Enhancer-bound LDB1 regulates a corticotrope promoter-pausing repression program

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Substantial evidence supports the hypothesis that enhancers are critical regulators of cell-type determination, orchestrating both positive and negative transcriptional programs; however, the basic mechanisms by which enhancers orchestrate interactions with cognate promoters during activation and repression events remain incompletely understood. Here we report the required actions of LIM domain-binding protein 1 (LDB1)/cofactor of LIM homeodomain protein 2/nuclear LIM interactor, interacting with the enhancer-binding protein achaete-scute complex homolog 1, to mediate looping to target gene promoters and target gene regulation in corticotrope cells. LDB1-mediated enhancer:promoter looping appears to be required for both activation and repression of these target genes. Although LDB1-dependent activated genes are regulated at the level of transcriptional initiation, the LDB1-dependent repressed transcription units appear to be regulated primarily at the level of promoter pausing, with LDB1 regulating recruitment of metastasis-associated 1 family, member 2, a component of the nucleosome remodeling deacetylase complex, on these negative enhancers, required for the repressive enhancer function. These results indicate that LDB1-dependent looping events can deliver repressive cargo to cognate promoters to mediate promoter pausing events in a pituitary cell type.

LDB1 | ASCL1 | MTA2 | looping | enhancer

Enhancers are distal regulatory elements that are involved in tissue-specific gene expression. Cell type-specific factors cooperatively bind to enhancers, correlating with their activation as exemplified by eRNA expression and the appearance of particular histone marks, e.g., histone H3 acetyl lysine 27 (H3K27ac) (1–4). Cell type-specific activation of enhancers mediates the determination and specification of specific cell types, but the underlying principles by which they interact with their cognate promoters either to activate or repress target gene promoters remain incompletely understood.

The pituitary gland is the one of the major endocrine organs and is responsible for the production and secretion of several hormones, regulating multiple physiological processes including body growth, metabolism, reproduction, and stress response. Adrenocorticotropic hormone, which regulates stress level, is a cleaved product of proopiomelanocortin (POMC) produced in the corticotrope. Numerous transcription factors have been reported to regulate *POMC* gene expression in AtT20 cells and to be involved in pituitary gland development (5). For example, the T-BOX transcription factor gene *T-Pit/Tbx19* is expressed during early stages of pituitary organogenesis and is required for corticotrope cell-type determination in vivo (6–8). Transcription factors in the basic helix-loop-helix (bHLH) family play essential roles in many, if not all, studied systems and in multiple developmental stages (9, 10). Although bHLH genes, such as *neurogenic differentiation factor D1* (*NeuroD1*), have been reported to regulate *POMC* gene expression from in vitro studies, they play only a minor and transient role in the pituitary development in vivo (11–13). Achaete-scute homolog 1 (ASCL1), a homolog of the *Drosophila achaete/scute* (*ac/sc*) bHLH transcription factor, has been shown to be critically involved in the development of several lineages in central and peripheral nervous systems and other organs (14–17). *Ascl1* also is expressed in the developing pituitary gland (8), and experiments in Zebrafish suggest its importance during early adenohypophysis development (18). Studies in *Drosophila* indicate that achaete recruits and functionally requires a cofactor, CHIP, to regulate target genes (19). Lim domain-binding (LDB)/nuclear LIM interactor (NLI)/cofactor of LIM homeodomain protein (CLIM), the mammalian homolog of CHIP, has been demonstrated to be a critical factor for multiple developmental systems and to be a component of LIM homeodomain codes that determine neuronal subtype specification in the spinal cord (3, 20, 21). Recently, using an engineered transcription unit as a model, LIM domain-binding protein 1 (LDB1) has been reported to be capable of mediating promoter:enhancer looping through LDB1 homodimerization (22–24).

Here, we report that ASCL1 is a major regulator of the corticotrope lineage in the pituitary gland, activating a large set of cell type-specific enhancers, marked by levels of H3K27ac and p300 occupancy, but also causing repression of another cohort of

Significance

The apparent importance of promoter:enhancer looping is well established; however, the molecular mechanisms of these interactions in gene activation vs. gene repression remain to be fully elucidated. Here, we report that LIM domain-binding protein 1 (LDB1) can function in transcriptional enhancer-mediated gene activation mainly at the level of transcription initiation by regulating promoter:enhancer looping, consequent to the recruitment to basic helix-loop-helix–bound enhancers in pituitary corticotrope cells. Intriguingly, LDB1 also mediates promoter:enhancer looping required for target gene repression, acting at the level of promoter pausing, by recruiting metastasis-associated 1 family, member 2 to these repressive enhancers. These findings shed light on a regulatory aspect of the molecular function of LDB1, providing a putative mechanism of enhancer-dependent transcriptional repression.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus database, www.ncbi.nlm.nih.gov/geo (accession no. GSE64516).

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mRNAs. Both ASCL1-directed activation and repression transcriptional programs are largely dependent on the recruitment on enhancers of LDB1, which is required for the looping of both activating and repressing enhancers to their cognate gene promoters. Although the effects of gene activation in this cell type occur primarily at the level of transcriptional initiation, the function of the LDB1-dependent repressive enhancer appears to result in promoter pausing of target genes and is largely the result of looping-induced delivery of negative cargo, including components of the nucleosome remodeling deacetylase (NuRD) complex, from these repressive enhancers. Together, our data reveal that enhancer-bound LDB1 has a role in mediating the actions of both activating and repressive enhancers in a cell type-specific gene-regulation program.

Results

Enrichment of bHLH-Binding Motif in Putative Active Enhancers in AtT20 Cells. To investigate the putative enhancer program of the terminally differentiated pituitary lineage, we conducted a survey of histone markers in a corticotrope-derived mouse cell line, AtT20 cells. ChIP followed by sequencing (ChIP-seq) of monomethylated histone H3 lysine 4 (H3K4me1), H3K27ac histone markers, and p300 protein occupancy mapped 78,608, 81,366, and 42,614 peaks, respectively. Prediction of putative enhancers (71,225) was based on the presence of H3K4me1 histone markers and by excluding annotated transcriptional start sites (TSSs) in the mouse genome (Fig. S1*A*). Motif enrichment analysis of all putative enhancers revealed the enrichment of the binding sites of several reported regulators of the *POMC* gene, including the T-BOX, bHLH, ETS, bZIP, and NR4A subfamily of nuclear receptors (Fig. S1*B*) (5–8, 11–13, 25–28). Further, we have defined the subset of the enhancers that have active chromatin markers (H3K27ac and p300) (Fig. 1*A*). Motif enrichment analysis of H3K27ac$^+$ p300$^+$ enhancers revealed enrichment of binding sites for the CCCTC-binding factor (CTCF), neurofibromin 1 (NF1), bHLH, FORKHEAD, X-BOX, and T-BOX families of transcription factors (Fig. 1*A*). Although members of the CTCF and NF1 family are general cellular transcription factors, the factors in the bHLH and other families may be critical for lineage development and enhancer programming in corticotropes. A predicted T-BOX factor, T-BOX 19 (TBX19), has been convincingly demonstrated to be essential for the development of the POMC lineage and the expression of the *POMC* gene (6, 7). The high enrichment of bHLH-binding sites on active enhancers prompted us to search for the bHLH factors critical for the development of murine POMC lineage. Persistent expression of *Ascl1* in the differentiated POMC lineage suggested that ASCL1 might be involved in POMC lineage development (8). Sequential ChIP performed using chromatin isolated from adult pituitary gland, first with anti-H3K27ac antibody to immunoprecipitate active regulatory regions and followed by precipitation using the anti-ASCL1 antibody, demonstrated the presence of ASCL1 on the active *POMC* promoter, supporting a potential functional role of ASCL1 in regulating genes of the corticotrope lineage (Fig. 1*B*).

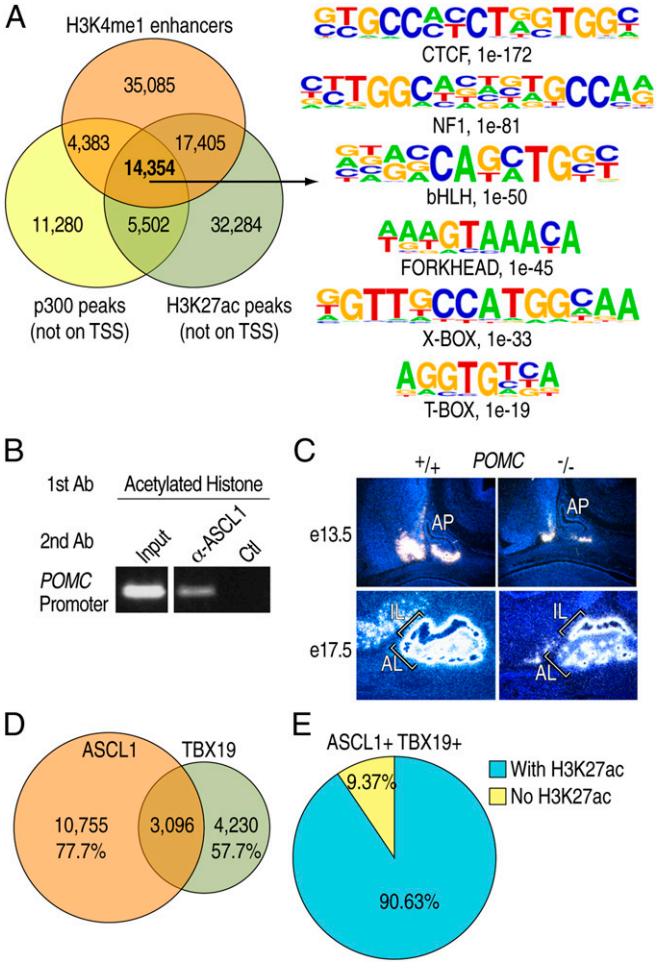

Fig. 1. ASCL1 is required for the development of the POMC lineage and, together with TBX19, is highly correlated with the H3K27ac marker. (*A*, *Left*) Venn diagram showing the intersection of H3K4me1, p300, and H3K27ac ChIP-seq peaks excluding TSSs. The number of the peaks in all possible combinations of the three markers based on ChIP-seq detected in AtT20 cells are indicated. (*Right*) The enriched motifs of enhancers with p300 and H3K27ac markers. The top-ranked enriched motifs are shown with *P* values. (*B*) Sequential ChIP of adult pituitary glands by anti-H3K27ac followed by anti-ASCL1 compared with no-antibody control (beads) to demonstrate that ASCL1 is recruited to the H3K27Ac$^+$ *POMC* promoter in vivo. The panel was sliced to remove unrelated content, as indicated by the blank space. (*C*) Null mutants of *Ascl1* exhibit reduced *POMC* transcripts in both the pituitary gland and ventral diencephalon at E13.5 and E17.5. AL, anterior lobe; AP, adenohypophysis; IL, intermediate lobe. (*D*) Venn diagram showing the intersection of ASCL- and TBX19-containing enhancers. Numbers of enhancers are indicated. (*E*) The majority of ASCL1$^+$ and TBX19$^+$ enhancers are decorated with the H3K27ac histone marker.

Function of ASCL1 in the Development of the Murine Pituitary Gland. To evaluate further the predicted role of *Ascl1* in the development of the POMC lineage, we first examined *Ascl1* expression in the developing pituitary. *Ascl1* first was detected in the evaginating oral ectoderm at embryonic day (E)9.5 and intensified at the ventral part of the nascent gland after E12.5 (Fig. S1*C*), subsequently declining in the anterior compartment on the initiation of terminal cell-type differentiation, and eventually becoming highly expressed in the intermediate lobe and low in the anterior lobe of the mature pituitary (Fig. S1*C*). Examination of pituitary-specific terminal differentiation markers and upstream regulators revealed that several lineages were affected at different regulatory steps in pituitary glands harboring an *Ascl1*-null mutation (Fig. 1*C* and Fig. S1 *D* and *E*). At E13.5 and E17.5, *Ascl1*$^{-/-}$ pituitaries exhibited a substantial reduction in *POMC* expression (Fig. 1*C*). Expression of *Tbx19* was decreased in E13.5 and E17.5 *Ascl1*$^{-/-}$ embryos (Fig. S1*E*). A significant decrease in *NeuroD1* expression at E13.5 and in *Nr4a1* expression at E17.5 was observed also (Fig. S1*E*). We next assessed the full gene-expression program affected in the *Ascl1*-null mutation on the development of the pituitary gland at E13.5, using a microarray approach. In addition to some pituitary-specific targets (Fig. 1*C* and Fig. S1 *D* and *E*), expression of a broad variety of other regulatory genes, including transcriptional factors/cofactors, components of cell cycle, cell death, several developmental signaling pathway, membrane receptors, and channel proteins, also were altered

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significantly in the absence of *Ascl1* (Fig. S1*F*, *Left*). Reduced expression of several targets, including *Chromogranin B*, *Hes6*, and *Manic Fringe* genes, was confirmed by in situ analysis, using E14.5 embryos (Fig. S1*F*, *Right*). In contrast, *Hes family bHLH transcription factor 1 (Hes1)*, which serves as an effector gene of the Delta/Notch signaling pathway and as a negative feedback target of *Ascl1* in other systems (29, 30), was not altered in the *Ascl1*$^{-/-}$ pituitary (Fig. S1*F*, *Right*). These results thus demonstrate a broad role of ASCL1 in mRNA expression during the development of the pituitary and POMC lineage.

ASCL1 Plays an Important Role in the Program of Corticotrope. To investigate the function of ASCL1 further, we generated AtT20 cell lines stably expressing ASCL1 fused to the biotin ligase recognition peptide (BLRP) in a cell line already expressing high levels of the biotinylating enzyme BirA. Streptavidin pulldown of chromatin followed by sequencing revealed that, when correlated with histone markers, 13,851 out of total 21,080 ASCL1 peaks were present on enhancers but only 700 (3.29%) peaks were present on promoters in those cells (Fig. 1*D*, Fig. S1*G*, *Right*, and Fig. S1*H*, *Right*). ChIP-seq of TBX19 protein, also known to be crucial for corticotroph development, revealed that 7,326 TBX19 peaks were present on H3K4me1-marked enhancers (Fig. 1*D*). In contrast to ASCL1, TBX19 occupancy on promoters was very high (3,481 peaks, 23.66% of total peaks) (Fig. S1*G*, *Right* and Fig. S1*H*, *Left*). We have noticed that, although the consensus binding site for ASCL1 is consistent with previous reports (31), TBX19 bound to a more relaxed TNNCA core site (Fig. S1*I*), suggesting that specificity of TBX19 binding may rely on homodimers or heterodimers with other proteins. Indeed, TBX19 was bound to *POMC* enhancer as a homodimers and to *POMC* promoter as a heterodimer with PTX1 (6, 32). Intersection of ASCL1- and TBX19-containing enhancers revealed 3,096 cobound peaks (Fig. 1*D*). More than 90% and more than 80% of the cobound enhancers were colocalized with H3K27ac and p300 peaks, respectively (Fig. 1*E* and Fig. S1*J*), in contrast to the lower association of those markers with other enhancers (Fig. S1 *J* and *K*), supporting the active role of those factors in determining the enhancer program in those cells.

To investigate the effect of *Ascl1* on gene transcription of their targets, global run-on sequencing (GRO-seq) experiments were performed, revealing down-regulation of 956 genes and up-regulation of 1,252 transcription units upon si*Ascl1* treatment. Among those dysregulated genes, ~40% of ASCL1-activated genes had ASCL1 enhancers within a median distance of 21 kb from the regulated promoter, whereas ~58% of the ASCL1-repressed genes had ASCL1-bound enhancers at a median distance of 52 kb (Fig. S1*L*). These results suggest that repressive enhancers may locate farther from their cognate gene promoters than activating enhancers.

LDB1-Mediated ASCL1 Function. To explore the molecular mechanisms that underlie transcriptional regulation by ASCL1, we used anti-FLAG antibody–conjugated beads to purify the FLAG–ASCL1–associated protein complex. Mass spectrometry analysis detected LDB1 peptides (Fig. 2*A*), consistent with biochemical and genetic interactions of *Achaete/Scute* and *Chip* in *Drosophila* (19). We therefore were interested in determining whether LDB1 was involved in ASCL1 function in the POMC lineage. Indeed, LDB1 was expressed in the pituitary gland and in POMC-producing AtT20 cells (Fig. S2*A*). ASCL1 was coimmunoprecipitated by specific anti-LDB1 antibody from AtT20 cells, further confirming the mass spectrometry results (Fig. 2*B*). Interestingly, in the reciprocal mass spectrometry analysis of the FLAG–LDB1 protein complex, we identified a dozen distinct peptides specific for ubiquitous members of the E-protein family bHLH transcription factors TCF4 and TCF12 (Fig. S2*B*), which are known to form heterodimers with cell type-specific bHLH transcription factors, including ASCL1 (10). These data suggest that LDB1 also interacts with TCF4 and TCF12, the common E-protein dimerization partners.

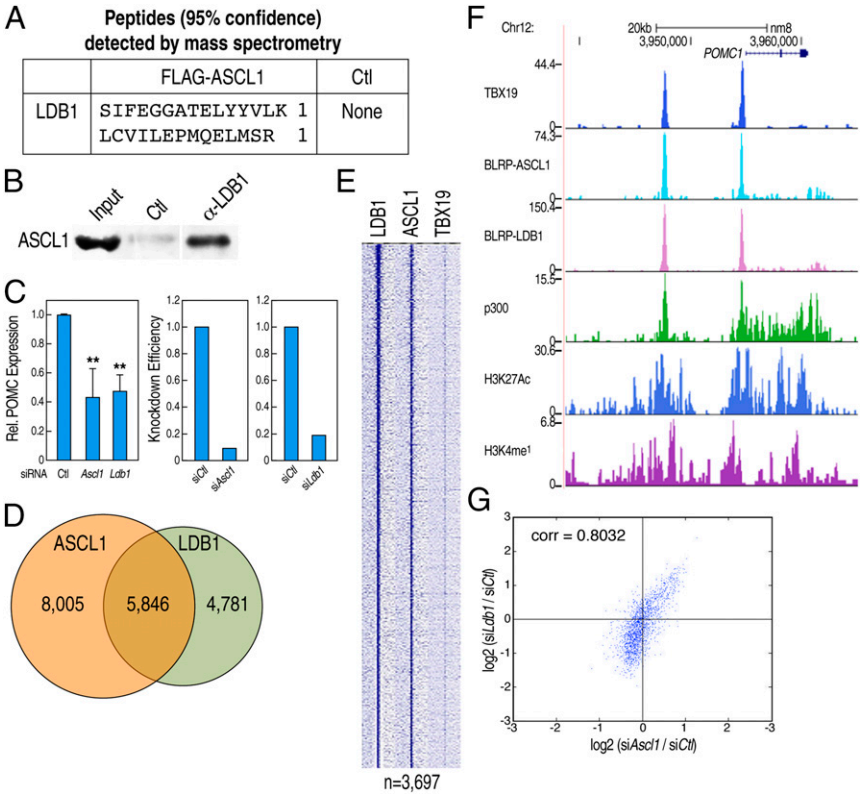

Fig. 2. The ASCL1/LDB1 complex coregulates a large gene program in AtT20 cells. (*A*) Mass spectrometry analysis of the FLAG–ASCL1 complex detected unique LDB1 peptides. The parental cell line was used as a control. (*B*) Coimmunoprecipitation of LDB1 followed by Western blotting confirmed endogenous LDB1–ASCL1 interaction in AtT20 cells. Beads alone served as a control. The panel was sliced to remove unrelated content, as indicated by a blank space. (*C*, *Left*) siRNAs against *Ascl1* and *Ldb1* down-regulate the expression of the *POMC* gene compared with AllStars Negative Control siRNA. (*Center* and *Right*) The knockdown efficiency of *Ascl1* (*Center*) and *Ldb1* (*Right*) in AtT20 cells. Data are presented as mean ± SEM ($n = 3$; $^{**}P < 0.01$). (*D*) Venn diagram showing 5,846 ASCL1 peaks on enhancers colocalized with LDB1. (*E*) Heatmap showing 3,697 ASCL1^{+} TBX19^{-} peaks on enhancers colocalized with LDB1. (*F*) Snapshot of the genome browser shows the binding of TBX19, ASCL1, LDB1, and p300, together with histone markers H3K27ac and H3K4me1, on the *POMC* locus. Strong bindings of all factors are shown on both the *POMC* promoter and enhancer. (*G*) The *Ldb1* transcriptional program was highly correlated with that of *Ascl1*. Genes in the proximity of cobound ASCL1 and LDB1 enhancers are shown. Data were obtained from the GRO-seq assay after knockdown of either the *Ascl1* or *Ldb1* gene compared with AllStars Negative Control siRNA. Corr, correlation coefficient.

ChIP in AtT20 cells showed that LDB1 and ASCL1 were corecruited to the same regulatory region in the POMC gene (Fig. S2*C*) and functioned as potent activators of the *POMC* regulatory regions in transient transfection assays (Fig. S2*D*). Additionally, knockdown of *Ascl1* and *Ldb1* using specific siRNAs repressed the expression of the *POMC* gene (Fig. 2*C*). Together, these data indicate that recruitment of LDB1 is required for *POMC* gene activation. To determine the global binding pattern of LDB1 in AtT20 cells, ChIP-seq was carried out in cell lines stably expressing the BLRP–LDB1 fusion protein. The analysis revealed 10,627 LDB1 peaks on enhancers with bHLH consensus binding sites highly enriched on those peaks (Fig. 2*D* and Fig. S2*F*); 5,846 (55%) LDB1 peaks on enhancers were co-occupied with ASCL1 (Fig. 2 *D* and *E* and Fig. S2*E*), consistent with the apparent physical interactions between the two factors. The percentage of LDB1 peaks on the promoter (5.61%) was comparable to the rate of the promoter binding for ASCL1 (Fig. S2*G*). The cobinding of LDB1 and ASCL1, together with TBX19, p300, H3K27ac, and K3H4me1, was observed on both the *POMC* promoter and enhancer (Fig. 2*F*). Analysis of GRO-seq results for genes in the proximity (<300 kb) of cobound LDB1 and ASCL1 enhancers revealed that the *Ldb1* transcriptional program was highly correlated with that of *Ascl1*(R = 0.80) (Fig. 2*G*); in contrast, functional correlation between *Ldb1* and *Tbx19* was low (R = 0.27) (Fig. S2*H*).

LDB1 Regulates Promoter:Enhancer Looping in LDB1-Activated Genes. LDB1 was postulated to play an important role in promoter:enhancer looping events and as a coactivator in several systems (21–24, 33–35). To investigate its roles in gene transcription and in promoter:enhancer looping events in the POMC lineage, we performed GRO-seq after *Ldb1* knockdown and observed that, of 3,378 genes down-regulated by si*Ldb1*, 927 transcription units are located in direct proximity to the LDB1-occupied enhancers. The expression levels of genes down-regulated by si*Ldb1* are shown in Fig. S3*A*. The tag density plot of GRO-seq data from *Ldb1*-knockdown cells revealed that nascent transcripts were highly concentrated at promoters of LDB1-activated genes in control cells and were massively reduced upon *Ldb1* knockdown, suggesting that the primary transcriptional role of LDB1-bound activating enhancers was to promote transcriptional initiation (Fig. 3*A*). The GRO-seq signal also was greatly reduced across the gene body regions, presumably mainly because of a drastic lower transcriptional initiation rate (Fig. 3*A*). To investigate further how LDB1-bound enhancers activate their target genes, we examined the looping interactions of representative enhancers in AtT20 cells by a promoter:enhancer chromosome conformation capture-based DNA annealing selection and ligation assay (PE3C-DSL; see *Material and Methods* and *SI Materials and Methods* for details) (1, 36), using oligonucleotide libraries synthesized to interrogate promoter and enhancer regions. We found that enhancer:promoter interactions between LDB1-bound enhancers and the adjacent promoters of *POMC* and *lung carcinoma myc related oncogene 1* (*Lmyc1*) genes were greatly diminished following knockdown of *Ldb1* (Fig. 3*B*). The significant change in the efficiency of enhancer–promoter ligation upon *Ldb1* knockdown also was observed when different restriction enzymes were used (Fig. S3*B*). Interestingly, LDB1-dependent interactions were not dependent on the presence of LDB1 on the promoters, as in the case of the *Lmyc1* locus. To confirm these results further, we also performed a conventional chromosome conformation capture (3C) assay for the *POMC* locus and observed that the relative ligation efficiency was reduced by >60% after knockdown of *Ldb1*, whereas internal controls showed comparable genomic DNA present in both samples (Fig. 3*C* and Fig. S3*C*).

LDB1 Represses the Target Gene by Regulating Promoter:Enhancer Looping and by Recruiting Metastasis-Associated Protein 2. GRO-seq data showed that a large number of genes [4,415; false-discovery rate (FDR) <0.01; fold change (FC) >1.2] are activated by *Ldb1* knockdown (Fig. S3*D*). Tag density analysis of the GRO-seq data revealed that LDB1-repressed transcription units had

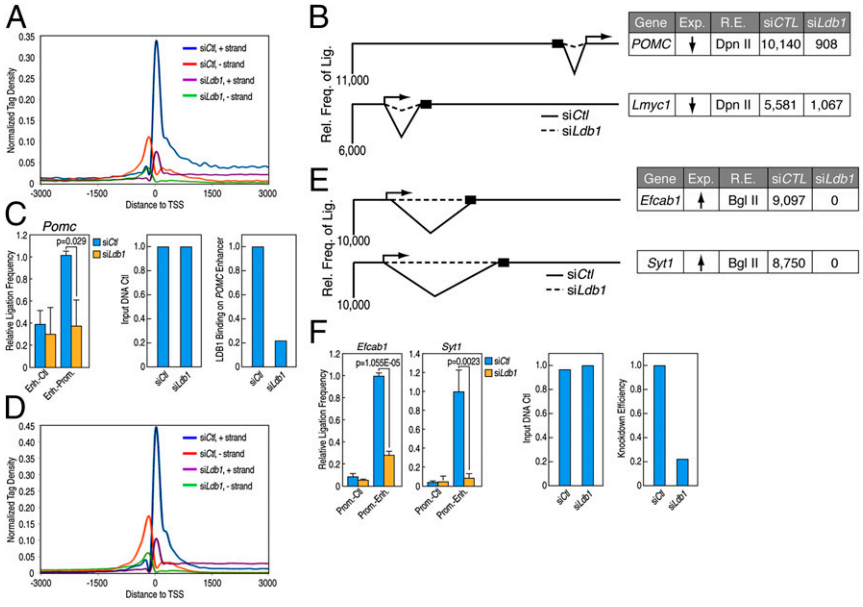

Fig. 3. LDB1 is required for enhancer:promoter looping in both gene activation and gene repression. (*A*) Tag density plot showing that LDB1 activates genes mainly at the transcriptional initiation level. A dramatic reduction of nascent RNA on the promoter region, along with reduced elongation, was detected upon knockdown of *Ldb1*. AllStars Negative Control siRNA was used as control. (*B*) Promoter:enhancer looping is reduced for LDB1-activated genes upon knockdown of *Ldb1* compared with AllStars Negative Control siRNA, as detected by PE3C-DSL (See *Material and Methods* and *SI Materials and Methods* for details). The numbers show read counts from deep sequencing. DpnII was used as the restriction digestion enzyme (R.E.). Exp., expression level. (*C*) 3C assays to confirm the promoter:enhancer interactions for LDB1-activated genes. (*Left*) The relative ligation efficiency is detected by quantitative PCR (qPCR). Data are presented as mean ± SEM; n = 3. (*Center*) Comparable amounts DNA were used for ligation as determined by qPCR. (*Right*) The reduction of LDB1 binding on the *POMC* enhancer upon knockdown of *Ldb1* was evaluated by ChIP assay with LDB1 antibody. AllStars Negative Control siRNA was used as control. (*D*) Tag density plot shows that LDB1 represses genes by regulating transcriptional pausing. Transcriptional elongation is enhanced in LDB1 knockdown cells compared with cells treated with AllStars Negative Control siRNA, suggesting a pausing-release effect. (*E*) Promoter:enhancer looping detected by PE3C-DSL is lost for LDB1-repressed genes upon knockdown of *Ldb1*, as compared with cells treated with AllStars Negative Control siRNA. DpnII was used as the restriction digestion enzyme (R.E.). Exp., expression level. (*F*) 3C assay to confirm the promoter:enhancer interaction in LDB1-repressed genes. (*Left*) The relative ligation efficiency is detected by qPCR. Data are presented as mean ± SEM; n = 3. (*Center*) Comparable amounts of DNA as determined by qPCR were used for ligation. (*Right*) Knockdown efficiency was evaluated by relative mRNA level, using qPCR. AllStars Negative Control siRNA was used as the control siRNA in all experiments.

higher nascent transcription signal in the gene body of *Ldb1*-knockdown cells than that of control cells; the reverse was observed for the promoter region, suggesting that knockdown of *Ldb1* caused the release of transcriptional pausing of LDB1-repressed genes (Fig. 3*D*). Further analysis showed that 1,446 LDB1-repressed genes were located within 300 kb of LDB1-occupied enhancers. Interestingly, the PE3C-DSL assay showed that LDB1-dependent enhancer:promoter interactions also are required for repressed genes, (Fig. 3*E* and Fig. S3*E*). A confirmation by the 3C assay showed that interactions between enhancers and promoters for *EF-hand calcium-binding domain-containing protein 1* (*Efcab1*) and *Synaptotagmin I* (*Syt1*) genes were reduced by >70% and >90%, respectively (Fig. 3*F* and Fig. S3*F*). Taken together, these data indicated that LDB1 is required for enhancer:promoter interaction in both the LDB1-activated and -repressed transcriptional programs.

Mass spectrometry analysis of LDB1-interacting proteins detected several members of the NuRD complex, suggesting that the NuRD complex may have a possible role in LDB1 function (Fig. 4*A* and Fig. S4*A*). Coimmunoprecipitation assays were performed to confirm the interaction between LDB1 and chromodomain helicase DNA-binding protein 4 (CHD4) and between LDB1 and metastasis-associated 1 family, member 2 (MTA2). A significant interaction between LDB1 and MTA2 was observed, although only a minimal signal of CHD4 above beads control was observed (Fig. 4*B*). To investigate any possible functional relationship of *Ldb1* and *Mtas,* GRO-seq was performed in AtT20 cells after specific knockdown of both *Mta1* and *Mta2* (Fig. S4*B*). Interestingly, we observed that 586 genes repressed by LDB1 also were repressed by MTA1/2 (Fig. 4*C*). To investigate MTA binding on these genes, we performed ChIP-seq using MTA2 antibody. A tag density plot of 369 cobound MTA2 and LDB1 enhancers in the proximity of genes repressed by both LDB1 and MTAs showed significant MTA2 binding. Interestingly, MTA2 occupancy on those enhancers was reduced significantly upon knockdown of LDB1 (Fig. 4*D*), as exemplified by the *protein LTV1 homolog* (*Ltv1*), or DNA repair and recombination protein RAD54-like (*Rad54l*) loci (Fig. 4*E*). As a control, the binding of MTA2 on all LDB1 enhancers was not changed after *Ldb1* knockdown (Fig. S4*C*). These two genes were repressed by LDB1 and MTA1/2, as demonstrated by the up-regulation of the prespliced nascent RNAs of both genes upon knockdown of *Ldb1* and *Mta1/2* (Fig. 4*F*). Our data thus indicate that the genes corepressed by LDB1 and MTA1/2 are in the proximity of the LDB1-bound enhancers and that MTA2 is recruited to those enhancers in an LDB1-dependent fashion.

Discussion

The role of LDB1 as a coactivator in development has been studied for more than a decade; however, the molecular mechanisms of its function remain poorly understood. Here, we report that LDB1 can function both for transcriptional enhancer-mediated gene activation in pituitary corticotrope cells, mainly at the level of transcription initiation, and for the actions of enhancers mediating target gene repression at the level of promoter pausing. LDB1 appears to mediate enhancer looping both for enhancers bringing activating cargo and for those bringing repressive cargo to the promoter. These findings shed light on the molecular function of LDB1 and point to the putative mechanism of enhancer-dependent transcriptional repression based on the recruitment of the NuRD complex or components of the NuRD complex at repressive enhancers.

CHIP has been reported to interact with *Drosophila* proneuronal ASC-Da bHLH factor (19). Our proteomic studies demonstrate that LDB1 interacts strongly with pan bHLH factors, the E-proteins. This observation potentially expands the function of LDB1 to the large bHLH family. Because LDB1 also has been reported to interact with other transcription factors, it is tempting to suggest a potentially general role for LDB1 in regulating cell type-specific, enhancer-dependent gene-expression programs. LDB1-mediated promoter:enhancer looping has been reported in the *β-globin* locus based on the homodimerization of LDB1 occupying both enhancer and promoter sites (22–24). Our data show that, in corticotropes, LDB1 can mediate looping to promoters without detectable promoter LDB1 binding. Because we have detected by ChIP-seq that LDB1 is bound predominantly to enhancers, our data clearly suggest that the majority of LDB1-mediated

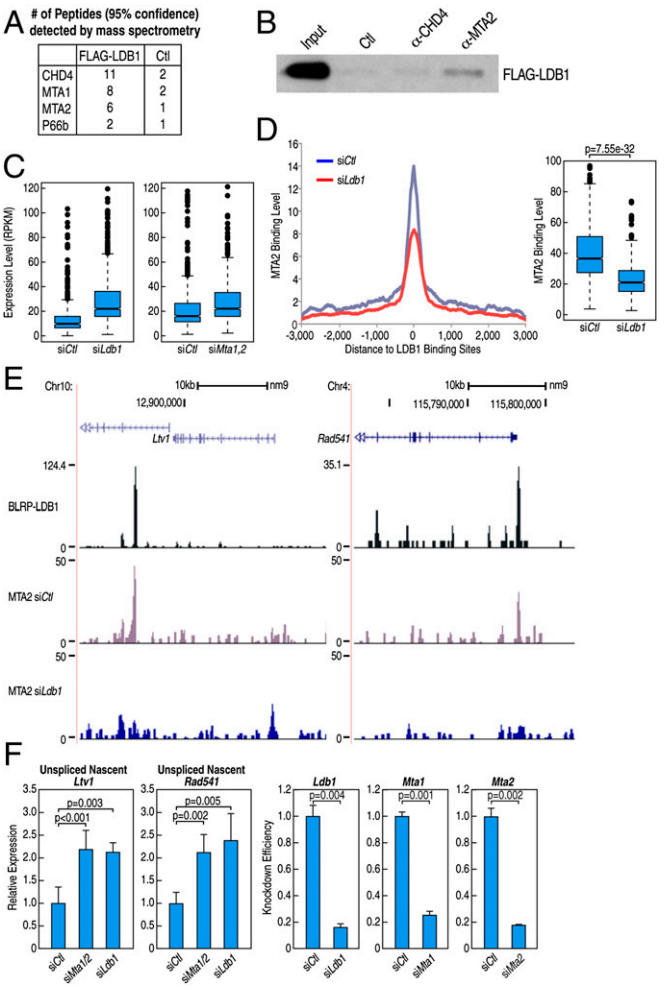

Fig. 4. MTA is involved in LDB1 repression. (*A*) Mass spectrometry of the FLAG–LDB1 complex shows the number of unique peptides detected for components of the NuRD complex. (*B*) Reciprocal coimmunoprecipitation of MTA2 and CHD4 confirms the interaction of FLAG–LDB1 with MTA2 as compared with the no-antibody condition. (*C*) Box plots showing 586 genes up-regulated by both si*Ldb1* (*Left*) and si*Mta1/2* (*Right*), compared with the AllStars Negative Control siRNA condition. (*D*) LDB1 regulates MTA2 recruitment in LDB1/MTA1/2 corepressed genes. (*Left*) Tag density plot of MTA2 recruitment on 369 cobound LDB1 and MTA2 enhancers in the proximity of LDB1-dependent repressed genes, showing a reduced recruitment of MTA2 upon *Ldb1* knockdown, based on ChIP-seq. (*Right*) The box plot shows a significant reduction in the median level of recruitment of MTA2 upon knockdown of *Ldb1* compared with cells treated with AllStars Negative Control siRNA. (*E*) Genome browser snapshots show that MTA2 recruitments on the LDB1-bound enhancer or promoter are lost upon knockdown of *Ldb1*. (*Left*) *Ltv1* locus. (*Right*) *Rad54l* locus. (*F, Left*) The two panels show relative levels of unspliced nascent *Ltv1* and *Rad54l* compared with the control condition. Unspliced nascent *Ltv1* and *Rad54l* transcripts are up-regulated upon knockdown of si*Mta1/2* or si*Ldb1* compared with cells treated with AllStars Negative Control siRNA. (*Right*) The three panels show the knockdown efficiency of *Ldb1*, *Mta1*, and *Mta2* detected by qPCR. Data are presented as mean ± SEM; $n = 6$.

looping events do not reflect enhancer:promoter-dependent LDB1 homodimerization.

In accord with the report in the *β-globin* locus suggests that LDB1 regulates transcriptional elongation (37), our data also show reduced nascent RNA signal in gene body regions by half upon LDB1 knockdown. However, our data argue that the primary role of LDB1 is to regulate transcriptional initiation.

In addition to gene activation, LDB1 appears to be equally required for the function of enhancers putatively mediating target gene repression. We have shown that LDB1 serves as a crucial component of looping mechanisms in enhancer-mediated gene repression. Furthermore, we show that LDB1 is involved in the recruitment of a NuRD complex component, MTA2, to the repressive enhancer, suggesting that this component is a part of the cargo that mediates the promoter-pausing events regulated by these LDB1-bound enhancers. These findings significantly expand our understanding of the role of LDB1 protein in gene regulation and also show, for the first time to our knowledge, that LDB1-dependent looping serves as the strategy for delivering repressive cargo to promoters exhibiting promoter pausing.

Materials and Methods

Animals. All experimental procedures were performed in accordance with University of California, San Diego Animal Care and Use Committee guidelines. Breeding pairs of *Ascl1*-knockout mice (14) were obtained from Jackson Laboratories. The day that plug was observed was designated E0.5.

PE3C-DSL Assay. The PE3C-DSL assay was designed to quickly screen potential promoter:enhancer interaction pairs based on 3D-DSL (1, 36). Several donor or acceptor probes were designed on promoters and enhancers using custom Perl scripts as described (1). The uniqueness of the probe sequences was verified by Bowtie alignment to the mouse genome mm8. Universal adaptor sequences that are compatible with HiSeq 2000 flow cell design were added to the probe ends for bridge amplification of the ligated products and for direct sequencing. Acceptors were phosphorylated at their 5′ ends, and both acceptors and donors were pooled in equimolar amounts for PE3C-DSL (for details, see *SI Materials and Methods*).

The other materials and methods are provided in *SI Materials and Methods*. siRNAs are listed in Table S1. PCR primers used for cloning and the gene-specific primer pairs are listed in Table S2. Sequences of ligated oligonucleotides from PE3C-DSL assay are listed in Table S3.

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