis in several respects. First, we discovered that Esco1 is selectively and ubiquitously recruited by cohesin, and moreover occupies its binding sites throughout the cell cycle. Comparable observations were made by Minamino et al. and reported during revision of this study (46). Second, we found that depleting either Esco1 or cohesin had similar effects on transcription, mainly involving the up-regulation of Esco1-proximal genes. Third, we identified two isoform-specific motifs (the A-box and B-box) that were crucial for Esco1's recruitment by cohesin and gene-silencing activity. As these motifs were also required for cohesion (see below), we sought to separate the two functions genetically. Our analysis of the Esco1$^{\Delta T}$ mutant suggest that, after being recruited to cohesin sites, Esco1 must also engage downstream mediator(s) of transcriptional repression that are otherwise dispensable for cohesion (42, 47). Future biochemical studies will be needed to identify these mediators and determine if their recruitment or activation occurs via Esco1-catalyzed acetylation.

Strikingly, the motifs that promote Esco1's colocalization with cohesin were also required for cohesion establishment, though not for its overall binding to chromatin or CoAT activity. These results reinforce the emerging concept that Smc3 acetylation is necessary but not sufficient to tether sister DNAs (26, 28, 45). Rather, this modification must be coordinated with other, as-yet poorly defined events at the replication fork, to convert cohesin into a stably bound and functionally cohesive state. It has been suggested that replication forks may need to slide through (or open and reclose) the cohesin ring without permitting its

Table 1. Comparison of Esco1- and cohesin-regulated gene expression

Threshold	ESCO1 regulated (up)	ESCO1 regulated (down)	RAD21 regulated (up)	RAD21 regulated (down)	ESCO1/RAD21 coregulated (up/up)	ESCO1/RAD21 coregulated (down/down)	ESCO1/RAD21 antiregulated (up/down)	ESCO1/RAD21 antiregulated (down/up)
FDR ≤ 0.05	483	65	917	189	245	27	58	16
FDR ≤ 0.05 and FC ≥ 1.5	214	25	503	69	73	8	10	6

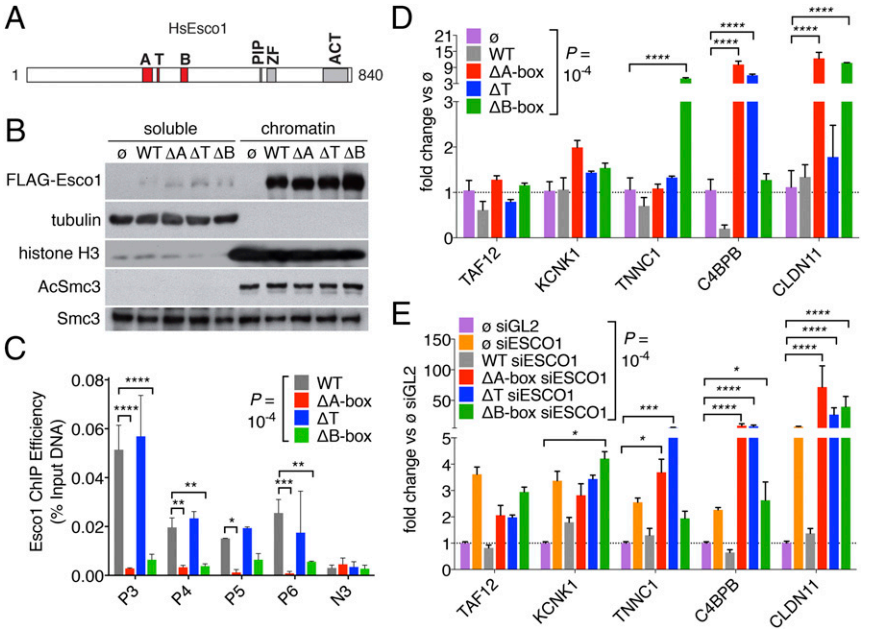

Fig. 4. Two motifs in Esco1 mediate its association with cohesin and gene-silencing activity. (*A*) Twenty-two Esco1 orthologs were aligned and organized into a phylogenetic tree (Fig. S4*A*). The A-box, B-box, and a third motif present in terrestrial vertebrates (T) are indicated (see also Fig. S4 *B*–*D*). (*B*) Soluble and chromatin fractions from HeLa cells expressing wild-type (WT) or mutant (ΔA, ΔT, ΔB) FLAG-Esco1 or no transgene (ø) were resolved by SDS/PAGE and blotted as shown. (*C*) Site-specific Esco1 binding was assessed by FLAG ChIP and quantitative PCR. Data are from two independent experiments. (*D* and *E*) N-terminal Esco1 mutants are defective in gene silencing and interfere with endogenous Esco1. Transcript levels were quantified before (*D*) or after (*E*) Esco1 knockdown as in Fig. 3*E*. Error bars indicate SEMs from triplicate RT-qPCR measurements of three to four biological replicates per condition. *P* values were computed as above.

dissociation from chromatin, thereby tethering sister DNAs via cointrapment (48, 49). Alternatively, cohesin rings loaded onto each sister DNA as it emerges from the fork might be licensed to oligomerize or catenate (6, 50). In either case, our results imply that the critical DNA-tethering reaction(s) require Esco1's ongoing association with cohesin, in addition to its acetylation of Smc3. Because Smc3 acetylation depends on cohesin's ATPase activity, chromatin loading (51), and Pds5 (19, 52, 53), our results suggest that Esco1 not only modifies cohesin soon after its topological linkage to DNA, but in fact remains bound to the cohesin-DNA complex, possibly through chromatin-specific interactions with Pds5 and Smc3's ATPase domain. Although Pds5's precise effects on cohesin are still enigmatic, one of its key roles is to provide a regulatory interface for fine-tuning cohesin's association

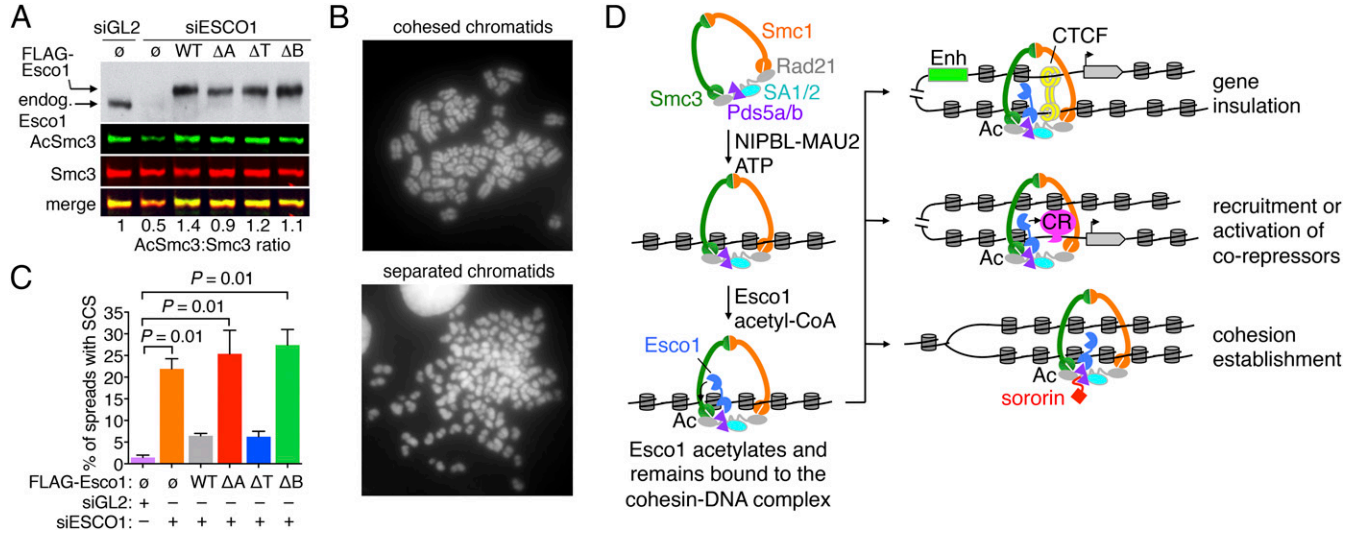

Fig. 5. Esco1's sustained association with cohesin-DNA complexes is required to convert Smc3 acetylation into functional cohesion. (*A*) HeLa cells expressing RNAi-resistant FLAG-Esco1 (WT, ΔA, ΔT, ΔB) or no transgene (ø) were transfected with control or Esco1-specific siRNAs. Whole-cell lysates were resolved by SDS/PAGE and blotted as shown. Quantitation of total and acetylated Smc3 was performed using infrared dye-coupled antibodies and two-channel imaging system. (*B* and *C*) Cells in *A* were treated with nocodazole for two hours and analyzed by chromosome spreading. The incidence of sister chromatid separation (SCS) was determined from 80 to 200 cells per condition in two separate experiments. *P* values were computed using one-way ANOVA and Holm–Sidak multiple comparison tests. Error bars indicate SEM. (*D*) A model for the role of Esco1's N-terminal domain in gene silencing and cohesion establishment. Cohesin recruits Esco1 during or shortly after its ATPase-dependent loading, via the latter's A-box and B-box motifs. In addition to acetylating Smc3's ATPase domain, Esco1 remains bound to the cohesin-DNA complex. Through this interaction, Esco1 is able to silence the expression of nearby genes, likely through stabilization of intrachromosomal loops organized by cohesin and CTCF (*Top*) or engagement of a transcriptional corepressor (CR, *Middle*). During DNA replication, both Smc3 acetylation and sustained Esco1 binding are required to establish cohesion between sister chromatids (*Bottom*). These observations suggest that interactions between Esco1 and the cohesin-DNA complex are required for essential DNA-tethering reaction(s) (for example, opening the cohesin ring to allow entry of a second chromatin fiber without dissociation from the first) or to stabilize the reaction products until the arrival of sororin.

with chromosomes (18, 27, 54). Smc3 acetylation and Esco1 binding might cooperate to produce a partially stabilized cohesin-DNA intermediate that can capture and tether a second chromatin fiber or cohesin-DNA complex. In addition to establishing nascent cohesion between sister chromatids, the same mechanism could be used to form intrachromosomal loops and large-scale domains (55–57).

Materials and Methods

ChIP was performed as described (58) with slight modifications. SOLiD sequencing and microarray hybridization were conducted at Memorial Sloan Kettering Cancer Center core facilities. Data analysis was performed with MACS2.0, Partek Genomics Suite, Prism, and R software packages. A LightCycler 480 (Roche) was used for quantitative PCR. Retroviral transduction, siRNA transfection, and chromosome spreading were performed as described (24). Detailed experimental procedures are provided in *SI Materials and Methods*.

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