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LDB1-mediated enhancer looping can be established independent of mediator and cohesin

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ABSTRACT

Mechanistic studies in erythroid cells indicate that LDB1, as part of a GATA1/TAL1/LMO2 complex, brings erythroid-expressed genes into proximity with enhancers for transcription activation. The role of co-activators in establishing this long-range interaction is poorly understood. Here we tested the contributions of the RNA Pol II pre-initiation complex (PIC), mediator and cohesin to establishment of locus control region (LCR)/β-globin proximity. CRISPR/Cas9 editing of the β-globin promoter to eliminate the RNA Pol II PIC by deleting the TATA-box resulted in loss of transcription, but enhancer–promoter interaction was unaffected. Additional deletion of the promoter GATA1 site eliminated LDB1 complex and mediator occupancy and resulted in loss of LCR/β-globin proximity. To separate the roles of LDB1 and mediator in LCR looping, we expressed a looping-competent but transcription-activation deficient form of LDB1 in LDB1 knock down cells: LCR/β-globin proximity was restored without mediator core occupancy. Further, Cas9-directed tethering of mutant LDB1 to the β-globin promoter forced LCR loop formation in the absence of mediator or cohesin occupancy. Moreover, ENCODE data and our chromatin immunoprecipitation results indicate that cohesin is almost completely absent from validated and predicted LDB1-regulated erythroid enhancer-gene pairs. Thus, lineage specific factors largely mediate enhancer–promoter looping in erythroid cells independent of mediator and cohesin.

INTRODUCTION

Gene regulation underlies the unique transcriptomes that determine developmental progression in complex organisms. This specialization is brought about by the activity of tens of thousands of transcriptional enhancers that are differentially active in particular cells and tissues (1–4). Enhancers function to increase the transcription of target genes over long linear distances. To accomplish this, enhancers engage in close physical contact with target promoters through chromosome folding or looping (5–7). How enhancers communicate with the proper target genes to establish and maintain cell-specific functions is a pressing question in modern biology with implications for understanding and treating genetic diseases (1,8–10).

Genome wide studies showed that architectural proteins CTCF and its frequent binding partner cohesin are important for partition of the genome into largely conserved topologically associating domains or TADs on the megabase scale (11–13). CTCF and cohesin also contribute to organization of sub-TAD domains that may represent 'insulated neighborhoods' within which enhancers function to activate their related target genes (13,14). These architectural proteins have also been reported to contribute directly to enhancer–promoter communication (15). The large mediator complex that is recruited to enhancers could also be a central player in connecting enhancers to promoters (16). In embryonic stem (ES) cells and in mouse embryonic fibroblasts, RNAi studies showed that cohesin and the mediator complex are necessary for enhancer–promoter interactions of pluripotency genes (17).

One prominent model envisions that mediator interacts with enhancer-bound activators, bridges to target promoters and recruits cohesin (17–19). Cohesin subunits form a ring structure capable of embracing two strands of chromatin and could contribute critically to the genesis of enhancer–promoter proximity at active genes. Alternatively, higher order genome folding might be influenced by cohesin, possibly via a loop extrusion mechanism and this folding might contribute to enhancers coming into proximity with target genes (20,21). In this regard, acute depletion of cohesin did not lead to global collapse of pluripotency gene expression in ES cells and representative genes maintained their association with enhancers (22). This result suggests the possibility of an indirect contribution of cohesin to enhancer function. Distinguishing these possibilities is challenging.

Numerous studies in individual model gene loci have shown that long-range enhancer interactions require cell

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type-specific proteins and protein complexes that bind to enhancers and promoters and can stabilize their interaction with each other (6,7). Studies of the β-globin locus using RNAi indicated that the erythroid factors KLF1, GATA1 and TAL1 as well as the more widely expressed factor LDB1 are necessary for β-globin genes to interact with and be activated by the locus control region (LCR) enhancer (23–26). GATA1, TAL1 and LDB1 are members of an erythroid complex including LMO2 that activates erythroid genes through occupancy of virtually all erythroid enhancers, including the β-globin LCR (25,27). The complex also occupies the β-globin promoter and LDB1 dimerization is required to establish proximity between the gene and LCR (28).

Here we tested the contributions of erythroid transcription factors and co-activators RNA Pol II pre-initiation complex (PIC), mediator and cohesin to establishment of β-globin/LCR interaction. We employed three systems, CRISPR/Cas9 editing of the β-globin promoter, rescue of LDB1 knock down (KD) erythroid cells by LDB1 and CRISPR/Cas9-mediated forced enhancer looping. Surprisingly we find that β-globin enhancer loops can be formed without the participation of the RNA Pol II PIC, mediator or cohesin. Moreover, active enhancers in erythroid cells, whether regulated by LDB1 or not, are largely depleted for cohesin. These results suggest that formation of long-range enhancer–promoter contacts depends largely on the interaction between specific transcription factors rather than on general regulators of gene expression.

MATERIALS AND METHODS

Cell culture

Wild-type (WT) and transgenic mouse erythroid leukemia (MEL) cells lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified incubator at 5% CO_2. MEL cell differentiation was induced by incubation cells with 2% Dimethylsulfoxide for 4 days.

Gene editing using CRISPR-Cas9

Guide RNA (gRNA) sequences with lowest off target hitscore were designed using the http://crispr.mit.edu/ website. Only one sequence of the core promoter and KLF1 binding site could be uniquely targeted in the β^{maj} promoter compared to β^{min}. Oligonucleotides with gRNA sequence (Supplementary Table S1) were cloned into CRISPR-Cas9 and gRNA expression vector pSpCas9(BB)-2A-GFP (PX458) (gift from Feng Zhang (Addgene plasmid #48138)) as described (29). MEL cells were transfected by electroporation using Nucleofactor (Lonza) following manufacturers suggestions. The top 0.1% of EGFP positive cells were sorted by FACS ARIA II (BD biosciences) and individual clones isolated. Clonal lines were genotyped by polymerase chain reaction (PCR) using EmeraldAmp GT PCR Master Mix (Takara) and target specific primers flanking the deletions (Supplementary Table S1) and deletions validated by sequencing (Supplementary Figure S1A). To address the specificity of genome editing, we checked top 25 intergenic potential off-target sites and found them to be located in inactive chromatin according to ChromHMM. We then sequenced the top five off-target sites located in coding regions of expressed genes (Krivega *et al.* (28)) in ΔCore and ΔCore/GATA cells and confirmed they were not mutated (Supplementary Figure S1B).

To generate DDΔ4/5-dCas9 overexpression vector, cDNA of HA-tagged DDΔ4/5 was cloned instead of VP64 domain in lenti dCAS-VP64_Blast plasmid (gift from Feng Zhang (Addgene plasmid # 61425)). Oligonucleotides with gRNA targeting β^{maj} promoter upstream region were cloned in lentiGuide-Puro (gift from Feng Zhang (Addgene plasmid # 52963)) as described (30). MEL cells were transfected with Escort™ IV transfection reagent (Sigma), selected in blasticidin and puromycin containing medium during 1 week. Stable clones were isolated and DDΔ4/5-dCas9 expression was checked by western blot hybridization.

Chromatin immunoprecipitation (ChIP)-qPCR

Chromatin immunoprecipitation (ChIP) was performed as described (28). EGS (Thermo Fisher Scientific) followed by formaldehyde was used for cross-linking chromatin for Med1, Med12 and KLF1 ChIP. Differences in DNA enrichment were determined by real-time qPCR using SYBR chemistry with the ABI 7900HT (Applied Biosystems). The comparative Ct method was used to calculate protein of interest relative enrichment over input DNA abundance. For ChIP primers see Supplementary Table S2. Primers with specificity for β^{maj} or β^{min} were employed. For antibodies see Supplementary Table S5.

Chromatin conformation capture assay (3C)

Chromatin conformation capture (3C) was performed using Bgl II cleavage as described (28). Relative cross-linking between HS2 and fragments of interest was analyzed by real-time qPCR with published TaqMan probes and primers (28). Interaction frequencies were normalized to interaction frequencies between two restriction fragments in the ERCC3 locus. Random ligation products of BglII digested BAC DNA containing the entire murine β-globin or ERCC3 loci of mouse strain 129 origin were used for primer efficiency normalization. Additional primers are listed in Supplementary Table S3.

Reverse-transcription qPCR

Reverse-transcription reaction was done as described (28). Briefly, RNA was extracted by RNeasy kit (Qiagen). Two micrograms of RNA were treated with DNaseI (Thermo Fisher Scientific) for 15 min at 25°C. RNA was reverse transcribed using SuperScript® III First-Strand Synthesis System following manufacturer's instructions (Thermo Fisher Scientific) with random hexamers (Life Technologies). RT-qPCR was performed using SYBR chemistry with the ABI 7900HT (Applied Biosystems). Data were normalized to the actin signal. For RT-qPCR primers see Supplementary Table S4. Primers with specificity for β^{maj} or β^{min} were employed.

Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation (Co-IP) was done as described (28). For nuclear extract preparation, induced MEL or E14.5 fetal liver cells were swollen in hypotonic buffer (10 mM Tris [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM Dithiothreitol and protease inhibitors) for 10 min on ice and centrifuged at 2000 × *g* for 10 min at 4°C. Pellets were re-suspended in two volumes of the hypotonic buffer, Dounce homogenized ten times with a loose pestle and centrifuged at 25 000 × *g* for 30 min at 4°C. The nuclear pellet was re-suspended in one volume of high salt buffer (20 mM Tris [pH 7.4], 0.6 M KCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT and protease inhibitors). Nuclei were briefly sonicated and rotated for 30 min at 4°C. Insoluble material was removed by centrifugation at 14 000 × *g* for 30 min. One miligram of nuclear extract was used for each immunoprecipitation. Protein complexes were precipitated in IP100 buffer (25 mM Tris [pH 8.0], 5 mM MgCl2, 10% glycerol, 100 mM KCl, 0, 1% NP-40 and 0.3 mM DTT) overnight at 4°C with antibodies against LDB1 or GATA1 and Dynabeads® Protein G (Thermo Fisher Scientific) (31). Complexes were washed once with IP500 buffer (500 mM KCl), twice with IP100 buffer and eluted by incubation with elution buffer (6 M urea, 0.05% sodium dodecylsulphate, 50 mM Tris [pH 8.0] and 5 mM EDTA) for 30 min at 37°C. Antibodies are listed in Supplementary Table S5.

Western blotting

Western blotting was done as described (28). Sample preparation, electrophoresis, transfer and hybridization followed Nupage protocols (Thermo Fisher Scientific). Antibodies are listed in Supplementary Table S5.

Bioinformatics analysis

All bioinformatic analysis used the UCSC mouse assembly mm9 and UCSC human assembly hg19. Binary heat maps were generated using the pybedtools.plotting.binary_heatmap function from pybedtools v0.7.7 (32), which handles combinatorial occupancy using BEDTools' multinter program with the -cluster option enabled. Active enhancers were called using the ChromHMM algorithm (33) and ENCODE data for uninduced MEL cells (available at https://doi.org/10.5281/zenodo.439534). Sites co-occupied by both Rad21 and SMC3 in MEL, K562, GM12878 and HepG2 cells according to ENCODE data were considered as cohesin occupied sites. ChIP-seq and ChromHMM datasets are listed in Supplementary Table S6. Jaccard index was calculated using the jaccard program with default options from BEDTools v2.25.0 (34). Looped enhancers were identified by intersecting ChromHMM predicted enhancers with intergenic interactors identified by promoter capture-Hi-C (35) using the intersect program with -wa option from BEDTools v2.25.0. Enhancer distribution among TADs and super-enhancers was determined by intersection of enhancers with TADs (11) and MEL cell super-enhancers (36) using the intersect program with -c option from BEDTtools v2.25.0. Distance between looped enhancers was determined by the closest program with -io option from BEDTools v2.25.0. Looped enhancers were assigned to interacting genes using the capture-Hi-C dataset (35). Expression level of the genes looped to enhancers was determined using RNA-seq data from induced MEL cells (28). Statistical analyzes and graph generation were performed using R and MS Excel. To identify transcription factor binding sites, 200 bp of DNA sequences around peak summits of enhancer occupied Rad21 and GATA1 were used for MEME-ChIP analysis with standard parameters (37). Med1 ChIP-seq reads were mapped against the mouse genome (mm9) using Bowtie2 (version 2.0.2) with default parameters. Uniquely mapped reads were used to call peaks by MACS2 (version 2.1.0) with default parameters.

RESULTS

Mediator is recruited to the β-globin locus by the LDB1 complex

Few studies address the mechanisms that regulate chromatin loop formation between enhancers and genes. Although LDB1 dimerization is a key component of β-globin promoter–enhancer long-range interaction (28,38), we wished to delve further into additional requirements for enhancer loops to form in the β-globin locus. To avoid possible indirect effects of protein KD approaches and to distinguish direct from indirect effects of co-activators, we deleted small elements in the β^{maj} globin gene promoter using CRISPR/Cas9 genome editing to eliminate RNA Pol II PIC formation and LDB1 occupancy. We established mouse erythroid MEL cell lines with a deletion of the β^{maj} globin gene core promoter (TATA box and initiator element–ΔCore MEL cells) or a deletion encompassing the core promoter along with the GATA1 binding motif (ΔCore/GATA MEL cells) (Figure 1A and Supplementary Figure S1A). A single gRNA was employed that uniquely targeted the β^{maj} and not the β^{min} gene. MEL cells are arrested at the early pro-erythroblast stage and can be induced by DMSO to differentiate to mature erythroid cells that produce abundant β-globin.

ChIP analysis showed that PIC occupancy was lost at the β^{maj} promoter upon deletion of the core element in induced ΔCore MEL cells as judged by drastic reduction in occupancy of the TBP subunit of the TFIID complex (Figure 1B). GATA1 and LDB1 still occupied the promoter after deletion of the core element. The signal for LDB1 was decreased, although not to a statistically significant extent (Figure 1C and D). However, deletion of the GATA site along with the core promoter strongly reduced LDB1 complex occupancy in induced ΔCore/GATA MEL cells. The CRISPR/Cas9 deletions did not compromise MEL cell differentiation (Supplementary Figure S2) and had no effect on GATA1 or LDB1 occupancy at the β-globin LCR HS2 (Figure 1C and D). This result supports the important role of GATA1 in defining LDB1 complex occupancy at the β^{maj} globin gene promoter (39).

The large mediator complex may bridge between enhancer-bound transcription factors and RNA Pol II machinery at target genes, contributing to their proximity (18). Previously published ChIP-seq data for Ter 119^{+} adult erythroid cells (40) and ChIP-qPCR using E14.5 mouse fetal

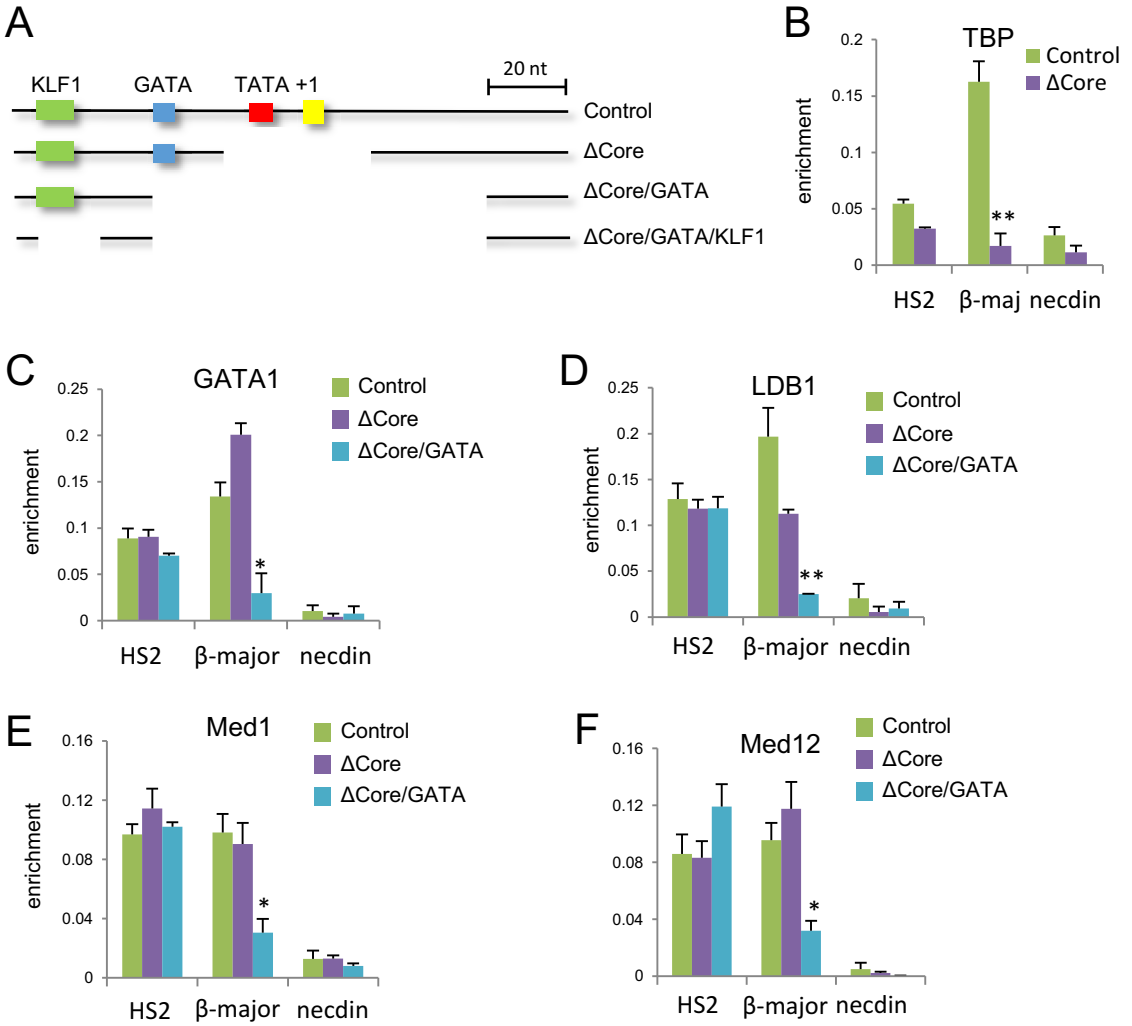

Figure 1. Mediator is recruited to the β-globin locus by the LDB1 complex. (**A**) Diagram of deletions in the endogenous β^{maj} globin gene in mouse erythroid leukemia (MEL) cells. Blue rectangle–GATA binding site, red–TATA box, yellow–initiator. Green rectangle–Klf1 site discussed in Figure 2. (**B**) Chromatin immunoprecipitation (ChIP) and qPCR were used to determine TBP occupancy at β^{maj} globin gene promoter in control (transformed with a vector lacking guide RNAs) and ΔCore induced MEL cell lines. (**C**) GATA1 and (**D**) LDB1 occupancy at LCR HS2 and β^{maj} globin gene promoter in control, ΔCore and ΔCore/GATA induced MEL cell lines. (**E**) Med1 and (**F**) Med12 occupancy at LCR HS2 and β^{maj} globin gene promoter in control, ΔCore and ΔCore/GATA induced MEL cell lines determined by ChIP-qPCR. Error bars indicate SEM of three biological replicates. (*) $P < 0.05$ and (**) $P < 0.01$ by Student's *t*-test.

liver erythroid cells confirmed mediator occupancy at the looped β-globin locus (Supplementary Figure S3A–C). It is also known that Med1 is recruited to the β-globin promoter with kinetics similar to TFIIB and to loop formation (41,42).

To investigate potential interplay between mediator and the LDB1 complex in establishing enhancer–promoter interactions at the β-globin locus, we checked chromatin occupancy of the mediator core subunit Med1 and the kinase module subunit Med12 in induced control, ΔCore and ΔCore/GATA MEL cells. Med1 and Med12 occupied the β^{maj} globin promoter as well as the LCR HS2 site in induced control cells (Figure 1E and F). Occupancy of these mediator complex components in the locus was not affected by the ΔCore deletion. By contrast, deletion of the GATA site in ΔCore/GATA cells severely affected mediator complex occupancy at the β^{maj} promoter. Thus, the PIC is dispensable for recruitment of mediator to the β^{maj} promoter and mediator can occupy the LCR HS2 element independent from target gene promoter occupancy, consistent with earlier work (42,43). These results suggest that mediator occupancy at the β^{maj} promoter depends on the LDB1 protein complex.

LDB1 and KLF1 each contribute to β^{maj} globin gene/LCR chromatin looping but the RNA Pol II PIC does not

To determine the importance of the RNA Pol II PIC and mediator to enhancer–promoter interaction, we carried out 3C using HS2 of the LCR as the anchor in WT cells and cells that have lost occupancy of these complexes due to deletions in the β^{maj} globin gene promoter. The β^{maj} and β^{min} globin genes were interrogated independently using gene-specific primers. Increased frequency of interaction between

HS2 and both β-globin genes was detected in induced compared to uninduced MEL cells (Figure 2A). LCR/β^{maj} interaction was not affected in induced ΔCore MEL cells that have lost PIC occupancy. However, loss of the PIC at β^{maj} was reflected in strongly reduced production of primary transcripts and reduced Pol II occupancy at the promoter (Figure 2B and C). In contrast, LCR/β^{maj} interaction was strongly decreased in induced ΔCore/GATA MEL cells that have lost promoter occupancy of the LDB1 complex and mediator (Figure 2A), with further reduction of transcripts and Pol II occupancy at β^{maj} (Figure 2B and C). This result is consistent with our demonstration that LDB1 is required for LCR/β^{maj} interaction (25,28).

Although elimination of the PIC, LDB1 and mediator complex occupancy by the ΔCore/GATA deletion at β^{maj} reduced interaction frequency strongly, it was not reduced to the level seen in uninduced cells (Figure 2A). To test the basis for residual looping to the LCR in ΔCore/GATA cells, we created an additional CRISPR/Cas9 mediated deletion of the β^{maj} promoter KLF1 (also known as EKLF) binding site within the context of the ΔCore/GATA mutation (Supplementary Figure S1). The KLF1 site is 30-nt upstream of the LDB1 complex-interacting site. KLF1 null erythroid fetal liver cells were used to show that KLF1 is required for LCR/β^{maj} interaction and a subset of genes is known to be co-regulated by KLF1 and LDB1, although mechanisms are unclear (23,44). Deletion of the KLF1 site further decreased LCR/β^{maj} interaction to a very low level (Figure 2A). This result suggests that LDB1 and KLF1 contribute to LCR/β^{maj} long-range interaction additively.

Interestingly, reciprocal consequences to the deletions at β^{maj} were observed at the β^{min} globin gene. Increased interaction frequency between the LCR and β^{min} was observed after ΔCore deletion at β^{maj}, as well as an increase in β^{min} primary transcripts and Pol II occupancy (Figure 2A–C). The ΔCore/GATA mutation at β^{maj} had no further effect on LCR/β^{min} proximity or transcription beyond that seen after the ΔCore mutation, likely reflecting that the LDB1 complex does not occupy the β^{min} promoter (Supplementary Figure S4). However, the β^{maj} ΔCore/GATA/KLF1 mutation boosted LCR/β^{min} proximity, β^{min} transcripts and Pol II occupancy even further (Figure 2A–C). The β^{min} promoter is known to be occupied by KLF1 (45). Moreover, loss of KLF1 at β^{maj} was accompanied by a gain at β^{min} as determined by ChIP (Figure 2D). These results strongly suggest that LCR/β^{min} looping depends on KLF1. They also are supportive of a β^{maj}/β^{min} competition model, although this issue is unresolved (45,46).

Overall, these data indicate that the RNA Pol II PIC is not required to establish LCR/β-globin proximity, which comports with our observation and those of others that enhancer–promoter interaction and transcription activation can be separated in this locus (28,47). However, in this system, we could not separate the roles of mediator and of LDB1 complexes in looping because both are lost together along with β^{maj} globin gene/LCR interaction upon ΔCore/GATA β^{maj} promoter deletion.

Mediator core module is not required to establish LCR/β-globin interaction

So far, the results leave unanswered whether the mediator complex is required to establish enhancer looping. To investigate further, we took advantage of LDB1 KD MEL cells ectopically expressing either WT LDB1 or a mutated form of LDB1 with a small internal deletion (LDB1Δ4/5) (28) (Figure 3A). Expressing the mutant LDB1 protein in the KD background fully supports LDB1 complex occupancy in the locus and establishment of long-range LCR/β-globin interactions upon induction but does not support transcription activation, separating these processes (28). Cells rescued by expression of the mutant LDB1 have greatly reduced Pol II at the β-globin promoter.

LDB1 KD drastically affected Med1 and Med12 occupancy both at LCR HS2 and at the β^{maj} globin gene promoter in induced MEL cells (Figures 3B and C). Expression of the full length LDB1 protein in these cells fully rescued mediator complex occupancy at both locations. By contrast, LDB1Δ4/5 protein did not restore Med1 occupancy at the LCR or at the β-globin promoter, even though it rescued looping between them (28). LDB1Δ4/5 was similarly unable to rescue Med12 at the LCR but, intriguingly, fully rescued Med12 occupancy at the β-globin promoter. We attempted to KD Med12 to test its function in looping but we were unable to obtain viable cells. As an alternative, we assayed by ChIP histone H3Ser10 phosphorylation (H3Ser10P), which is a product of the mediator kinase module enzymatic activity and is a modification associated with active transcription (48,49). The H3Ser10P mark was detected along with Med12 occupancy at the looped but transcriptionally inactive β-globin promoter (Figure 3D). This result raises the possibility that H3Ser10 phosphorylation by Med12 occurs before transcription activation at the β-globin promoter. Overall, we conclude that LCR/β-globin looping can be established without the mediator core module. However, we could not rule out a role for the mediator kinase domain in loop formation in induced cells in this system.

The ChIP data of Figure 3B and C, as well as that in the β-globin promoter CRISPR/Cas9 mutation system suggest that the LDB1 complex recruits Mediator to the β-globin locus. Interaction of the LDB1 complex component GATA1 with MED1 has been reported (50). To ask whether an interaction between Med12 or Med1 and LDB1 exists, we performed co-IP experiments using nuclear extract from induced MEL cells (Figure 3E). Antibody against LDB1 specifically pulled-down Med12 without interaction with Med1. Under the stringent conditions employed, we did not observe interaction of GATA1 with Med12 or Med1. The ability of LDB1 to pull down MED12 is not affected by the 4/5 deletion (Figure 3F). The LDB1–MED12 interaction was also strongly detected in nuclear extracts from E14.5 mouse fetal liver that is primarily made up of erythroid cells (Figure 3G). These data support interaction between LDB1 and Med12 independent from the Mediator core module and GATA1 but do not rule out a potential contribution of other LDB1 complex members to Med12 interaction.

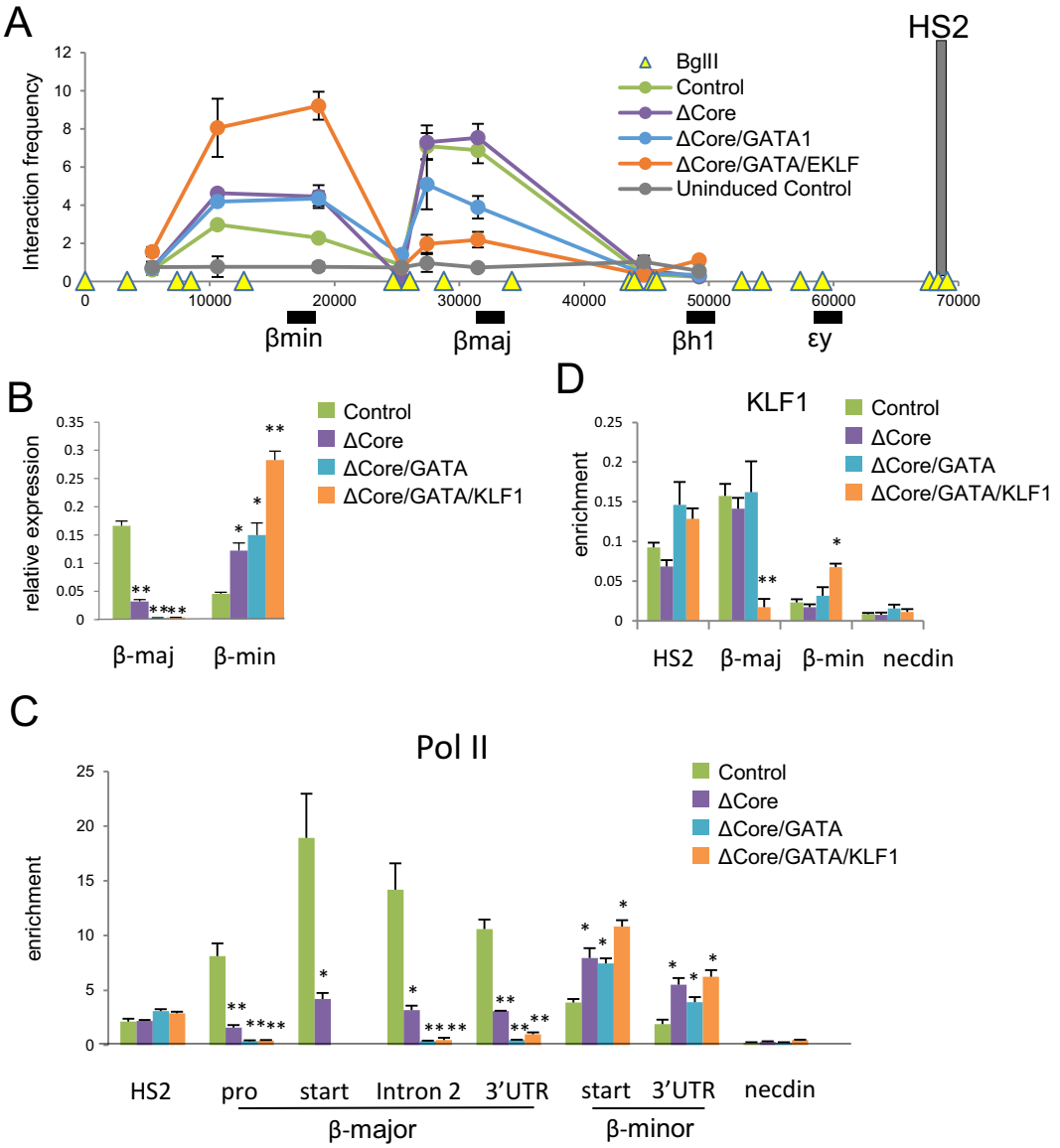

Figure 2. β^{maj} globin gene expression affects β^{min} globin gene activation by a looped enhancer. (**A**) Interaction frequency determined by chromatin conformation capture (3C) between locations across β-globin locus using HS2 enhancer as the anchor (gray bar) observed for induced ΔCore, ΔCore/GATA and ΔCore/GATA/KLF1 MEL cell lines and uninduced and induced control MEL cells. Yellow triangles along bottom of X axis indicate BglII restriction sites. (**B**) Primary transcripts of β^{maj} and β^{min} globin genes were examined by RT-qPCR after deletion of Core, Core/GATA or ΔCore/GATA/KLF1 elements in the β^{maj} globin gene promoter in induced MEL cells. (**C**) RNA PolII occupancy at β^{maj} and β^{min} globin genes in control, ΔCore, ΔCore/GATA and ΔCore/GATA/KLF1 induced MEL cell lines determined by ChIP-qPCR. (**D**) KLF1 occupancy at β^{maj} and β^{min} globin genes in control, ΔCore, ΔCore/GATA and ΔCore/GATA/KLF1 induced MEL cell lines determined by ChIP-qPCR. Error bars indicate SEM of three biological replicates. (*) $P < 0.05$ and (**) $P < 0.01$ by Student's *t*-test.

Forced β-globin/LCR looping does not require mediator or cohesin

LCR looping and β-globin transcription activation can be forced in uninduced erythroid cells by tethering LDB1 or its dimerization domain (DD) to the β-globin promoter using a Zn-finger peptide (51). The forced loop is supported by LDB1 complex binding in the LCR, which is also known to occur in uninduced MEL cells (25). We adopted this approach using the CRISPR/Cas9 system instead of a Zn-finger peptide to further test the role of mediator and cohesin in establishment of an enhancer loop. As before (Figure 3A), we sought to separate looping and transcription by using the Δ4/5 version of the LDB1 DD fused to dCas9 (DDΔ4/5-dCas9). We used a single guide RNA specifically targeting the β^{maj} globin gene promoter and not the β^{min} promoter or other globin promoters (Figure 4A). As predicted, β-globin transcription was not activated in uninduced cells targeted with DDΔ4/5-dCas9 (Figure 4B). Control cells, expressing DDΔ4/5-dCas9 and an empty gRNA vector, illustrate the level of β^{maj} transcription and promoter RNA Pol II normally obtained after induction (Figure 4B and C).

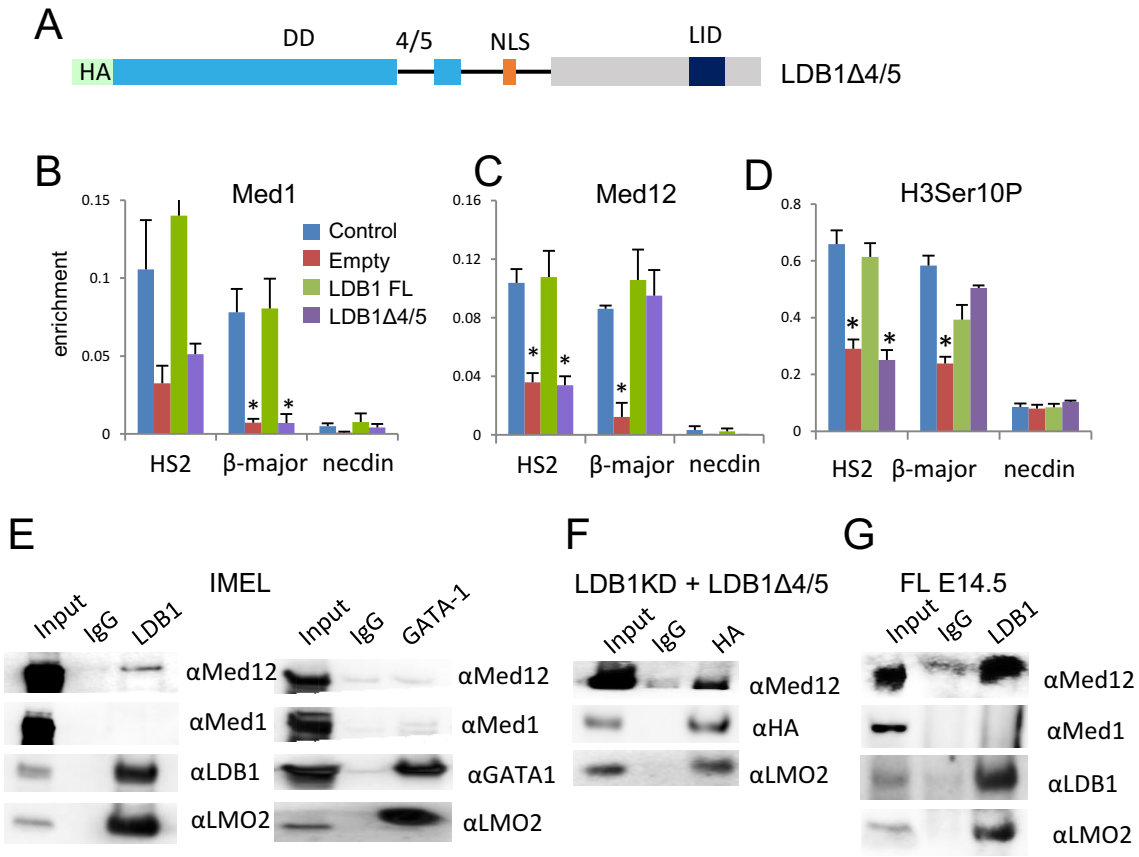

Figure 3. Enhancer–promoter interaction does not require mediator core module. (**A**) Diagram of mutant HA tagged LDB1 expressed in the background of LDB1 KD MEL cells. The region designated 4/5 was deleted from the dimerization domain of LDB1. Med1 (**B**), Med12 (**C**) and H3Ser10P (**D**) occupancy determined by ChIP-qPCR at LCR HS2 and the β^{maj} globin gene promoter in control, LDB1 KD (containing empty expression vector), LDB1 KD overexpressing full length LDB1 and LDB1 KD overexpressing LDB1 Δ4/5 induced MEL cell lines. Error bars indicate SEM of three biological replicates. (*) $P < 0.05$ and (**) $P < 0.01$ by Student's *t*-test. (**E**) Co-immunoprecipitation (Co-IP) of endogenous proteins using nuclear extract from induced MEL cells with antibodies against LDB1 or GATA1. Immunoprecipitation material was analyzed by western blot with Med12, Med1, LDB1, GATA1 and LMO2 antibodies. (**F**) Nuclear extract from LDB1 knock down (KD) cells expressing LDB1 Δ4/5 were used for co-IP as in panel E. (**G**) Nuclear extract from E14.5 mouse fetal liver cells was used for co-IP as in panel E.

Tethering DDΔ4/5-dCas9 to the β^{maj} promoter fully rescued LCR/β^{maj} loop formation in uninduced cells to the level seen in induced control cells (Figure 4D). ChIP analyzes showed that the LCR and β-globin gene in a forced loop in uninduced cells is devoid of Med1 and Med12, whereas, these proteins occupy the normally looped locus of induced control cells (Figure 4E and F). Thus, the absence of mediator in the targeted locus in uninduced cells correlates with the absence of transcription at the LDB1 DDΔ4/5-looped but inactive locus.

We used this system to ask if cohesin is involved directly in normal loop formation or in forced looping between the LCR and β-globin gene. We carried out ChIP experiments using antibodies to cohesin complex components Rad21 and Smc3 (Figure 4G and H). Neither of these proteins could be detected at LCR HS2 or at the β-globin gene whether these sites were occupied by mediator or not. By contrast, cohesin occupied its known site, 3′HS1, flanking the β-globin locus. We confirmed the absence/presence of cohesin at these sites in fetal liver erythroid cells (Supplementary Figure S3D and E). We conclude that a chromatin loop involving the β-globin LCR and gene can be formed in the absence of mediator and that cohesin is not recruited whether the LCR loop forms normally upon induction or whether it is forced in uninduced cells.

Only a few predicted enhancers in erythroid cells are occupied by cohesin

In either naïve or primed ES cells, about a quarter of cohesin-occupied sites genome wide corresponded to active enhancers and promoters (17). Studies using ChIA-PET analysis of human ES cells or human erythroid K562 cells indicated that up to about 12% of the cohesin-anchored (Rad21 or Smc1) loops genome wide connect enhancers and promoters (14,52,53). From these data, it is less clear what fraction of total enhancers in these cells are occupied by cohesin and thus might generally use cohesin in looping to target genes.

To examine cohesin involvement with respect to the totality of the enhancer landscape in a particular cell type, we first defined six chromatin states including active enhancer and weak enhancer using ChromHMM (33,54) (see 'Materials and Methods' section). Hidden Markov modeling of

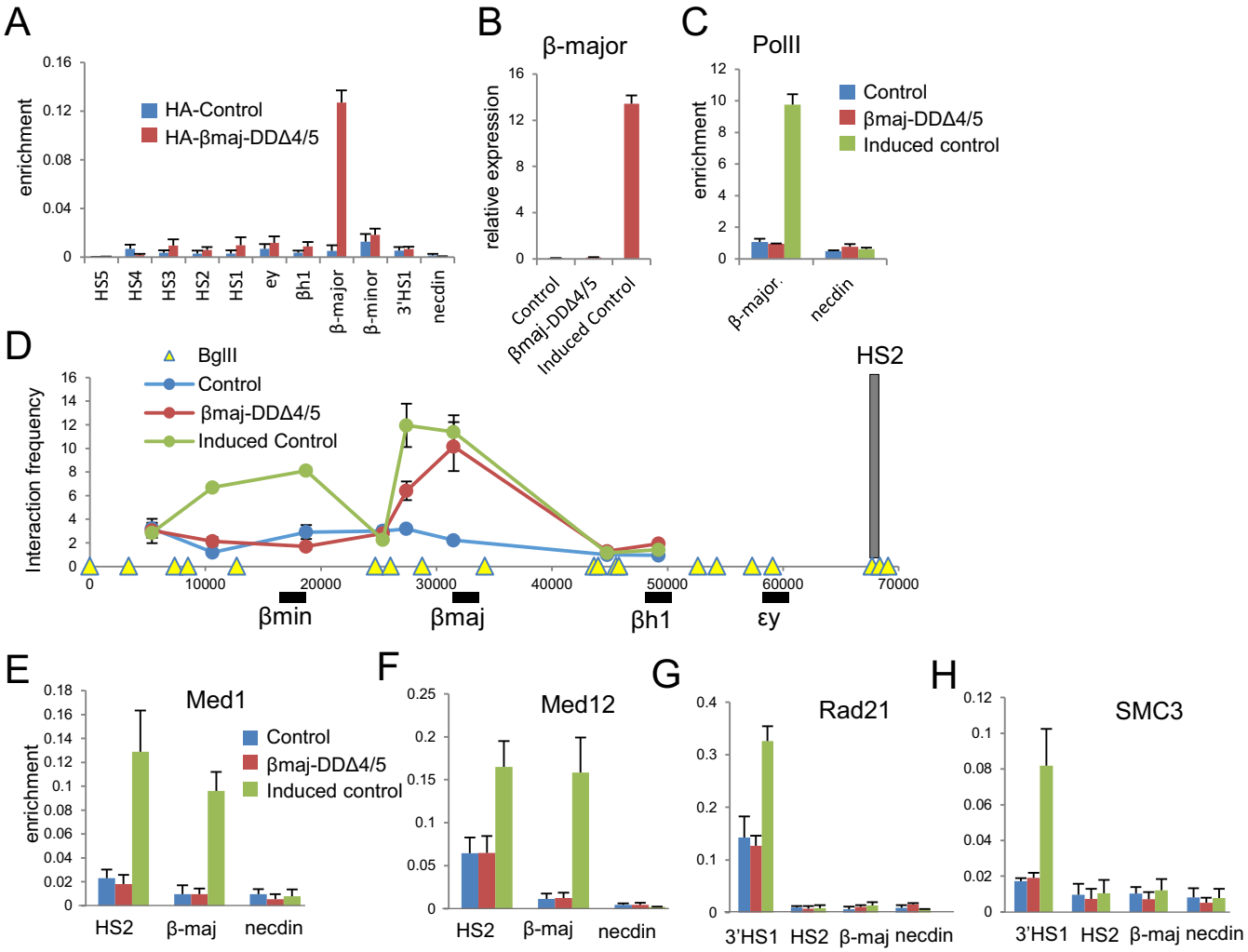

Figure 4. Forced looping is independent from transcription activation, cohesin and mediator complexes. (**A**) ChIP-qPCR determination of HA-DDΔ4/5-dCas9 occupancy in uninduced MEL cells overexpressing DDΔ4/5-dCas9 with an empty gRNA vector (HA Control) or β^{maj} promoter gRNA (HA-βmaj-DDΔ4/5 cells). (**B**) Expression of the β^{maj} globin gene in uninduced Control and βmaj-DDΔ4/5-expressing cells and in induced Control MEL cells. (**C**) PolII occupancy at β^{maj} globin gene promoter in uninduced Control and βmaj-DDΔ4/5-expressing cells and in induced Control MEL cells. (**D**) Interaction frequency determined by 3C between locations across the β-globin locus using the HS2 enhancer as the anchor (gray bar) for uninduced Control and βmaj-DDΔ4/5-expressing cells and for induced Control MEL cells. Yellow triangles along bottom of X axis indicate BglII restriction sites. Med1 (**E**), Med12 (**F**), Rad21 (**G**) and SMC3 (**H**) occupancy determined by ChIP-qPCR at LCR HS2 and the β^{maj} globin gene promoter in uninduced Control and βmaj-DDΔ4/5-expressing cells and in induced Control MEL cells. Error bars indicate SEM of three biological replicates.

enhancer chromatin in erythroid cells revealed about 50,000 enhancers, comparable to numbers for other cell types (55–57). Next, we determined co-localization in MEL cells of cohesin subunits Rad21 and SMC3 (Supplementary Figure S5) with predicted enhancers. Co-localization was defined as overlap of at least 1-nt of a ChIP-seq called peak with an enhancer called by ChromHMM. We included GATA1 occupancy in the analysis as a proxy for residence of the LDB1 complex.

Figure 5A shows a binary heat map comparing these data. Intriguingly, we found that cohesin subunits can be found overlapping only about 7% of active enhancers (3300 out of 48 041 enhancers) in MEL cells, with a Jaccard index of 0.025 indicating low similarity in genome-wide distribution of the two features. Cohesin was further excluded (overlap of about 3%, Jaccard index 0.016) from GATA1-occupied enhancer regions that are known to be involved in regulation of erythroid gene expression through the LDB1 complex (27). Co-localization of cohesin with the ChromHMM weak enhancer class yielded a similar overlap of cohesin (4%, Jaccard index 0.0048, Supplementary Figure 5). These results reveal a weak overlap between predicted enhancers in erythroid cells and cohesin complex occupancy.

To investigate the nature of enhancers either occupied or not occupied by cohesin in erythroid cells, we first examined CTCF occupancy. CTCF is a key DNA-binding partner of cohesin but cohesin may have a CTCF-independent role in transcription regulation (58). 'Cohesin no CTCF' (CNC) sites have been observed to be associated with liver-specific gene expression and to stabilize transcription factor binding at enhancers (59). Overlap of CTCF-occupied sites de-

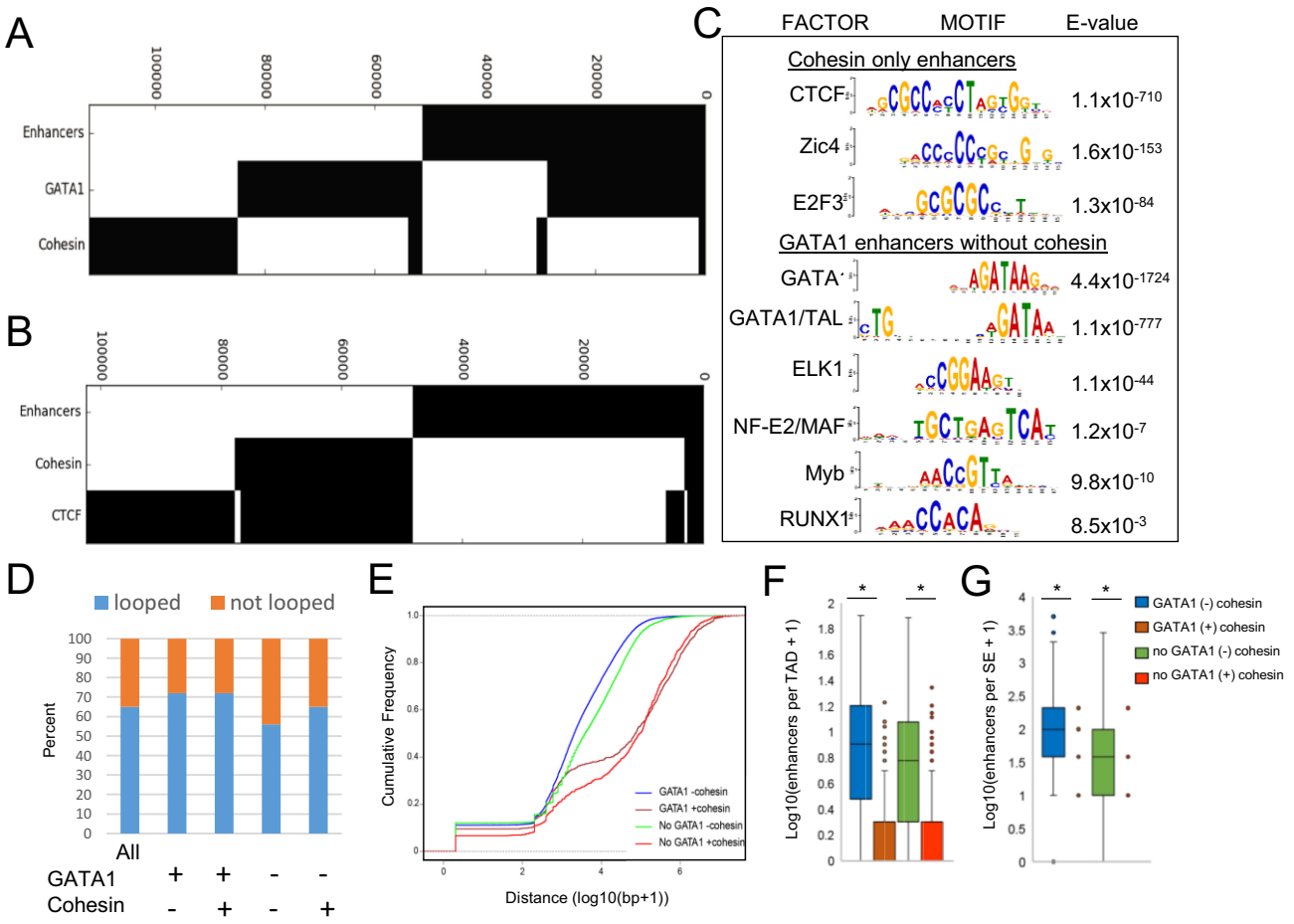

Figure 5. Weak overlap of cohesin with LDB1-regulated erythroid enhancers. (**A**) Binary heat map depicting overlap of ChromHMM-called enhancers in erythroid cells, GATA1 occupancy and cohesin occupancy (defined as sites jointly occupied by Rad21 and Smc3). See 'Materials and Methods' section and Supplementary File 3 for datasets. Each column on the x-axis represents a unique genomic location; black in a column indicates factor occupancy or an enhancer at that genomic location. (**B**) Binary heat map depicting overlap of enhancers, cohesin occupancy and CTCF occupancy. (**C**) Table depicting top transcription factor binding motifs enriched at cohesin peaks in cohesin occupied enhancers and GATA1 peaks in GATA1-no cohesin occupied enhancers. Associated *e*-values determined by MEME analysis. (**D**) Percent of all called enhancers in erythroid cells that are looped to at least one gene. Enhancers are divided into groups that do or do not have GATA1 occupancy (as a proxy for LDB1 complex occupancy) and within these groups, whether or not they are occupied by cohesin. See text for further details. (**E**) Cumulative frequency distribution of distances between enhancers with GATA1 (with or without cohesin) and enhancers without GATA1 (with or without cohesin). Box plots of enhancer distribution among (**F**) topologically associating domains (TADs) and (**G**) super-enhancers. (*) $P < 10^{-10}$ by Mann–Whitney U-test.

termined by ChIP-seq (Supplementary Table S6) revealed that 86% of cohesin occupied predicted enhancers in erythroid cells were co-occupied by CTCF (Figure 5B). Thus, very few cohesin-only sites occurred inside (or outside) enhancer chromatin. Also, only 8% of predicted enhancers in erythroid cells were occupied by CTCF in the absence of cohesin. As expected, there was extensive overlap of CTCF and cohesin sites in the genome at locations not defined as enhancers and many CTCF sites without cohesin. Therefore, in erythroid cells, there is only a small overall representation of CNC sites either within or outside enhancer chromatin.

Consistent with CTCF occupancy at the cohesin occupied enhancers, a search for known transcription factor binding motifs centered upon the cohesin-occupied enhancers using MEME showed strong enrichment of the CTCF motif (Figure 5C) along with motifs for Zic4 and E2F3, transcription factors known to interact with enhancers (60,61). Notably, at GATA1 enhancers without cohesin, the motifs associated with GATA1 and LDB1 (GATA1, GATA1/TAL1) were enriched as well as those for known erythroid regulators Myb, RUNX1 and NF-E2, the latter of which is known to occupy the β-globin LCR. The KLF1 motif was not enriched among these factors, possibly because it may typically appear further away from enhancers than the 200 nt search limitation in MEME.

Next, to investigate looping interactions of predicted enhancers either occupied or not occupied by cohesin, we intersected the data with promoter capture-Hi-C data for erythroid cells (35). The results showed that a large fraction (64%) of the enhancers was looped to at least one gene in erythroid cells (Figure 5D). Enhancers occupied by GATA1 looped with the same frequency whether or not they were co-occupied by cohesin (*P*-value 0.1451 by Chi-square test, odds ratio 1.0854), suggesting the predominant importance of the LDB1 complex in loop formation for these enhancers.

However, cohesin contributed significantly to looping by enhancers without GATA1, as these enhancers interacted more frequently than those without cohesin (*P*-value 0.0001 by Chi-square test, odds ratio 1.5105).

To investigate clustering by predicted enhancers in erythroid cells, we analyzed the distance distribution between enhancers with and without cohesin. Enhancers without cohesin localize close to each other (median distance 1.5 and 2.5 kb between GATA1 co-occupied enhancers or enhancers without GATA1, respectively). Enhancers with cohesin are more distant from each other (median distance 20 and 28 kb between GATA1 co-occupied enhancers or enhancers without GATA1, respectively) (Figure 5E). Consistent with this distribution pattern, enhancers without cohesin more frequently colocalize in the same TAD or super-enhancer domain then enhancers with cohesin (11,62) (Figure 5F and G). Close localization of GATA1 occupied enhancers without cohesin is typified by the clustered elements of the β-globin LCR and the α-MRE that regulate β- and α-globin genes, respectively (40,63) (and see below). Overall, enhancers in erythroid cells extensively loop to genes in the absence of cohesin. Those enhancers that interact with cohesin are substantially co-occupied by CTCF and tend to be further apart than non-cohesin enhancers in erythroid cells whether or not they are co-occupied by GATA1.

Long-range looping of erythroid genes is largely independent of cohesin

Next, we focused on the genes that were observed to loop to GATA1-occupied enhancers and 'no GATA1' enhancers and examined their expression levels. Genes that looped to one or more GATA1 enhancers with no cohesin (Figure 6A, group 1) were expressed at a significantly higher level than those looped to one or more GATA1 enhancers with cohesin (Figure 6A, group 2) ($P < 0.05$ by Mann–Whitney U-test). A third group, those genes looped to two or more GATA1 enhancers at least one of which had cohesin and one did not (Figure 6A, group 3) was also expressed more highly than the genes in group 1 ($P < 0.05$ by Mann–Whitney U-test), most likely because these genes looped to higher numbers of enhancers. Among genes looped to enhancers without GATA1, similar differences were observed (Figure 6A, groups 4–6). Thus, in erythroid cells, enhancers that interact with cohesin, whether or not they are GATA1-occupied, are looped to genes with overall lower expression levels that those that do not involve cohesin.

So far, we have considered all genes expressed in erythroid cells. To ask what kind of enhancer–promoter connections are most typical of erythroid specific genes, we determined the distribution of a curated list of 775 erythroid genes (available at http://doi.org/10.5281/zenodo.189503) in groups 1–3 of Figure 6A. Erythroid genes were enriched in groups 1 and 3 compared to all genes looped to GATA1-occupied enhancers ($P < 0.0001$ by Chi-squared test). Most erythroid genes (70%) looped to enhancers with GATA1 but without cohesin (group 1) and an additional 15% looped to enhancers occupied by GATA1 with and without cohesin (group 3). Of interest, there were no erythroid genes looped exclusively to GATA1 plus cohesin-occupied enhancers (group 2).

Several examples (out of 513 group 1 erythroid genes, Figure 6A) depicted in Figure 6C–E illustrate the absence of cohesin and CTCF called peaks, according to ENCODE data, from known LDB1-regulated erythroid enhancers and the erythroid target genes they contact (35). Below each is the chromatin state map called by ChromHMM keyed as in Figure 6B. Called peaks for GATA1 correlate with enhancer chromatin in these loci. *Runx1* (Figure 6C) and *LMO2* (Figure 6D) results are depicted for uninduced MEL cells, where these genes are actively transcribed (64,65). In induced MEL cells, the looped active β-globin gene and LCR region are similarly devoid of cohesin (Figure 6E). ChIP-qPCR validation of the cohesin ENCODE data is shown in Supplementary Figure S6. Of note, there are numerous (8,505 total in group 4, Figure 6A and see Supplementary Figure S7 for examples) genes that loop to non-LDB1 regulated enhancers in erythroid cells in the absence of cohesin and CTCF. These looping interactions presumably depend on as-yet-unidentified transcription factors and/or other mechanisms. We conclude that erythroid genes predominantly interact with enhancers regulated by GATA1/LDB1 in the absence of cohesin.

DISCUSSION

Our data contribute to understanding how enhancer–promoter interactions are established. We found that long-range enhancer looping at the β-globin locus does not depend on RNA Pol II PIC formation, suggesting that looping occurs before PIC assembly, consistent with data indicating looping precedes transcription activation (28). Nor is the RNA Pol II PIC required for mediator recruitment, which, instead, is dependent on the LDB1 complex in the β-globin locus. However, such recruitment of mediator is not required to establish an LCR/β-globin loop. Moreover, we found that only a small minority of active enhancers in erythroid cells is engaged by cohesin. We provide evidence that the potential for the cohesin ring complex to contribute directly to the establishment of enhancer–promoter proximity in erythroid cells is limited; in such cases, the ability of erythroid cell type specific transcription factors to mediate these interactions through homo- or heterotypic interactions is highlighted.

We found that the mediator core (Med1) is dispensable for enhancer loop formation. However, the mediator kinase module (Med12) can be stabilized at the β-globin promoter independently from the mediator core. The mediator kinase module is known to reversibly associate with the full complex and it can function independently from mediator core as it may be doing at the β-globin gene (18). The mediator kinase module may have a role in modulation of chromatin loops and may be recruited to the β-globin promoter for a specific function related to looping or transcription activation. The phosphorylation of histone H3Ser10 by the mediator kinase module may be such an early event in β-globin gene activation that occurs before PIC formation (66). Another possibility is that the mediator kinase module may regulate the function of transcription factors bound to the gene promoter (18). Finally, based on direct interaction between Med12 and activating long-noncoding RNAs involved in enhancer–promoter contacts (67), it is reasonable

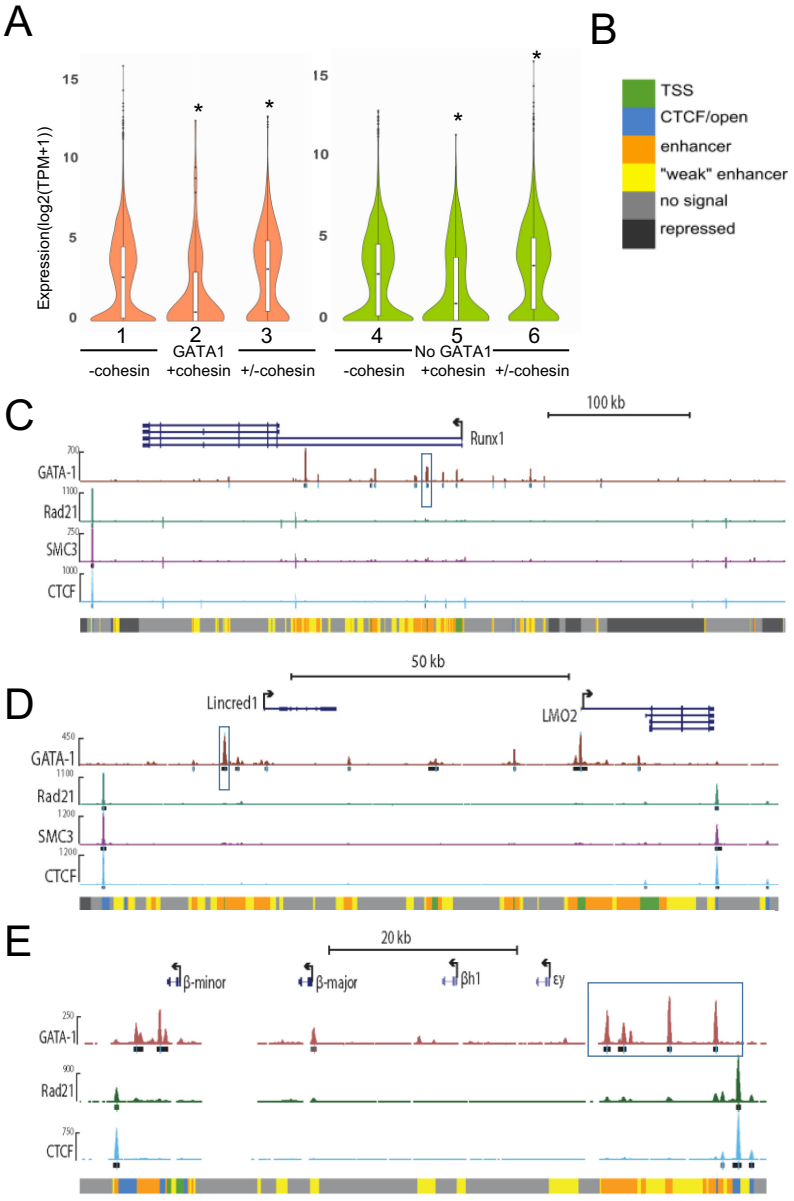

Figure 6. Erythroid genes loop to GATA1-occupied enhancers without cohesin. (**A**) Violin plots comparing expression level distribution of genes that are looped to called enhancers in erythroid cells and separated into groups depending on whether the contacted enhancers show occupancy of GATA1 or cohesin. See text for further explanation. Groups 1–3 and 4–6 are mutually exclusive. (*) $P < 0.05$ by Mann–Whitney U-test. (**B**) Color coding of biological states in the ChromHMM model. RefSeq gene models are depicted for (**C**) *Runx1*, (**D**) *Lmo2* and (**E**) β-globin. Blue rectangle marks previously validated erythroid enhancers. ChIP-seq signal tracks are from ENCODE with called peaks shown below. Chromatin states called by ChromHMM are shown below ChIP-seq.

to propose that interaction of LDB1 with MED12 Mediator kinase module may be a link between transcription factors involved in looping and long-noncoding RNAs (68).

Interestingly, when an LCR/β-globin loop was forced in uninduced cells by CRISPR/dCas9 targeting of LDB1 DDΔ4/5, neither Med1 nor Med12 was detected at the LCR or at the gene. This result is consistent with a model whereby LCR proximity is an early and mediator-independent step in locus activation that precedes migration to the nuclear interior and localization in transcription factories, events that are poorly understood but that subsequently allow association of mediator and RNA Pol II and transcription initiation (28). Our results begin to enumerate the mechanistic steps by which looping contributes to β-globin activation.

Cohesin appears to be variably associated with enhancers in diverse cell types, according to published reports. When active enhancers in cerebral cortex neural cells were called genome wide using ChromHMM, as we have done here, investigators found no evidence for cohesin binding to them

(69). On the other hand, in the developing limb bud between 35% and 65% of enhancers showed evidence of cohesin binding, depending on the method used to designate the enhancers (70). Consistent with these observations, we found that in different human cell types cohesin occupancy of enhancer chromatin varied from 4% in human K562 erythroid cells to 30% in human HepG2 liver cells (Supplementary Figure S8). Thus, cohesin-associated and -independent enhancers vary among cell types and cohesin-independent enhancers appear to be a general phenomenon including among those regulated by LDB1 in erythroid cells.

Our data supporting the role of lineage specific transcription factors in enhancer loop formation do not rule out that mediator and cohesin help to stabilize enhancer–promoter loops, but argue that for many enhancers these complexes are not necessary for loops to form. Interestingly, the erythroid gene loci illustrated in Figure 6 have strong CTCF/cohesin co-occupied sites in flanking regions, suggesting they reside in insulated neighborhoods (14,53,71). Such insulating loops are likely to contribute to enhancer stability and perhaps to loop formation through a loop extrusion mechanism (20,21). However, they are not sufficient for loop formation since RNAi experiments perturbing levels of LDB1 complex members alone result in loss of LCR/β-globin looping (23–25,72).

In erythroid cells, cohesin-occupied enhancers represent a small minority of all enhancers and no erythroid genes are observed to be interacting with them. While we provide evidence that erythroid genes substantially depend on the LDB1 complex for enhancer-gene interactions, unbiased investigations of additional contributors to looping are needed as our understanding of components required for LDB1-mediated enhancer loops to form or be stabilized likely remains incomplete. Furthermore, recent reports describe LDB1 involvement in long-range gene regulation in select non-erythroid cells (73–75). In these cases, it will be important to understand the LIM only or LIM-homeodomain proteins and DNA binding factors distinct from those in erythroid cells that cooperate with LDB1. In addition, mechanistic studies using highly directed CRISPR/Cas9 editing are needed to distinguish direct versus indirect influences of CTCF/cohesin co-occupied sites on long-range enhancer function.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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