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# Enhancer–promoter entanglement explains their transcriptional interdependence

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**Enhancers not only activate target promoters to stimulate messenger RNA (mRNA) synthesis, but they themselves also undergo transcription to produce enhancer RNAs (eRNAs), the significance of which is not well understood. Transcription at the participating enhancer–promoter pair appears coordinated, but it is unclear why and how. Here, we employ cell-free transcription assays using constructs derived from the human *GREB1* locus to demonstrate that transcription at an enhancer and its target promoter is interdependent. This interdependence is observable under conditions where direct enhancer–promoter contact (EPC) takes place. We demonstrate that transcription activation at a participating enhancer–promoter pair is dependent on i) the mutual availability of the enhancer and promoter, ii) the state of transcription at both the enhancer and promoter, iii) local abundance of both eRNA and mRNA, and iv) direct EPC. Our results suggest transcriptional interdependence between the enhancer and the promoter as the basis of their transcriptional concurrence and coordination throughout the genome. We propose a model where transcriptional concurrence, coordination and interdependence are possible if the participating enhancer and promoter are entangled in the form of EPC, reside in a proteinaceous bubble, and utilize shared transcriptional resources and regulatory inputs.**

transcription activation | enhancer–promoter contact | eRNA

Signal-dependent transcriptional activation of RNA polymerase II (Pol II)-transcribed genes is a hallmark of metazoan gene regulation (1, 2) and is largely governed by gene-distal enhancers (3–5). Although mechanistic details of enhancer-mediated promoter activation are still unclear (6), one way enhancers are thought to activate genes is by delivering Pol II and coregulators to the target promoters (7, 8). Enhancers undergo transcription on a genome-wide basis, producing enhancer RNAs (eRNAs) (9–12). Although locus-specific functions of eRNAs have been proposed, a generalizable purpose and role for eRNAs in transcription regulation remain elusive (13, 14).

A growing body of evidence suggests that transcription at enhancers and target promoters is concurrent, producing eRNAs and mRNAs simultaneously (12, 15–19). Also, eRNA levels correlate with target gene expression genome-wide in human and mouse (12, 15–22), in *Caenorhabditis elegans* (23), in *Drosophila* (24) and in sea urchin (25), suggesting coordinated transcription at enhancers and promoters across Metazoa. Targeted expression of specific eRNAs induce specific target genes (26–32), while targeted silencing of enhancer transcription represses specific target genes (31, 33–35) providing credence to the model of enhancer–promoter transcriptional coordination. These results suggest that i) specific eRNAs execute specific regulatory roles to stimulate target gene expression and/or ii) there is operational coordination of transcription between an enhancer and its target promoter. However, a general mechanism for such specific regulatory impact of eRNAs or a rationale for such coordinated transcription at enhancers and promoters are unclear.

Although there are a few reports to the contrary (36, 37), it is currently accepted that enhancer-mediated activation of target gene expression involves direct enhancer–promoter contacts (EPC) (6, 38–42). The magnitude of EPC correlates with enhancer and promoter transcription (43–45). Similarly, loss of EPC correlates with postinduction repression of transcription (46). Importantly, EPC and target gene expression also correlate with enhancer transcription producing eRNAs (44, 47). These observations strongly argue for a role of EPC in ensuring enhancer–promoter transcriptional coordination.

Using our cell-free assays, we demonstrated that the "act of transcription" at the enhancer and promoter is "mutually stimulatory" (48). Here, we extend our study to demonstrate that transcription activation at a participating enhancer–promoter pair is dependent on i) the mutual availability of the enhancer and promoter, ii) state of transcription at both the enhancer and promoter, iii) local abundance of both eRNA and mRNA, and iv) direct enhancer–promoter contact (EPC). These results suggest "transcriptional codependency" between enhancers and target promoters, explaining evolutionarily conserved enhancer–promoter transcriptional coordination.

## Significance

Transcription at the enhancer and target promoter is highly coordinated, but it is unclear why. Using unique cell-free assays, here we were able to demonstrate that transcription at the enhancer and target promoter is interdependent, which relies on the physical availability of both the enhancer and the promoter, on the act of transcription at both the enhancer and promoter, on the abundance of transcripts from both the enhancer and promoter, and on direct enhancer–promoter contacts. We argue with supporting evidence that such interdependence and regulatory specificity can be explained if the enhancer and the promoter are entangled within a transcriptional bubble that both provides shared resources for transcription and is regulatable by the transcript levels generated.

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## Results

**Physical Codependency between Enhancer and Promoter.** As discussed above, transcription at enhancers and target promoters appears to be concurrent and coordinated. To study enhancer-mediated transcriptional activation, we developed cell-free assays where various components of transcription reactions can be practically controlled. We generated a composite template (CompF) that encompasses the enhancer, promoter, and intervening estrogen response elements (EREs) of the estrogen-responsive and ERα-regulated human *GREB1* gene for in vitro transcription (IVT) and enhancer–promoter looping assays (48). When we analyzed estrogen receptor alpha (ERα)-mediated transcription of eRNA and mRNA from 320 independent IVT reactions from our previous studies on chromatinized CompF and plotted their qRT-PCR determined expression datapoints in triplicate, we observed a striking correlation between the two, with a $R^2$ value of 0.96 (*SI Appendix*, Fig. S1), suggesting that our assays can be exploited to study the mechanism behind enhancer–promoter transcriptional coordination. This observation underlies the basis for positing that transcriptional coordination between the enhancer and promoter involves mutual dependency. We had demonstrated using a template lacking the enhancer (CompFΔF1) in our IVT system that the enhancer was necessary for promoter activation (48). To test promoter-dependence of enhancer transcription, we employed the same strategy by using a CompF template lacking the promoter (CompFΔF6). As Fig. 1*A* shows, whether the template lacked the enhancer or the promoter, ERα-dependent activation of transcription did not occur based on comparison of reactions 4 and 7 to reaction 2. These results demonstrate that transcriptional activation at the enhancer or the promoter require their mutual presence, suggesting physical codependency. Transcriptional activation is restored when the enhancer fragment is supplemented to CompFΔF1 (reaction 5) or the promoter fragment to CompFΔF6 (reaction 8).

Several EREs were identified for the *GREB1* gene (49) before its enhancer was functionally characterized (50); these EREs are present in the CompF construct as F2, F3, and F5 (48). In our assays, these EREs do not seem to contribute to *GREB1* promoter transcription, consistent with the fact that these elements do not significantly contact the promoter in MCF-7 cells (50) nor do they produce significant E2-inducible transcripts (17). To test if these intervening EREs become functional upon the loss of F1, we conducted a series of looping assays with the CompFΔF1 and CompFΔF6 templates. These assays demonstrated that while the enhancer and the promoter in the wild-type (WT) CompF engaged in physical contact with each other, both the enhancer in CompFΔF6 and the promoter in CompFΔF1 failed to loop to their reciprocal terminus; i.e., to F5 and F2, respectively (Fig. 1*B*). This observation is consistent with a scenario where supporting enhancers contribute to transcriptional activation of a gene only when the predominant enhancer is operational (51). Taken together, these results suggest that ERα-dependent transcription activation requires EPC, underlining the physical codependency between the enhancer and the promoter (Fig. 1*C*).

**Transcriptional Codependency between Enhancer and Promoter.** We argued before that the "act of transcription" at the enhancer and promoter is mutually stimulatory (48), consistent with the suggestion that the process of transcription can activate neighboring genes (13, 52). To investigate this mutualism in the context of codependency demonstrated above, we sought to examine transcription at the promoter when enhancer transcription is specifically activated or repressed or vice versa. Since full or half

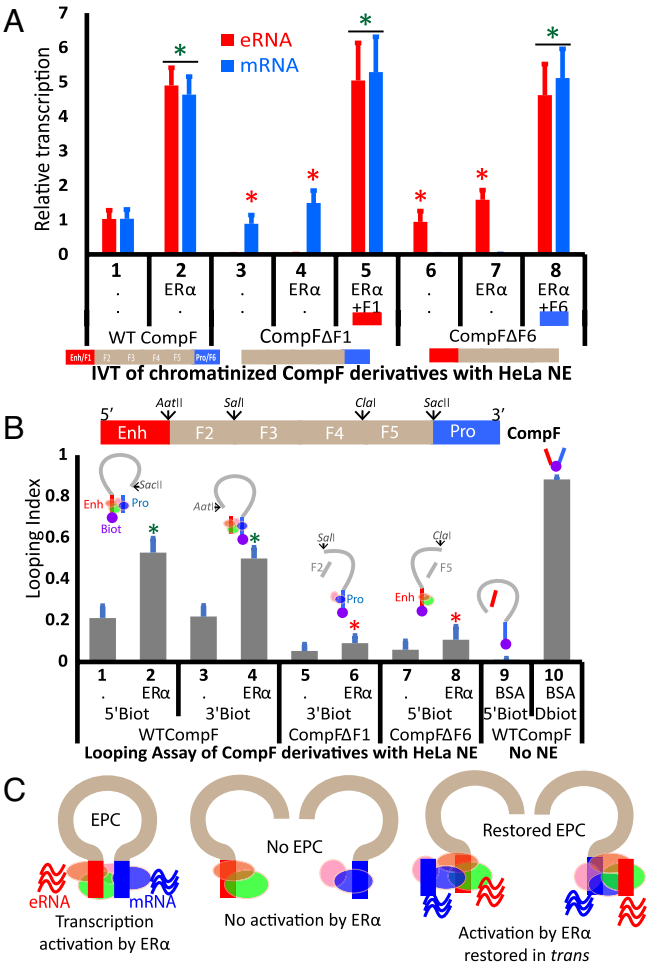


**Fig. 1.** Mutual physical dependency between the enhancer and promoter. (*A*) ERα activates transcription at both the enhancer and the promoter from the WT CompF, whereas CompF derivatives lacking the enhancer (ΔF1) or the promoter (ΔF6) fail to support ERα-induced transcription activation at both the enhancer and promoter. Adding equimolar F1 to CompFΔF1 (reaction 5) and F6 to CompFΔF6 (reaction 8) restores EPC in trans, restoring activation. (*B*) Looping assay demonstrates that the promoter in CompFΔF1 and the enhancer in CompFΔF6 fail to make contact with their opposite terminus. Templates biotinylated at one end (either 3′ or 5′ as indicated) were digested with indicated restriction enzymes after the looping reaction (see inlet schematics) and pulled down with M280 beads. qPCR quantified enrichment of the "cleaved, unbiotinylated terminus" of the opposite end is reported as the looping index. A 5′biotinylated CompF without NE serves as a negative control for the enrichment of the unbiotinylated end (reaction #9), while a doubly biotinylated (DBiot) CompF with biotin on both termini served as the maximal retention of the cleaved fragment. (*C*) Schematic summary of (*A*) and (*B*) that ERα-mediated activation occurs on templates with EPC, but not without. Green asterisk denotes significant enhancement compared to reaction 1 ($P$ = <0.001); red asterisk denotes significant reduction compared to reaction 2 ($P$ = <0.001); with two-tailed Student's $t$ test.

EREs are present in both the enhancer and promoter regions of *GREB1*, we introduced a GAL4-binding sequence into either the enhancer or the promoter of CompF, generating CompF-F1gal and CompF-F6gal derivatives, respectively, such that the enhancer or the promoter can be selectively activated or repressed. We employed commercially available classical recombinant activator GAL4-VP16 (53) as the alternative to ERα in IVT assays. As a potent repressor, we subcloned the GAL4 DNA binding domain (DBD)-conjugated Sin3A-interacting domain (GAL4-SID) (54) sequences into a bacterial expression vector pET302 and purified the GAL4-SID as a 6xHis-tagged recombinant protein (*SI Appendix*, Fig. S2). We reasoned that IVT assays on CompF-F1gal and CompF-F6gal in the presence of GAL4-VP16 would be able to activate transcription

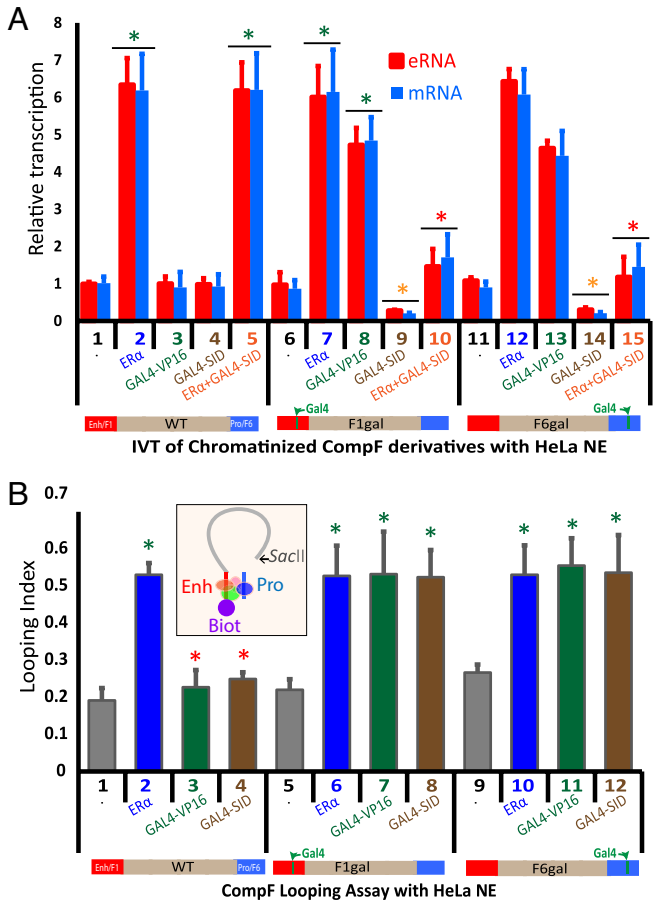


**Fig. 2.** Mutual transcriptional dependency between the enhancer and promoter. (*A*) GAL4-VP16 and GAL4-SID activate and repress transcription from both the enhancer and the promoter, even as they are targeted to only the enhancer (F1gal) or the promoter (F6gal). GAL4-SID abrogates ERα-mediated transcription activation from both the enhancer and promoter; compare reactions 9 and 14 to 6 and 11, respectively. (*B*) Looping assays showing that like ERα, GAL4-VP16, and GAL4-SID stimulate EPC. Inlet depicts the assay schematic. Green asterisk denotes significant enhancement compared to reaction 1 ($P$ = <0.001); red and orange asterisks denote significant reduction compared to reaction 2 ($P$ = <0.001) and reaction 1, respectively ($P$ = <0.05) with two-tailed Student's $t$ test. Inlet in (*B*) depicts assay schematic.

specifically at the enhancer and the promoter, respectively. Likewise, IVT assays on these two CompF derivatives in the presence of GAL4-SID should repress transcription specifically at the enhancer (CompF-F1gal) or the promoter (CompF-F6gal).

Fig. 2*A* presents these results. Expectedly, GAL4-VP16 and GAL4-SID activated and repressed transcription, respectively, from the respective enhancer and promoter that contained GAL4 binding sequences: see eRNA levels (red) in reactions 8 and 9 for activation and repression, respectively, compared to reaction 6 on CompF-F1gal; and see mRNA levels (blue) in reactions 13 and 14 for activation and repression, respectively, compared to reaction 11 on CompF-F6gal. Activation by GAL4-VP16 is somewhat less (~fivefold) compared to by ERα (~sixfold), possibly because we inserted only one GAL4-binding sequence into the enhancer or the promoter, while the *GREB1* enhancer and promoter together contain multiple full or half EREs. Interestingly, the mRNA levels in reactions 8 and 9 were comparable to the eRNA levels, even as the promoter was not targeted. Likewise, the eRNA levels in reactions 13 and 14 matched the mRNA levels, even as the enhancer was not targeted. These results clearly demonstrate concerted, interdependent transcriptional activation and repression between both the enhancer and the promoter, even as only one of them is targeted for activation or repression. This interpretation is strengthened by the observations that ERα-mediated activation of "both" the enhancer and promoter is suppressed when GAL4-SID is targeted to the enhancer only (reaction 10, compared to reaction 7) or to the promoter only (reaction 15 compared to reaction 12).

We rule out the possibility that the GAL4-fusion proteins nonspecifically bind at the enhancer and promoter while being targeted to only one of them. We demonstrate using DNA pulldown-coupled immunoblotting that GAL-VP16 and GAL4-SID bind strongly to the F1gal and F6gal fragments but not to WT fragments (*SI Appendix*, Fig. S3). Interestingly, both these proteins promote EPC despite binding to the enhancer or promoter they are targeted to, similar to ERα (Fig. 2*B*; compare reactions 7 and 8 to 6, and 11 and 12 to 10), as would be expected if there was transcriptional codependency between the enhancer and promoter.

**Transcript Abundance Codependency between Enhancer and Promoter.** To better understand the role of transcription in maintenance of transcriptional mutualism at the enhancer and promoter, we sought to examine the consequence of targeted transcript degradation. For instance, what would happen if nascent eRNAs (or mRNAs) were specifically destroyed during transcription? We reasoned that an IVT reaction in the presence of homologous antisense oligonucleotides (ASO) and RNase H could be used to specifically degrade the targeted nascent transcripts. Therefore, we conducted IVT on chromatinized CompF templates in the presence of RNase H and enhancer-specific or promoter-specific ASOs (Fig. 3*A*). We observed a slightly increased level of both eRNA and mRNA in the presence of RNase H, consistent with a model where RNase H stimulates transcription elongation (55). In contrast, we observed significant loss of eRNA in the presence of Enh ASO and RNase H (red bars; compare reactions 7 to 3) and of mRNA in the presence of Pro ASO and RNase H (blue bars; compare reactions 9 to 3); no such loss of transcript was observed in the presence of control oligonucleotides and RNase H (compare reaction 5 to 3). Unexpectedly, Enh ASO and Pro ASO diminished the mRNA (blue bar, reaction 7) and eRNA (red bar, reaction 9) levels, respectively. Two arguments indicate that this cross-destruction of transcripts is not due to the ASOs nonspecifically cross-annealing to the transcripts during IVT. First, there is no sequence homology between the Enh ASO and mRNA and Pro ASO and eRNA; and second, Enh ASO and Pro ASO failed to cause cross-destruction of mRNA and eRNA, respectively, when incubated with preextracted IVT-derived RNA and RNase H (*SI Appendix*, Fig. S4). We also ruled out the possibility that the ASOs could somehow impede the transcription reaction itself: the same ASOs and RNase H did not interfere with ERα-activation of chromatinized 4×ERE-E4 template (56) (*SI Appendix*, Fig. S5).

The clue to the cross-diminishment of the transcripts comes from the looping assay. Both the Enh ASO and Pro ASO caused significant loss of EPC in the presence of RNase H, while the control oligonucleotides had no effect (Fig. 3*C*; compare reactions 7 and 9 to 3 and 5). Our interpretation is, the ASO-targeted destruction of nascent target transcripts collapses the EPC, consequently abrogating the coordination of transcription between the enhancer and promoter. This interpretation holds that RNA species are important for EPC sustenance. Indeed, eRNAs have been recently proposed to contribute to phase separation of transcription coregulators (57, 58).

We demonstrated that ASO-mediated transcript destruction also has a mutual effect in MCF7 cells, similar to the cell-free observations detailed above. We transfected MCF7 cells with

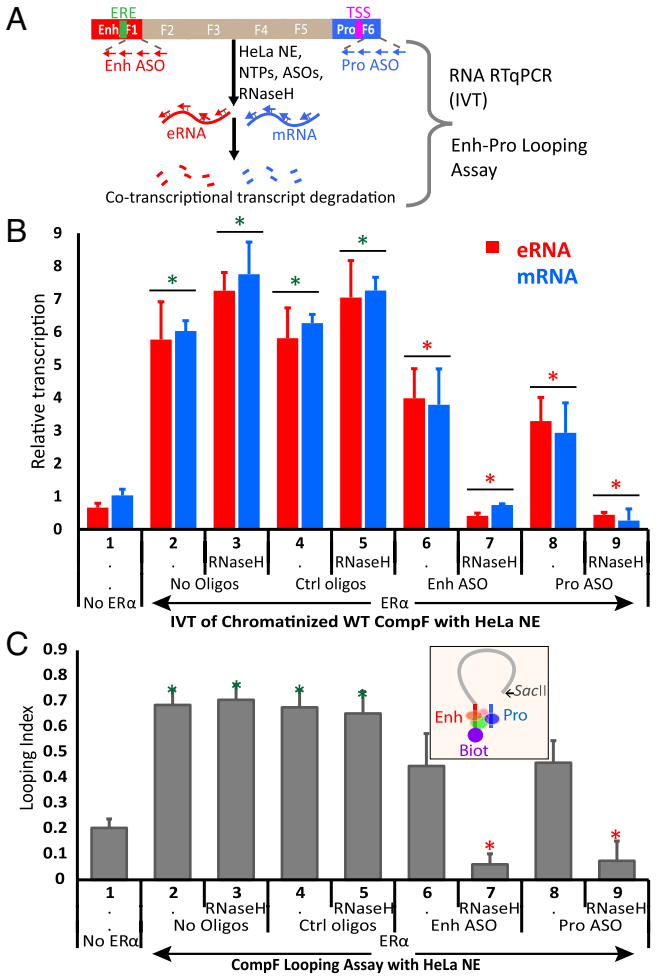


**Fig. 3.** Transcript abundance dependency between the enhancer and promoter. (*A*) Schematic showing targeted destruction of eRNA or mRNA using complementary ASO and RNaseH during IVT. (*B*) qRT-PCR data showing that Enh and Pro ASOs reduce the transcript levels in the presence of RNaseH (reactions 7, 9) while control oligos do not (reactions 4, 5). Loss of transcripts in reactions 6 and 8 is likely because endogenous RNaseH in the NE is utilized. (*C*) Looping assay showing that ASO-mediated destruction of transcripts disrupts EPC. Inlet depicts the assay schematic. Green asterisk denotes significant enhancement compared to reaction 1 ($P$ = <0.001); red asterisk denotes significant reduction compared to reaction 3 ($P$ = <0.001) with two-tailed Student's $t$ test.

control oligos as well as Enh and Pro ASOs and examined E2-induction of *GREB1* transcription the following day. Both the Enh ASO and the Pro ASO caused comparable loss of both eRNA and mRNA (*SI Appendix*, Fig. S6*A*). As with the cell-free looping assays, the 3C assay in MCF7 cells revealed that the Enh ASO and the Pro ASO each caused loss of EPC: E2 stimulation failed to induce the *GREB1* enhancer to contact both the preTSS and postTSS regions of the promoter in cells transfected with the Enh ASO and Pro ASO (*SI Appendix*, Fig. S6*B*).

**EPC Is Necessary for Enhancer–Promoter Transcriptional Coordination.** The results described above demonstrate that the transcriptional status of both the enhancer and promoter are closely tied to EPC, suggesting that EPC likely plays an important role in transcriptional coordination between the enhancer and the promoter. We have previously demonstrated EPC in our trans-Interaction assay, where the enhancer and the promoter fragments come in contact with each other when coincubated with nuclear extract (NE) and ERα (48). In this assay, one of either the enhancer or promoter fragment is biotinylated and is then immobilized on streptavidin beads, while the unbiotinylated version of the other fragment is retained on the beads upon EPC (Scheme A. EPC; Fig. 4*A*). Transcription factors, RNA Pol II and accessory factors still are retained bound to the streptavidin-immobilized fragments upon washing, and transcription ensues upon addition of NTPs (data shown). We reasoned that EPC can be prevented if the biotinylated enhancer and promoter fragments are immobilized on streptavidin beads separately, incubated with NE and ERα separately, washed, and then mixed together (Scheme B, No EPC; Fig. 4*A*), because the molar excess of the beads over the fragments would preclude interfragment interactions.

When we conducted transcription using either of the two schemes, we observed expected ERα-mediated activation of transcription on both the enhancer and promoter in the "EPC" scheme, but no mutual activation was observed where no EPC is expected to occur (Fig. 4*B*), demonstrating that EPC is important for mutual transcriptional activation of the enhancer and promoter. When we employed the gal-mutant of enhancer (F1gal) and the wt promoter (F6) in these experiments, GAL4-VP16 and GAL4-SID robustly activated and repressed transcription, respectively, mutually from both the enhancer and promoter in the "EPC" scheme. However, no mutual coactivation or corepression was observed in the "no EPC" scheme, where only the enhancer showed an expected GAL4-fusion response (Fig. 4*C*).

To test the transcript abundance model, we formed the transcription complexes as in Fig. 4*A* and added the Enh ASO or Pro ASO and RNase H to the beads; transcription was initiated by addition of NTPs. While the control oligos did not reduce the transcript levels, the Enh ASO and Pro ASO drastically reduced the transcript levels mutually in the EPC scheme. However, the Enh and Pro ASOs showed only specific, but not mutual, transcript reduction in the "no EPC" scheme (Fig. 4*D*; compare reaction 4 to 7). These results clearly demonstrate that EPC is central to enhancer–promoter transcriptional coordination.

**Phase-Separation Likely Regulates Enhancer–Promoter Coordination.** The results described thus far demonstrate that enhancer–promoter transcriptional coordination and interdependence depends on i) physical presence (Fig. 1) of and physical contact (Fig. 4) between the enhancer and the promoter; ii) transcription at the enhancer and the promoter (Fig. 2) and iii) the abundance of both eRNA and mRNA (Fig. 3). As had been theorized earlier (59), transcriptional components undergo liquid–liquid phase separation (LLPS) into dynamic condensates that effectuate transcription (60–65). Therefore, we investigated if our cell-free assay system also undergoes phase separation. The polyalcohol 1,6 hexanediol (1,6HD) dissolves LLPS (66) and has been shown to disrupt transcriptional condensates (57, 63, 67); therefore, we challenged our assays with 1,6HD. As Fig. 5*A* shows, 1,6HD inhibited ERα-inducible transcription of both the enhancer and the promoter of the CompF construct in vitro, while its less-disruptive isomer 2,5 hexanediol (2,5HD) had a very mild effect. Similarly, the CompF-based looping assay showed that 1,6HD disrupted EPC, while 2,5HD had only a mild effect (Fig. 5*B*). Importantly, 1,6HD reduced the recruitment of the RNA Pol II and CDK7, an integral component of Pol II transcription initiation machinery, to EPC, as revealed by combination pulldown followed by immunoblotting (Fig. 5*C*). Both 1,6HD and 2,5HD abolished the recruitment of SRC-3, an essential ERα-dependent coactivator for *GREB1* transcription (48), while promoting the recruitment of the RNA exosome complex component EXOSC10. All three assays used identical buffer and reaction conditions. Therefore, the differential effects of 1,6HD on the recruitment and retention of transcriptional components to the EPC suggest that in our cell-free assays the EPC is encompassed

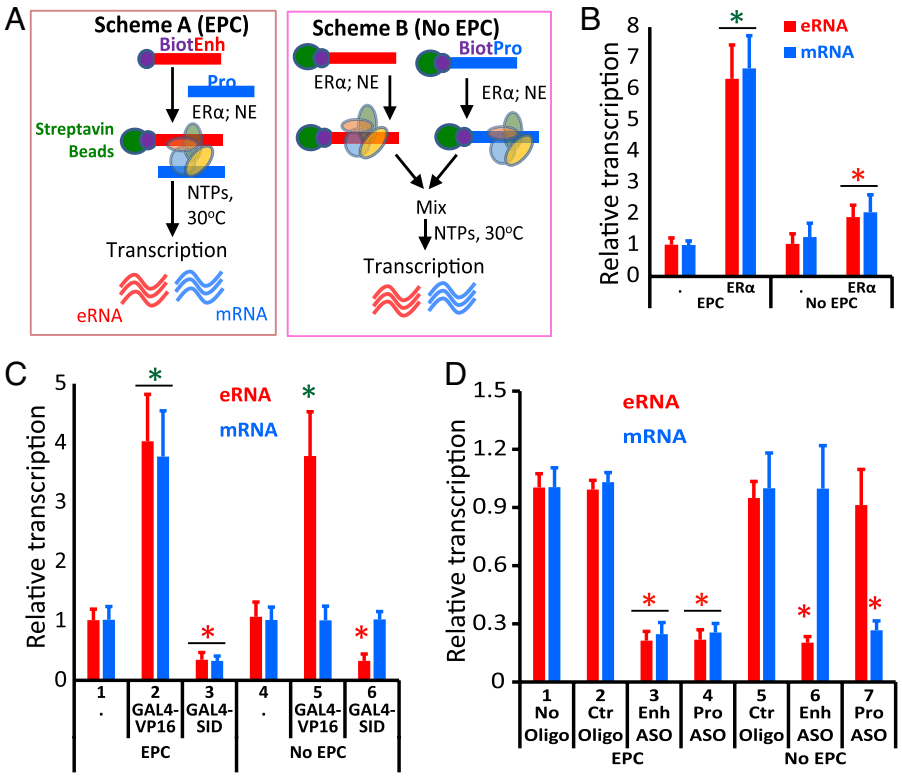


**Fig. 4.** EPC is central to enhancer–promoter transcriptional coordination. (*A*) Schemes A and B showing the strategy for EPC and no EPC, respectively, using chromatinized templates. (*B*) qRT-PCR on reactions outlined in (*A*) showing mutual transcriptional activation of both enhancer and promoter by ERα in the EPC scheme but not in the "No EPC" scheme. (*C*) qRT-PCR data on reactions as outlined in (*A*), but with F1gal fragment (enhancer) and wt F6 fragment (promoter) and without ERα. Results show mutual activation (reaction 2) and mutual repression (reaction 3) of both enhancer and promoter by GAL4-VP16 and GAL4-SID, respectively, in the EPC scheme. No mutual response is observed in the "No EPC" scheme (compare reaction 5 to 2, reaction 6 to 3). (*D*) qRT-PCR data on reactions as outlined in (*A*) showing transcription reduction. All reactions had NE and ERα, with 100 nM E2; therefore reaction 1 is equivalent to reaction 2 in (*B*). ASOs and RNaseH were added to the beads before NTPs. Enh and Pro ASOs cause mutual transcript loss in the EPC scheme but not in the "no EPC" scheme, where they cause targeted transcript loss. Green asterisk denotes significant enhancement compared to reaction 1 ($P$ = <0.001); red asterisk denotes significant reduction compared to reaction 2 ($P$ = <0.001) with two-tailed Student's $t$ test.

within a transcriptional bubble that undergoes LLPS and holds the regulatory components in proximity, disruption of which accompanies reduced EPC and reduced transcriptional activation.

## Discussion

A striking observation in many genome-wide transcriptome studies is the very high correlation in transcription between enhancers and target promoters across Metazoa (12, 15–25). This begets two primary questions: why do enhancers transcribe, and what is the basis for their correlation with promoter transcription. Initially, enhancer transcription was thought of as an incidental, "noisy" byproduct of active enhancers mapping to open chromatin structures (68, 69); the discovery of eRNAs as the substrate of nuclear RNA exosome complexes (70) and in the light of genome-wide RNA surveillance by the RNA exosomes (71) gave credence to the idea that eRNAs represent transcriptional noise. Since the "act of transcription" can cause transcription in the genic neighborhood (52), eRNAs were also thought of as a byproduct of "necessary" transcription. Recent studies have revealed that eRNAs have diverse post-transcriptional functions that fall into three major categories: promoting EPC, recruiting transcriptional coregulators, and facilitating transcript elongation by regulating promoter-proximal pause release (13). However, these ideas still do not fully explain why enhancers transcribe. The questions that still persist are whether eRNAs are made for a purpose that is essential for proper gene expression, or does the cell use them in some way since the cell invests resources in their noisy making?

Together with enhancer–promoter transcriptional correlation, transcriptional concurrence (12, 15–19), and correlation of EPC with transcription (44, 45, 47, 72), the remarkable precision and specificity in activation or repression of target genes upon the perturbation of enhancer transcription (30–32, 73) strongly suggest that transcription at the enhancers and target promoters must be a highly coordinated event. The strongest argument for EPC-coupled transcriptional coordination is that both the enhancer and promoter exist in a microenvironment where they can utilize shared resources, such as transcription coregulators, nucleotides, cofactors, etc (6). In our work presented here, we go further to demonstrate that transcription between an enhancer and its target promoter is interdependent.

This interdependence comes in three layers: i) physical interdependence, where the physical contact between promoter and enhancer elements are necessary; ii) transcriptional interdependence, where transcriptional status of either the promoter or enhancer mirrors that of the other (borrowing a term from quantum mechanics, this scenario can be called "enhancer–promoter entanglement"); and iii) transcript abundance, where loss of transcripts at the enhancer or promoter inhibits transcription at both the enhancer and promoter. In the context of physical interdependence, a recent study observed over threefold higher expression of eRNA—not lower—when the target promoter was deleted (74). This finding is actually in support of our physical interdependence model; because the enhancer in question contacts alternate promoters in the same gene locus when its preferred promoter is lost, thereby maintaining EPC and transcriptional coordination (74). Likewise, in enhancer perturbation screening studies (30–32, 73), it has been observed that enhancer activation stimulates

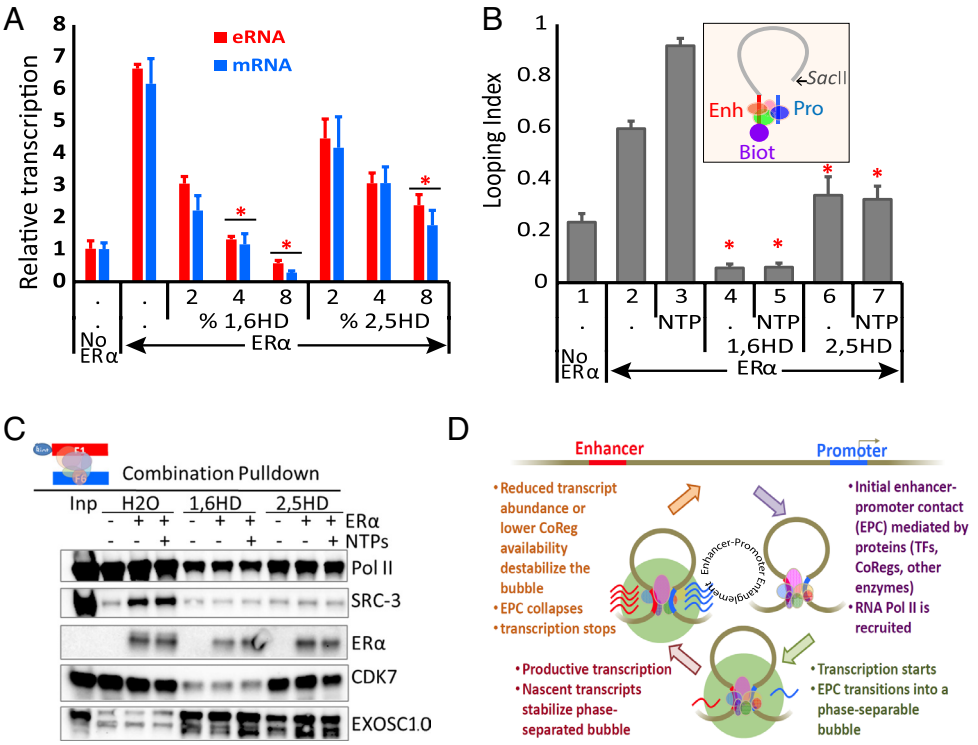


**Fig. 5.** Enhancer–promoter cell-free transcription reactions likely undergo phase-separation. (*A*) IVT of chromatinized WT CompF with HeLa NE in the absence or presence of final 2, 4, and 8% (v/v) of 1,6 hexanediol (1,6HD) and 2,5 hexanediol (2,5HD) as indicated. (*B*) Looping assay on 5′biotinylated CompF with HeLa NE in the absence or presence of 1,6HD and 2,5HD at a final concentration of 8% v/v as indicated. Inclusion of NTPs in reactions 3, 5, and 7 enables transcription, maximizing EPC in reaction 3. Inlet depicts the assay schematic. (*C*) Combination pulldown of chromatinized biotinylated F1 and unbiotinylated F6 in the absence or presence of 1,6HD or 2,5HD (8% v/v). Pulled down nucleoprotein complexes were analyzed by immunoblotting. All reactions (IVT, looping, and combination pulldown) were conducted in identical assay conditions. Red asterisks denote significant reduction compared to the reaction with ERα without HD ($P$ = <0.001) with two-tailed Student's $t$ test. (*D*) Model depicting transcriptional coordination and interdependence between enhancer and promoter. The enhancer and its target promoter are brought together by protein complexes they assemble, forming EPC. Upon transcription initiation, the nascent transcripts promote the EPC transition into a phase-separable bubble. The enhancer and promoter coexist and cotranscribe as an EPC in the transcriptional bubble, thereby explaining enhancer–promoter transcriptional coordination and concurrence. Stability of the EPC transcriptional bubble is dependent on nascent eRNA and mRNA made at the EPC, thereby explaining enhancer–promoter transcriptional interdependence, reflecting "entanglement." Coregulator (CoReg) exhaustion, by the proteasome complex associated with transcription, or eRNA depletion by RNA exosome complex can modulate the stability of the bubble.

promoter activation and enhancer repression causes promoter repression. Therefore, our transcriptional interdependence model is supported by genome-wide transcriptomic studies. Nevertheless, we acknowledge that our results are based on cell-free studies on a single-gene locus, *GREB1*, and generalization of our model will require greater depth and width in transcriptomic investigations.

Thus far, there is overwhelming precedence in transcriptomic literature that enhancer–promoter interactions correlate with transcriptional activation. Importantly, our results (Fig. 2) that EPC is formed in the presence of GAL4-SID correlating with repression implies that long-range interactions are employed in distal transcriptional silencing. Indeed, a growing number of reports suggest that silencers loop to promoters to effectuate repression (reviewed in ref. 75). Also, an enhancer can function as a silencer (76–78)—possibly if it gains enrichment of repressors or corepressors (79). Taken together, our data support the idea that the *GREB1* enhancer assumes silencer function when bound with GAL4-SID and executes promoter silencing via directly contacting the promoter.

It is important here to dissect the consequence of transcript abundance in finer detail. Note that in the experiments we describe (Fig. 3), transcription per se is not impacted initially; for the ASOs to have the desired effect, transcription must have initially taken place so that nascent transcripts could be destroyed. Here, the reasons behind the loss of target transcripts and nontarget transcripts are different. ASOs/RNAse H degrades nascent target transcripts, leading to loss of transcription in the transcriptional microenvironment encompassing the EPC, which leads to loss of production of the nontarget transcript.

But why should destruction of target transcripts shut off overall transcription at the EPC? To help explain this question, we propose a model of an "EPC transcriptional bubble" (Fig. 5*D*); also see ref. 6. Our model (Fig. 5*D*) proposes that i) the enhancer and promoter utilize common transcriptional resources, providing a basis for concurrence in enhancer–promoter transcription; ii) transcription at the enhancer and promoter is mutually stimulatory (48) and therefore coordinated, and iii) nascent transcripts generated at the enhancer (eRNA) and the promoter (mRNA) contribute to phase-separation and stability of the "EPC transcriptional bubble," together ensuring sustained transcription and thereby explaining enhancer–promoter transcriptional interdependence. Support for the "transcriptional bubble" model comes from four lines of recent research: First, transcripts derived from the enhancer and the promoter exist in such close proximity that they can be ligated inside the cells into a hybrid product, and these hybrids are interpreted as the evidence of EPC (80). Second, TFs and coactivators undergo LLPS to form phase-separable transcription condensates at super-enhancers (60, 61, 67). Third, eRNA-containing condensates were suggested to form at E2-induced enhancers (57), while low synthetic eRNA levels of an IVT template demonstrably promote phase-separation of coactivators (58). Interestingly, Henninger et al. (58) observed that higher synthetic eRNA levels disallow coactivator condensation. If this scenario plays out inside the cells, then the transcriptionally active EPCs might have a mechanism to limit eRNA abundance inside the bubble. Indeed, the nuclear RNA exosome is present at the *GREB1* EPC (Fig. 5*C*) and is expected to regulate eRNA abundance and the consequent transcriptional

behavior of the EPC. Fourth, we demonstrate that our cell-free reactions are sensitive to the presence of a known phase-disruptor 1,6HD, suggesting that the biochemical microenvironment encompassing EPC and housing the transcription reactions likely undergoes LLPS. Greater association of the RNA exosome complex component EXOSC10 with the EPC in the presence of LLPS-disruptors likely hints at the possibility of scavenging of the chromatin-bound transcripts upon dissolution of the transcriptional condensates, giving credence to the notion of a protective transcriptional "bubble." We are currently developing additional methodologies to conclusively test this transcriptional bubble model that enables entanglement of the participating enhancer–promoter pairs. We conclude that eRNAs are made because enhancer transcription is a must for promoter-driven transcription and propose that cells evolved biochemical ways to variously utilize the eRNAs since they have already been transcribed. The precision of EPC will depend on the biochemical compatibility between the proteins assembled at the enhancer and the promoter and will require comprehensive characterization of locus-specific EPC-proteomes.

## Materials and Methods

**Antibodies and Other Reagents.** The following antibodies and reagents were used: M280 Streptavidin Dynabeads™(Invitrogen 60210); SRC-3 antibody (Custom mouse monoclonal produced in Monoclonal Antibody/Recombinant Protein Expression Core Facility at Baylor College of Medicine); GAL4DBD antibody (Sigma-Aldrich G3042); SIN3A antibody (Bethyl Lab A300-724A); ERα antibody (Santa Cruz Biotechnology HC-20; sc-543); HDAC1 antibody (Bethyl Lab A300-713A); NiNTA Agarose (Qiagen 30210); B-PER (ThermoFisher 90078); BL21(DE3) pLysE Chem Comp cells (Sigma-Aldrich CMC-0015); Magic Media (ThermoFisher K6815); OneTaq HotStart DNA Pol (NEB M0481L); GAL4-VP16 protein (Sigma-Aldrich SRP-2017); TURBO DNA-free kit (Invitrogen/Ambion AM1907); SensiFAST SYBR Hi-ROX One-Step kit (Bioline BIO-73005); SYBR Green PCR Master Mix (Applied Biosystems 4309155); 1,6 hexanediol (Sigma-Aldrich 240117); and 2,5 hexanediol (Sigma-Aldrich H11904). NTPs were from Promega; dNTPs were from Invitrogen. Primers and ASOs were obtained from Sigma-Millipore or Integrated DNA Technology. See *SI Appendix* for lists of primers and ASOs used.

**Templates for IVT and Looping Assays.** The construction of pCR2.1-CompF and production of CompF by PCR from this parental plasmid have been described elsewhere (48). The ΔF1 and ΔF6 versions of CompF (used in Fig. 1) were PCR-amplified from the parental pCR2.1-CompF. Biotinylated and unbiotinylated primers were selectively combined to generate the singly- and doubly biotinylated fragments used in Fig. 1*B*. GAL4-binding sequence "CGGAAGACTCTCCTCCG" was inserted to replace the deviant full ERE "ggggcacactgaccc" in the F1 region of CompF to generate CompF-F1gal. The GAL4-binding sequence was inserted into "tacctgtgtggagagcactgtgacccagcaaaacacttcagg" (half-ERE underlined) in the F6 region to generate CompF-F6gal. Mutations were generated using Agilent QuickChange SDM kit. F1 and F6 fragments, wt or gal versions (used in Fig. 5), were amplified from the corresponding pCR2.1-CompF derivatives. 4×ERE-E4 template (used in *SI Appendix*, Fig. S5) has been described elsewhere (56). All fragments were amplified with OneTaq DNA polymerase and purified with Bio-Rad PCR Kleen Spin columns. See details of the primers used.

**IVT.** NE preparation from HeLa S3 cells, IVT and looping assays, and chromatin reconstitution of the IVT templates were conducted as described (48). All IVT assays involved 0.2 pmoles of chromatinized template, 50μg NE, and 1 pmole of activator (ERα or GAL4-VP16) or repressor (GAL4-SID) where indicated. All reactions contained 100 nM E2. The template and all reagents as indicated were mixed to a volume of 45 μL at RT to allow the formation of preinitiation complex. Transcription was initiated by addition of 5 μL NTPs mix (5 mM) and shifting the reactions to 30 °C. IVT reactions described in Fig. 3*A* were carried out slightly differently. The 5′biotinylated CompF chromatin was first immobilized on M280 streptavidin beads. Preinitiation complex was formed with NE without or with ERα, and the unbound proteins were washed with Buffer D. Protein-bound CompF-chromatin on beads were resuspended in 45 μL buffer mix that provided the final composition of 12 mM HEPES-KOH (pH 7.9), 12% glycerol, 60 mM KCl, 12 mM MgCl2, 0.12 mM EDTA, 0.3 mM DTT, 1 mM ATP, 0.9 mM acetyl CoA. control oligos, ASOs, and RNase H were added as indicated, and transcription was initiated with NTPs and shifting the reactions to 30 °C. After 45 min, transcription was terminated with 250 μL TriReagent. RNA was extracted, digested with DNase with the Ambion DNase-free kit as per the manufacturer's instruction, and the RNA was used in qRT-PCR. Aqueous solutions of 1,6HD, and 2,5HD were added to the reactions prior to NTPs at final concentrations as indicated.

**Use of ASOs.** Four ASOs each were used for the sets of control (random sequences), Enh (reverse complement to the enhancer sequence downstream of the ERE) and Pro (reverse complement to the sequence downstream of TSS). Each ASO was 110 nt long. For each set, a mixture of the four ASOs was used to a final quantity of 2 pmoles per IVT /looping reactions of 50 μL. Typically, ASOs were added to the reactions before the addition of NE.

**Looping Assay.** Buffer and reagent compositions and assay conditions for looping assays were identical to the IVT reactions described above, except that unchromatinized templates were used as described (48) as incomplete restriction digestion due to the chromatin structure interferes with the assay. Templates were biotinylated either at the 5′ or the 3′ end as indicated (Fig. 1*B*). Assays were otherwise identical to IVT reactions. After 45 min of incubation at 30 °C, nucleoprotein complexes were crosslinked with 0.1% and purified over Bio-Rad PCR Kleen columns. Eluates were digested with indicated restriction enzymes (Fig. 1*B*) and pulled down with M280 streptavidin beads. Bound beads were washed and analyzed by qPCR. Efficiency of digestion was monitored for all assays and was found to be >80% as before (48) and was considered while calculating looping index. See ref. 48 for details of looping index calculation. For experiments in Fig. 5, 0.2 pmoles of chromatinized biotinylated F1 or F6 fragments were bound to 10 μL of M280 streptavidin beads. For Scheme A, unbiotinylated chromatinized F6 was added to biotinylated F1, NE and ERα were added as indicated, and the bound complexes were pulled down and washed with Buffer D. For Scheme B, Biotinylated F1 and F6 were bound to 10 μL of M280 streptavidin beads separately, each then were bound to NE and ERα as indicated, they were washed separately, and the beads were pooled (mixed). Control oligos or ASOs (2 pmoles total of four ASOs) and RNase H were added, followed by NTPs (5 mM) for 45 min at 30 °C. RNA extraction and qPCR were as described above. Aqueous solutions of 1,6HD and 2,5HD were added to the reactions at final concentrations as indicated.

**Combination Pulldown.** Three pmoles chromatinized Dbiotinylated F1 was immobilized on 20 μL of M280 streptavidin beads and incubated with chromatinized unbiotinylated F6 fragments in the presence of 0.5mg NE, without or with 30 pmoles ERα and 0.5 mM NTPs as indicated. All reactions contained 100 nM E2 and 0.2 mM ATP. Binding was carried out at 30 °C. After 30 min, 1,6HD and 2,5HD were added to the reactions to final concentration of 8% as indicated. The nucleoprotein complexes were pulled down 10 min later with magnetic stands, washed in Buffer D, and denatured with SDS sample buffer. Samples were analyzed by immunoblotting.

**Preparation of GAL4-SID.** The parental SID-GAL4 sequence in a mammalian expression vector was a kind gift from Dr. Donald Ayer, University of Utah (54). It was amplified with primers for N-terminal SID and C-terminal GAL4DBD. A PmlI (CACGTG) site was introduced at the 5′ end and an AvrII (CCTAGG) site at the 3′ end during PCR. The amplicon was subcloned into pET302 at PmlI/AvrII, in frame after the 6xHis. A TEV cleavage site was introduced after the 6xHis but was not required in purification. The final sequence is

MHHHHHHVENLYFQGAAAVRMNIQMLLEAADYLERREREAEHGYASMLP EFTMKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTK RSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLT GLFVQDNVNKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTRIR* (verified by sequencing the plasmid).

The protein was expressed in BL21(DE3)pLysE with IPTG induction in Magic Media. The induced cells were extracted with P-BER extraction buffer following the manufacturer's instructions. SID-GAL4 formed inclusion bodies that were dissolved in 1:1 mixture of 8M guanidine-HCl (GnHCl) and P-BER. Dissolved protein was bound to Ni-NTA column in Buffer D supplemented with 4M GnHCl and washed in-column with decreasing GnHCl concentration in Buffer D. The protein was eluted with Buffer D with 0.25M imidazole. Fractions with clean single-band

23 kDa SID-GAL4 were pooled and dialyzed against Buffer D. The protein concentration was estimated with Bio-Rad Protein Assay.

**MCF7 Transcription and 3C Assay.** E2-induction of transcription and looping (3CqPCR) experiments were carried out as described (48). MCF7 cells were hormone-deprived for 3 d. On day 3, cells were transfected with control oligos or ASOs (100 pmoles each oligos; a mix up four control oligos of random sequence, or four Enh ASOs complementary to the eRNA, or four Pro ASOs complementary to the mRNA). Transfection was with TransIt-X2 reagent according to the manufacturer's protocol. Next day, cells were induced with 100 nM E2 for 45 min. RNA was extracted with TriReagent, and RTqPCR was conducted after DNase digestion. For 3C, cells were x-linked with 1% formaldehyde after 45 min E2 induction, lysed, digested with *Pst*I, ligated, deproteinized, and analyzed by qPCR.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

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