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CtBP and Associated LSD1 Are Required for Transcriptional Activation by NeuroD1 in Gastrointestinal Endocrine Cells

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Gene expression programs required for differentiation depend on both DNA-bound transcription factors and surrounding histone modifications. Expression of the basic helix-loop-helix (bHLH) protein NeuroD1 is restricted to endocrine cells in the gastrointestinal (GI) tract, where it is important for endocrine differentiation. RREB1 (RAS-responsive element binding protein 1), identified as a component of the CtBP corepressor complex, binds to nearby DNA elements to associate with NeuroD and potentiate transcription of a NeuroD1 target gene. Transcriptional activation by RREB1 depends on recruitment of CtBP with its associated proteins, including LSD1, through its PXDLS motifs. The mechanism of transcriptional activation by CtBP has not been previously characterized. Here we found that activation was dependent on the histone H3 lysine 9 (H3K9) demethylase activity of LSD1, which removes repressive methyl marks from dimethylated H3K9 (H3K9Me2), to facilitate subsequent H3K9 acetylation by the NeuroD1-associated histone acetyltransferase, P300/CBP-associated factor (PCAF). The secretin, β-glucokinase, insulin I, and insulin II genes, four known direct targets of NeuroD1 in intestinal and pancreatic endocrine cells, all show similar promoter occupancy by CtBP-associated proteins and PCAF, with acetylation of H3K9. This work may indicate a mechanism for selective regulation of transcription by CtBP and LSD1 involving their association with specific transcription factors and cofactors to drive tissue-specific transcription.

NeuroD1 is a basic helix-loop-helix (bHLH) transcription factor that was originally identified as an activator of insulin gene transcription (1) and as a neurogenic differentiation factor in *Xenopus* (2). Cells expressing NeuroD1 arise from endocrine precursor cells in both the intestine and pancreas that transiently express the bHLH transcription factor neurogenin 3 (Neurog3). Neurog3 appears to be important for initiating endocrine differentiation in the gastrointestinal tract and directly increases NeuroD expression. NeuroD expression occurs as a relatively late event in maturing enteroendocrine cells and pancreatic β cell precursors. Lineage tracing studies examining the cell fate of Neurog3- and NeuroD-expressing cells suggest that Neurog3⁺ cells are multipotential, giving rise to both endocrine and nonendocrine cell lineages (3). In contrast, cells expressing NeuroD are restricted to an endocrine cell fate, giving rise to most enteroendocrine cells while in the pancreas primarily contributing to the β cell lineage (4). In addition to driving an endocrine cell fate, NeuroD may have other functions, including inhibiting cell cycle activity as cells differentiate (5). The genes encoding secretin (Sct), insulin (Ins I and Ins II), and glucokinase (Gck) are the only direct transcriptional targets of NeuroD identified thus far in endocrine cells of the intestine and pancreas (6–8).

By itself, NeuroD1 is a relatively weak activator of transcription whose activity depends on its association with other promoter-associated transcription factors (9–11) and the coactivators p300 (5, 12, 13) and PCAF (P300/CBP-associated factor) (14). We previously identified the zinc finger protein RREB1, which binds to DNA sequences adjacent to the NeuroD1-binding E box at the secretin gene promoter and physically associates with NeuroD1 to increase NeuroD1-dependent transcription (15). RREB1 was first described as a DNA binding protein that interacted with a Ras-responsive element in the calcitonin gene promoter (16). Others suggested that RREB1 promoted p53 transcription following DNA damage (17). The ability of RREB1 to increase transcription

(15–18) was not expected, since it lacks an intrinsic transcription activation domain (15) and was identified as a component of the multiprotein corepressor complex containing CtBP (19) and LSD1 (20). The majority of studies suggest a role for RREB1 in transcriptional repression of a number of genes (21–26).

One member of the CtBP complex, the histone lysine demethylase LSD1, has dual functions to either repress (27, 28) or increase (29, 30) transcription. In the present work, we examined the potential role of CtBP and LSD1 in regulation of NeuroD1-dependent transcription in gastrointestinal endocrine cells. Our results show that CtBP and its associated proteins, LSD1 and CoREST, cooccupied promoters with NeuroD1 at actively transcribed genes. We found that active transcription involving CtBP depends on the presence and enzymatic activities of LSD1 and NeuroD-associated PCAF.

MATERIALS AND METHODS

Cells, reagents, and antibodies. The human duodenal cell line HuTu 80 was obtained from ATCC. Antibodies used included those to CtBP1 (sc-11390 or sc-17759; Santa Cruz Biotech), LSD1/KDM1 (ab17721; Abcam), CoREST (ab24166; Abcam), tubulin (T6074; Sigma-Aldrich), acetylated H3K9 (H3K9Ac) (9649; Cell Signaling), dimethylated H3K4 (H3K4Me2) (9726S; Cell Signaling), H3K9Me2 (9753; Cell Signaling), H3 (ab1791; Abcam), PCAF/KAT2B (ab12188; Abcam), NeuroD1 (sc-1084; Santa Cruz Biotech), and RNA polymerase II (Pol II) (MMS-129R). RREB1 antibody was described previously (15).

Plasmids. Expression plasmids for CtBP (31), LSD1 (29), NeuroD1 (11), and the glutathione *S*-transferase (GST)–androgen receptor (AR) DNA-binding domain (DBD) fusion protein (29) were described previously. The GST-RREB1 fusion plasmid included amino acids (aa) 447 to 1195 of RREB1 (18). The two PXDLS-like motifs in RREB1 at aa 899 to 901 and 934 to 936 were changed from PXD to AXA to generate GST-RREB1-MUT.

Reporter plasmids. The reporters pREP4-Secretin-Luc and pREP4-Cck-Luc contained secretin (32) and Cck (33) promoter fragments cloned into pREP4-Firefly-Luc (34). pREP7-RSV-Renilla-luc was used to normalize transfection efficiency (34).

shRNA-expressing plasmids. For stable expression of small hairpin RNA (shRNA), previously published shRNA coding sequences for CtBP (19), NeuroD (35), CoREST (36), or PCAF (37) were cloned into the pREP4 plasmid containing the U6 promoter, and for transient expression, the shRNA coding sequences were cloned into the pBS-U6 vector (38). The CtBP shRNA expression plasmid was previously shown to knock down both CtBP1 and CtBP2 (19, 39). To generate the shRNA-resistant CtBP1 expression plasmid, we introduced silent mutations (indicated in bold) at the shRNA coding sequence (aa 83 to 91), by site-directed mutagenesis using a primer pair (sense, AG**A** GAA GA**T** C**T**C GA**A** AAA TT**T** AA**G** GC**T** CTC CGC ATC AAT CGT CCG GAT T; antisense, **A**GC **C**TT **A**AA **T**TT **T**TC **G**AG **A**TC **T**TC **T**CT GGT GAG AGT GAT GGT GTG GTA).

Generation of stable HuTu 80 cells expressing shRNA. Cells were transfected with pREP4-U6 control, pREP4-U6-CtBP shRNA-, pREP4-U6-NeuroD shRNA-, pREP4-U6-PCAF shRNA-, or pREP4-U6-CoREST shRNA-expressing plasmids with Lipofectamine according to the manufacturer's protocol (Invitrogen). After 48 h, the transfected cells were selected with hygromycin (500 μg/ml) for 10 days. RREB1 and LSD1 knockdown (KD) cells were generated by transduction with lentivirus containing shRNAs targeting RREB1 (clone no. V3LHS_390533 and V2LHS_241472; Open Biosystems) or LSD1 (20), followed by selection with puromycin (2 μg/ml). Puromycin-resistant cells were grown to 60 to 70% confluence for subsequent experiments.

ChIP assays and reverse transcriptase (RT) PCR. Chromatin immunoprecipitation (ChIP) was performed as described previously with endogenous DNA (11). Nuclear lysates prepared from cells treated for 10 min with 1% formaldehyde were sonicated to an average size of 300 to 1,000 bp. Soluble chromatin fragments were precleared with normal IgG and immunoprecipitated by overnight incubation with antibody. ChIP assays were analyzed either by quantitative real-time PCR using SYBR green quantitative PCR (qPCR) mix according to the manufacturer's protocol (Thermo Scientific) or by gel electrophoresis. For re-ChIP, the antibody-bound chromatin from the first ChIP was eluted by incubating immunoprecipitates with 10 mM dithiothreitol (DTT) at 37°C for 20 min. The eluted chromatin was diluted 10-fold in re-ChIP buffer, immunoprecipitated overnight with a second ChIP antibody, and analyzed similarly to ChIP. All ChIP and re-ChIP experiments were done a minimum of three times. Gel data are representative gels from at least three independent experiments that resulted in the same findings. RNA isolated using the RNeasy Plus minikit (Qiagen) was reverse transcribed using the SuperScript II first-strand synthesis system (Invitrogen) with levels determined by quantitative PCR using SYBR green incorporation. Transcript abundance was normalized to β-actin expression. Primer sequences used for detecting promoter fragments of interest or for cDNA amplification are available upon request.

RESULTS

RREB1 recruits CtBP1 to increase NeuroD1-dependent transcription. We previously identified RREB1 as a DNA binding protein that interacted with the basic helix-loop-helix protein NeuroD1 to increase transcription (15). RREB1 contains two evolutionarily conserved PXDLS-like CtBP-binding motifs, at aa 899 to 903 and 934 to 938 (Fig. 1A). The function of RREB1 as a member of the large CtBP corepressor complex has not been characterized (19). Coimmunoprecipitation experiments showed that CtBP1 and RREB1 associate at their native levels (Fig. 1B, lanes 1 to 3). Moreover, CtBP1 directly binds to wild-type RREB1 *in vitro* but not to RREB1 with mutations in the CtBP-binding motifs (Fig. 1B, lanes 6 and 7). The association of RREB1 with a corepressor complex at a NeuroD-occupied site was not anticipated, since RREB1 functionally interacts with NeuroD to increase rather than repress transcription (15).

To determine whether CtBP has a role in transcription of the secretin gene, we knocked down endogenous CtBP in HuTu 80 cells, a human duodenal cell line that expresses the NeuroD and secretin genes and several other neuroendocrine genes (40, 41), by stably expressing a short hairpin RNA (shRNA) (19) previously shown to knock down both CtBP1 and CtBP2 (19, 39). Depletion of the CtBP1 protein (Fig. 1C, left) significantly reduced expression of SCT mRNA (Fig. 1C, right) compared to results with an empty shRNA vector. The reduction in secretin transcript expression in CtBP KD cells was unanticipated, suggesting that CtBP might increase transcription of this gene, in contrast to its major function as a corepressor. ChIP assays showed that CtBP depletion reduced RNA Pol II occupancy at the secretin promoter but not at a control gene, the RPL-30 gene, further implying that CtBP contributes to NeuroD-dependent transcription (Fig. 1D).

We then examined whether CtBP has a direct effect on transcription of the secretin gene using transient expression assays with HuTu 80 cells. Initial experiments showed that CtBP knockdown or overexpression had little effect on standard luciferase reporter genes. We switched to pREP4-Luciferase, a self-replicating episomal reporter that forms a chromatin structure comparable to that with stably integrated reporter genes (42) and has been used in similar studies (34, 43, 44). We observed a significant reduction of pREP4 secretin-luciferase reporter expression in CtBP knockdown cells (Fig. 1E) with little effect on expression of a pREP4-Cck-luciferase reporter, indicating that this effect was specific for the secretin gene and not the Cck gene, which is not directly regulated by NeuroD. The role of CtBP in NeuroD1-dependent transcription was further confirmed by the rescue of secretin-luciferase expression in cells coexpressing an shRNA-resistant CtBP1 (CtBP1-WT[R]) with the CtBP shRNA described above (Fig. 1E).

To determine whether the SCT gene is a direct target of CtBP, we examined the endogenous enhancer for the presence of CtBP by ChIP using primers that can amplify promoter sequence within 470 bp upstream of the transcription start site, including sequences close to the NeuroD1 (−80) and RREB1 (−127/−108) binding sites (40). Results show that CtBP1 antibody precipitated sequences from the secretin promoter but not from the β-actin promoter (Fig. 2A), indicating that CtBP1 occupancy was promoter specific.

As expected, we identified the presence of RREB1 at the Sct promoter by ChIP. In RREB1 ChIP, the promoter region was relatively enriched compared to sequences at kb −3.7, suggesting that RREB1 preferentially bound to the promoter region, close to its known binding site (Fig. 2B, left). Since CtBP binds directly to RREB1 but not to DNA, we next determined by re-ChIP experiments if RREB1 and CtBP1 cooccupy the promoter region. Chromatin fragments were first precipitated with RREB1 antibody (first ChIP), followed by reprecipitation with CtBP1 antibody (second ChIP). The CtBP1-bound chromatin fragments obtained
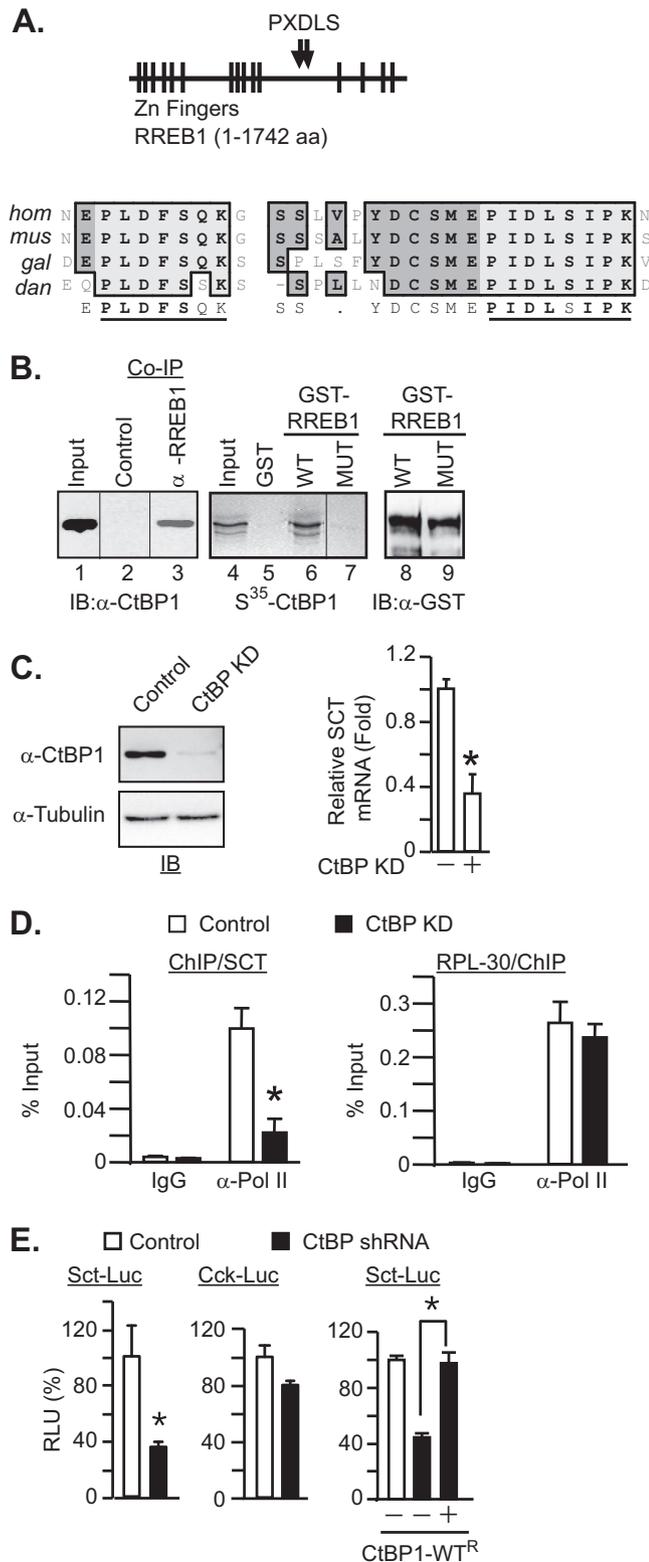
FIG 1 CtBP increases NeuroD1-dependent transcription of the secretin gene. (A) Location of CtBP binding (PXDLS) motifs conserved in human (hom) RREB1 (residues 897 to 906 and 927 to 946), mouse (mus), chicken (gal), and zebrafish (dan), with a consensus sequence underlined. (B) Interaction between CtBP1 and RREB1. Coimmunoprecipitation (Co-IP) of endogenous CtBP1 with RREB1 antibody from HuTu 80 cell extracts (lane 3). Input: 2.5% of the extracts used for IP. CtBP1 interacts directly with RREB1 through PXDLS motifs. *In vitro* binding of
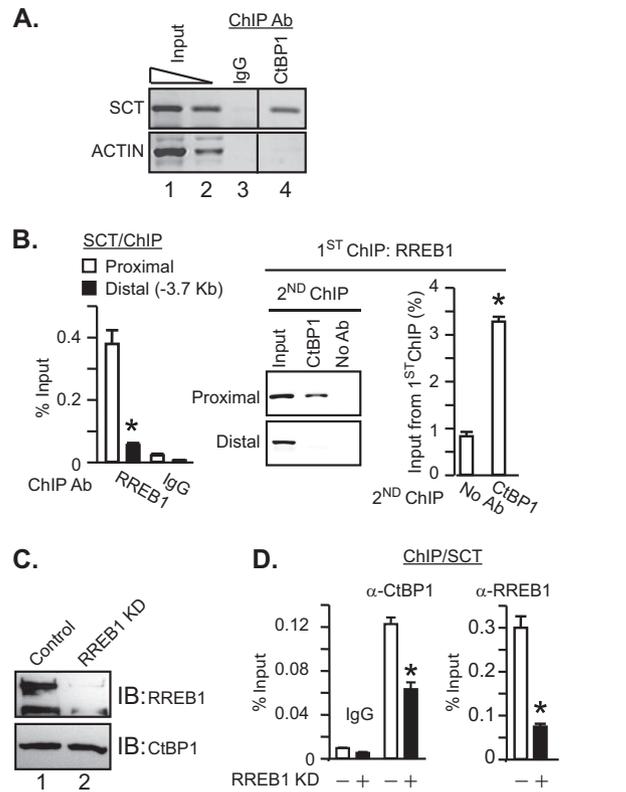

FIG 2 Promoter occupancy of CtBP1 depends on RREB1. (A) ChIP for CtBP1, showing occupancy at the secretin gene promoter but not a control (actin) promoter. (B) Chromatin fragments immunoprecipitated by RREB1 antibody (first ChIP) were examined for relative abundance of promoter (-469 to -335) versus distal (-3811 to -3610) sequences of the SCT gene (left). Following ChIP for RREB1, CtBP promoter cooccupancy with RREB1 was assessed by re-ChIP. The first ChIP (RREB1) DNA was reprecipitated with CtBP1 antibody (middle and right) and detected by qPCR or gel electrophoresis. Each re-ChIP result is expressed as a percentage of the chromatin input that copurified with RREB1 in the first ChIP. Gels shown (A and B) are representative of a minimum of three independent ChIP experiments. (C) Effect of RREB1 KD on RREB1 (top) or CtBP1 (bottom) protein expression. (D) Effect of RREB1 KD on SCT promoter occupancy by CtBP1 (left) or RREB1 (right). ChIP was done using CtBP1 antibody or RREB1 antibody in control or RREB1 KD cells. Results are shown as means ± SEM ($n \geq 3$); ∗, $P < 0.005$.

from the second ChIP were enriched for the promoter region of the SCT gene compared to the kb -3.7 distal site ([Fig. 2B], middle), indicating that CtBP and RREB1 cooccupied the promoter region.

To determine whether the presence of CtBP was dependent on RREB1, we examined the secretin promoter for the presence of

S³⁵-labeled CtBP1 to GST-RREB1 fusion proteins containing the wild type (WT) (lane 6) but not to mutant (MUT) (lane 7) PXDLS motifs is shown. Input: 3% of the materials used for binding assay. Lanes 8 and 9 show GST immunoblot of WT and MUT GST fusion proteins. (C) Effect of CtBP KD on the CtBP1 protein (left) or on secretin mRNA expression (right; measured by qPCR, normalized to β-actin) in HuTu 80 cells. (D) Effect of CtBP KD on RNA Pol II occupancy. ChIP at the SCT promoter (left) or a control region of the RPL-30 gene (right) was performed using RNA Pol II antibody (Ab) in control or CtBP KD cells. (E) Effect of CtBP depletion on Sct (left) and Cck control (middle) reporter genes. Rescue of Sct reporter activity in CtBP-depleted cells by transfected shRNA-resistant CtBP1 (right) is shown. Results are shown as means ± SEM ($n \geq 3$). ∗, $P < 0.025$.
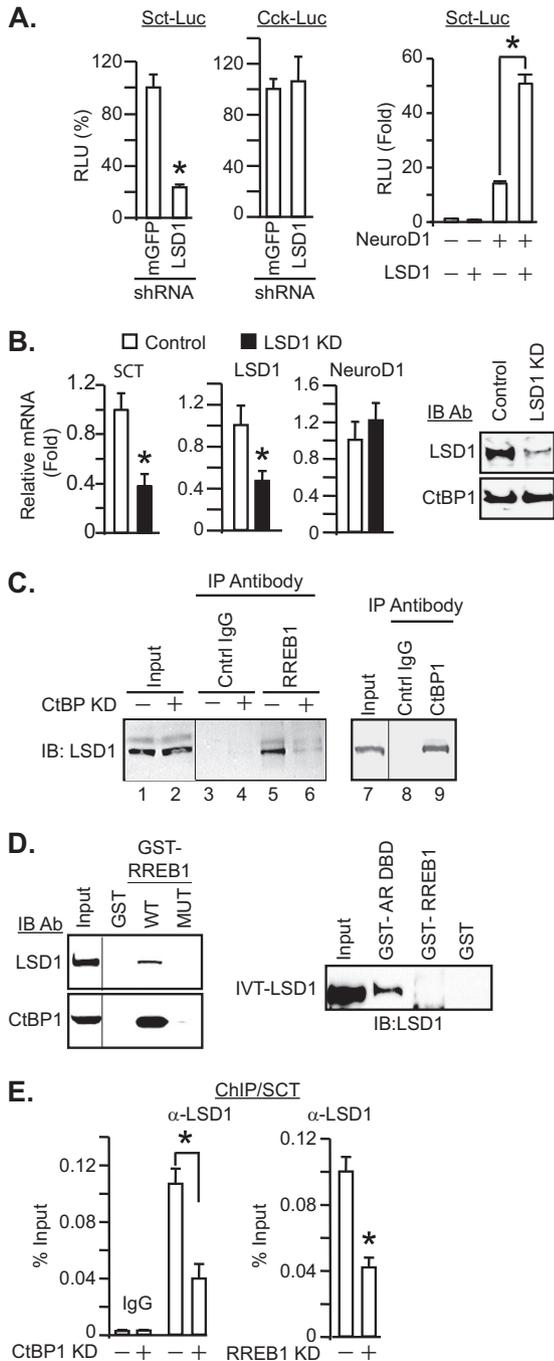
FIG 3 The CtBP complex recruits LSD1 to the promoter to increase transcription. (A) Effects of LSD1 knockdown on Sct (left) and Cck control (middle) reporter gene activity and LSD1 overexpression (right) in HuTu 80 and HEK 293 cells, respectively. (B) Effects of LSD1 depletion on endogenous SCT, LSD1, and NeuroD1 mRNA, measured by qPCR (left). Immunoblot showing effects of LSD1 depletion on LSD1 and CtBP1 protein expression (right). Results are shown as means ± SEM ($n \geq 3$); ∗, $P < 0.015$. (C) Effect of CtBP KD on the association of LSD1 with RREB1 (lanes 5 and 6). Co-IP of endogenous LSD1 with RREB1 in control (−) or CtBP KD (+) HuTu 80 cells. A positive control shows co-IP of endogenous LSD1 with CtBP1 (lane 9). Input: 1% (lanes 1 and 2) or 7% (lane 7). (D) Interaction of RREB1 with LSD1 in nuclear extracts (left) or with LSD1 made by *in vitro* transcription/translation in reticulocyte lysates (right). Nuclear extracts were incubated with GST-RREB1 fusion proteins containing WT or MUT CtBP binding motifs. Bound proteins were examined by immunoblotting for CtBP1 and LSD1 (left). IVT-LSD1 was

CtBP in RREB1-depleted cells. Depletion of RREB1 reduced CtBP1 occupancy of the promoter without reducing the expression level of the CtBP1 protein, suggesting that the presence of CtBP1 depends on RREB1 (Fig. 2C and D). Thus, binding of RREB1 to its target DNA may recruit the CtBP and associated proteins to this promoter.

CtBP recruits LSD1 for transcriptional activation. The histone demethylase LSD1 (KDM1A) is one component of the CtBP complex that functions to either increase or repress transcription. The dual functions of LSD1 prompted us to examine its role in secretin gene transcription in more detail. Knockdown of LSD1 with an shRNA significantly (Fig. 3B, right) reduced transcriptional activity of a secretin reporter gene but not of a Cck reporter, indicating that LSD1 contributes to transcription of the secretin gene (Fig. 3A, left). Reduced expression of Sct mRNA in LSD1-depleted cells further suggested a role for LSD1 in Sct gene expression. LSD1 depletion had no effect on NeuroD or CtBP expression, indicating that the effects of the LSD1 shRNA were due to targeting LSD1 (Fig. 3B). To further determine the functional role of LSD1 in NeuroD1-dependent transcription, we cotransfected 293 cells with the secretin reporter and expression plasmids for NeuroD1 and LSD1 (Fig. 3A, right). As we showed previously, this reporter shows minimal expression in the absence of NeuroD, despite the presence of other regulatory elements and the proteins that bind to them (5, 7, 11, 15, 45). NeuroD1 alone significantly increased (~15-fold) the minimal reporter gene expression seen in its absence. NeuroD-dependent transcription was further enhanced, to ~50-fold above the basal level, with expression of LSD1, whereas LSD1 had no effect on basal transcription of the reporter in the absence of NeuroD1 (Fig. 3A).

Endogenous LSD1 coimmunoprecipitated with both RREB1 (lane 5) and CtBP1 (lane 9), indicating that they associate at their native levels in nuclear extracts (Fig. 3C). However, CtBP knockdown eliminated LSD1 coimmunoprecipitation with RREB1 (lanes 5 and 6) without affecting the level of LSD1 (Fig. 3C, lanes 1 and 2), suggesting that the interaction of RREB1 with LSD1 was CtBP dependent. We confirmed the CtBP dependency of the association between LSD1 and RREB1 by the ability of LSD1 in nuclear extracts to bind to a GST-RREB1 fusion protein but not to a GST-RREB1 fusion protein with mutations in both the CtBP-interacting (PXDLS) motifs (Fig. 3D, left). *In vitro*-translated (IVT) LSD1 did not bind to the GST-RREB1 fusion protein, while retaining its ability to associate with the GST-AR DBD (29). These observations indicate that LSD1 does not directly interact with RREB1 (Fig. 3D, right). We further examined the role of CtBP in bringing LSD1 to the promoter by comparing ChIP for LSD1 in control, CtBP knockdown, and RREB1 knockdown HuTu 80 cells. Depletion of CtBP significantly reduced LSD1 occupancy at the secretin promoter (Fig. 3E, left), indicating that LSD1 occupancy was dependent on CtBP. Since the presence of CtBP at the SCT promoter was dependent on RREB1, depletion of RREB1 reduced the presence of LSD1 as expected (Fig. 3D, right), further

incubated with GST–androgen receptor DNA-binding domain (AR DBD) (a positive control), GST-RREB1, or GST alone (right). Bound proteins were detected by immunoblotting for LSD1. Input: 10% of the materials used for binding assay. (E) LSD1 ChIP at the SCT promoter with extracts of CtBP KD (left), RREB1 KD (right), and wild-type HuTu 80 cells. Results are shown as means ± SEM ($n \geq 3$); ∗, $P < 0.01$.
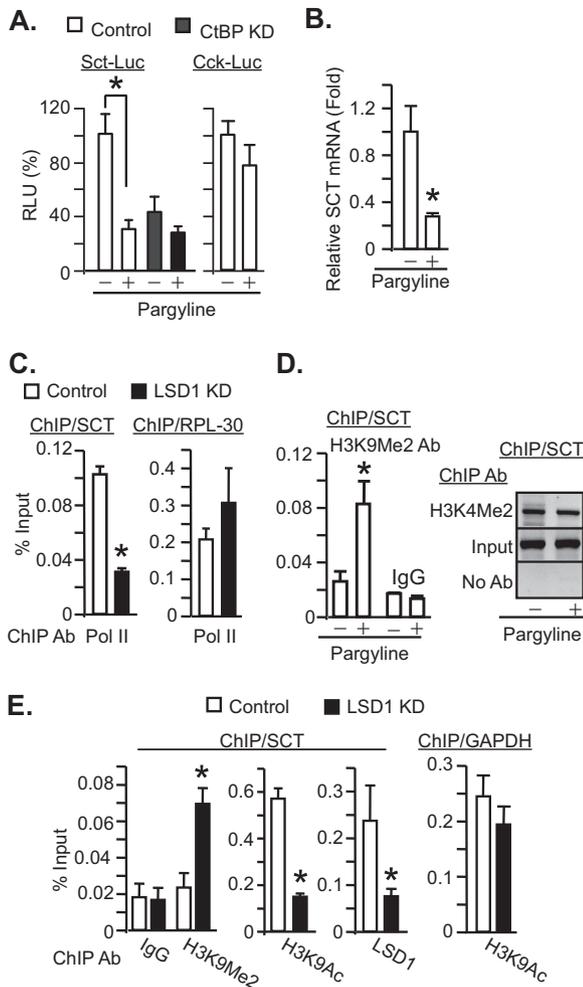
FIG 4 LSD1 derepresses secretin gene expression by H3K9me2-specific demethylase activity. (A) Effect of pargyline on transcriptional activity of a NeuroD-dependent (Sct) or -independent (Cck) promoter. Results are shown as means ± SEM ($n \geq 3$). ∗∗, $P < 0.001$. (B) Effect of pargyline on endogenous SCT mRNA expression, shown by RT-qPCR (normalized to β-actin). (C) Effect of LSD1 KD on RNA Pol II occupancy. ChIP at the SCT promoter (left) or a control region of the RPL-30 gene (right) was performed using RNA Pol II Ab in control or LSD1 KD cells. (D) ChIP for H3K9Me2 (left) or H3K4Me2 (right) modification at the secretin promoter in control and pargyline-treated cells. (E) Effect of LSD1 KD on H3K9Me2 or H3K9Ac modification at the SCT promoter. ChIP for H3K9Ac at the GAPDH gene was used as a control. Results are shown as means ± SEM ($n \geq 3$); ∗, $P < 0.05$.

suggesting that RREB1 recruits members of the CtBP complex to the SCT promoter. However, LSD1 does not directly interact with CtBP (46), suggesting that its presence at the promoter may depend on interactions with another protein in the CtBP complex.

H3K9Me2-specific demethylase activity of LSD1 is required for increased transcription associated with CtBP. To further establish the importance of LSD1 enzymatic activity for NeuroD1-dependent transcription, we treated cells with pargyline, an inhibitor of LSD1 (29, 47). Pargyline inhibited both transcriptional activity of a secretin gene reporter (Fig. 4A) and expression of the endogenous gene (Fig. 4B), suggesting that the enzymatic activity of LSD1 may be important for secretin gene transcription in enteroendocrine cells. Moreover, the drug failed to exert similar in-

hibitory effects on transcription in CtBP KD cells, confirming the dependence of the effects of LSD1 on its association with CtBP (Fig. 4A). Depletion of LSD1 by RNA interference (RNAi) reduced RNA Pol II occupancy at the secretin gene promoter (Fig. 4C) comparably to the effects of pargyline on SCT mRNA levels (Fig. 4B), whereas LSD1 depletion did not reduce Pol II occupancy at an unrelated control (RPL-30) gene, further suggesting that SCT transcription was dependent on LSD1.

Demethylation H3K4Me1 and H3K4Me2 by LSD1 results in repression of transcription (19). LSD1 can also demethylate H3K9Me2, resulting in increased transcription from removal of repressive histone marks (29). Demethylation of H3K9 may contribute to the observed activation of gene transcription by CtBP. To further characterize whether the H3K4 or H3K9 demethylase activity of LSD1 was associated with increased transcription, we examined the methylation status of H3K9 and H3K4 by ChIP at the SCT promoter in control and pargyline-treated HuTu 80 cells (Fig. 4D). Pargyline treatment increased H3K9Me2 at the SCT gene promoter, suggesting that the enzymatic activity of LSD1 is required to remove repressive marks at H3K9. Depletion of LSD1 was associated with a significant increase in H3K9Me2, confirming that the effects of pargyline were likely mediated by LSD1 (Fig. 4E). As expected for an actively transcribed gene, we observed methylation of H3K4. Pargyline treatment had little effect on ChIP for H3K4Me2 at the SCT promoter compared to results with untreated cells, suggesting that H3K4 may be nearly fully methylated and that, as expected, the H3K4 demethylase activity of LSD1 is inactive at the promoter of an actively transcribed gene. In addition, LSD1 depletion was associated with reduced acetylation of H3K9 at the Sct promoter. The latter modification is seen with actively transcribed genes. LSD1 depletion had no effect on H3K9-Ac at a control (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) gene, which is not normally occupied by LSD1 (Fig. 4E).

Acetylation of H3K9 by PCAF (KAT2B) is required for transcriptional activity. The experiments shown in Fig. 4 indicate that removal of the repressive H3K9Me2 marks at H3K9 is dependent on the H3K9 demethylase activity of CtBP-associated LSD1, possibly allowing for subsequent H3K9 acetylation and active transcription. H3K9 is a known substrate for the HAT (48) activity of PCAF. In addition, PCAF has been previously shown to bind directly to NeuroD1 and to increase transcription (14). As expected, ChIP for PCAF confirmed its presence at the SCT promoter (Fig. 5A). To determine whether PCAF occupancy of the promoter was dependent on NeuroD1, we examined NeuroD1 knockdown cells by ChIP for the presence of PCAF. Depletion of NeuroD1 reduced PCAF promoter occupancy, suggesting association with NeuroD1 as one potential mechanism for bringing PCAF to the CtBP-occupied SCT promoter (Fig. 5A).

We further evaluated the functional role of PCAF in cells stably expressing a PCAF shRNA in HuTu 80 cells by ChIP for RNA Pol II (Fig. 5B and C). PCAF knockdown significantly reduced RNA Pol II occupancy, suggesting that PCAF was required for active transcription of the SCT gene. The observed reduction was accompanied by a significant reduction in H3K9 acetylation at the SCT promoter but not at the control gene (GAPDH), suggesting that PCAF acetylates H3K9 to promote transcription of the SCT gene (Fig. 5B). Depletion of PCAF significantly reduced transient expression of a secretin reporter gene, confirming the importance of PCAF for Sct gene expression (Fig. 5C).
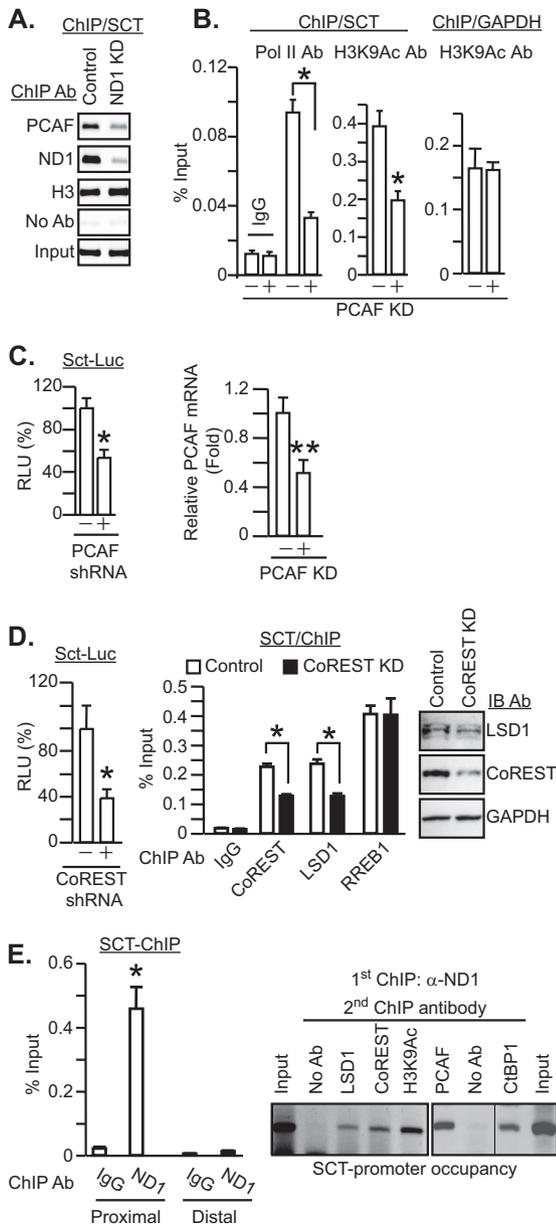
FIG 5 Multiple proteins of the CtBP complex cooccupy the promoter with NeuroD and PCAF. (A) Effect of NeuroD1 KD on SCT promoter occupancy by PCAF and NeuroD1 by ChIP. (B) Effect of PCAF depletion on RNA Pol II occupancy (left) or H3K9Ac modifications (middle) at the SCT promoter. ChIP for H3K9Ac at the GAPDH gene was used as a control (right). (C) Effect of PCAF depletion on transient expression of an Sct reporter. HuTu 80 cells were cotransfected with the reporters and a PCAF shRNA-expressing or control plasmid (left). Effect of PCAF shRNA on endogenous PCAF mRNA (right). (D) Effect of CoREST depletion on transient expression of an Sct reporter. HuTu 80 cells were cotransfected with the reporters and a control plasmid or a CoREST shRNA-expressing plasmid (left). Effect of CoREST KD on CoREST, LSD1, and RREB1 ChIP at the SCT promoter (middle) is shown. Immunoblots show CoREST, LSD1, or GAPDH protein expression in CoREST knockdown cells (right). (E) Cooccupancy of NeuroD and indicated proteins at the Sct promoter. Chromatin fragments immunoprecipitated by NeuroD1 antibody (first ChIP), showing the relative abundance of amplified promoter (-469 to -335) versus distal (-3811 to -3610) sequences of the SCT gene (left). The NeuroD1-bound chromatin fragments were reprecipitated for re-ChIP with the indicated antibodies, followed by amplification of immunoprecipitated SCT promoter DNA fragments (right). Results are shown as means ± SEM ($n \geq 3$). *, ($P \leq 0.002$); **, $P < 0.05$.

Proteins at NeuroD1 target promoters during active transcription. In addition to CtBP, RREB1, and LSD1, we examined the SCT promoter for the presence of the transcriptional corepressor, CoREST, another component of the CtBP complex that directly interacts with LSD1 (20, 49–51). ChIP experiments identified CoREST occupancy at the secretin promoter (Fig. 5D). Depletion of CoREST by a specific shRNA reduced the expression of an Sct reporter gene, suggesting that its presence contributes to transcriptional activation by NeuroD1. CoREST depletion also reduced promoter occupancy by LSD1 but had no effect on promoter occupancy by RREB1 (Fig. 5D). The CoREST shRNA had little effect on LSD1 protein expression (Fig. 5D, right), suggesting that CoREST is necessary for retaining LSD1 in the RREB1/CtBP complex at the Sct gene.

We next determined if CtBP1, LSD1, PCAF, and CoREST cooccupied the SCT promoter with NeuroD1 by re-ChIP. Following immunoprecipitation with the first ChIP NeuroD1 antibody, NeuroD-bound chromatin fragments underwent the second ChIP with antibodies against LSD1, CoREST, CtBP1, PCAF, and H3K9Ac (Fig. 5E). The NeuroD1-bound chromatin fragments obtained from the first ChIP were highly enriched for promoter region sequences of the SCT gene compared to distal sequences at kb -3.7, indicating that NeuroD bound to the promoter region, close to its established E box binding site. Each of the CtBP-associated proteins examined in the second ChIP cooccupied the SCT promoter with NeuroD1, as did PCAF. The presence of a strong activation mark, H3K9Ac, at the NeuroD1-occupied promoter indicates that the presence of CtBP1 and associated proteins occurs in an environment favoring active transcription. In addition, cooccupancy of CtBP1, LSD1, and CoREST with NeuroD1 suggests that they potentially contribute directly to NeuroD-dependent transcription.

Although NeuroD1 is expressed in most endocrine cells of the intestine and pancreas (4), relatively few genes that are directly activated by NeuroD1 have been identified. Besides the Sct gene, the mouse insulin I, insulin II, and β-glucokinase (Gck) genes have been identified as direct transcriptional targets of NeuroD1 (1, 6, 12). We examined the promoters of these additional NeuroD1 target genes in endocrine cells for the presence of proteins identified earlier as occupying the secretin promoter. We identified NeuroD1, RREB1, CtBP1, and PCAF by ChIP at the Gck and Sct promoters in the murine STC1 enteroendocrine cell line (Fig. 6A, left). The same proteins also occupied the Sct, insulin I, and insulin II promoters in the pancreatic β cell line, βTC6 (Fig. 6B, left). All four promoters in the murine cell lines show acetylation of H3K9, much like findings for HuTu 80 cells. Occupancy of both promoters by RNA Pol II further indicates that the Sct and Gck genes were actively transcribed in STC1 cells (Fig. 6A, right), as were both insulin genes in βTC6 cells (Fig. 6B, right). Likewise, the Sct gene was actively transcribed in βTC6 cells (52), which we previously showed expressed high levels of Sct transcripts (32). Thus, promoter cooccupancy of NeuroD1-regulated endocrine genes by CtBP and associated proteins may have a more general role for NeuroD-dependent transcription in the GI tract.

DISCUSSION

We previously identified RREB1 as a DNA binding protein that potentiated relatively weak transcriptional activation by NeuroD1. Other studies have shown that RREB1 may either activate (15–18) or repress (21–26) transcription, depending on the
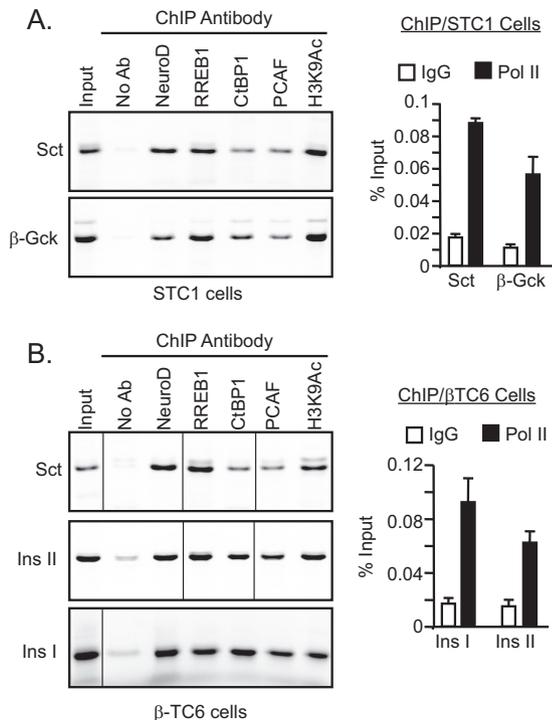
FIG 6 Presence of CtBP and associated proteins at the promoters of other NeuroD activated genes. ChIP showing the presence of indicated proteins and H3K9 acetylation at NeuroD1 target genes in STC1 enteroendocrine cells (A) or the pancreatic β cell line, βTC6 (B). RNA Pol II occupancy at the indicated promoters, shown by qPCR (right panels).

context. The discovery that RREB1 was a component of the large multiprotein CtBP complex containing multiple histone and chromatin modifying activities suggested that repressive and possible activating effects of RREB1 on transcription are the result of epigenetic modifications. Although RREB1 has been identified as a component of the CtBP complex, only one prior study has described functional interaction between RREB1 and CtBP at the HLA-G gene by electrophoretic mobility shift assay (EMSA). In this case, the interaction resulted in transcriptional repression of the HLA-G gene (53).

In the present work, we have shown that CtBP binds directly to RREB1 through two evolutionarily conserved PXDLS motifs between Zn fingers 11 and 12 to cooccupy the SCT promoter with NeuroD1. Depletion of RREB1 reduces CtBP occupancy, suggesting that RREB1 contributes to the recruitment of CtBP to specific DNA sequences. Thus, RREB1 may function similarly to ZEB1, a DNA binding protein with two PXDLS motifs that associate with the hydrophobic cleft of CtBP, bringing it and other proteins in the CtBP complex to specific DNA sequences to repress transcription (46, 54). RREB1 appears to recruit CtBP to the Sct promoter, resulting in increased transcription. The binding of RREB1 to DNA adjacent to the NeuroD binding site, as well as the physical interaction between the two proteins, may provide a measure of promoter selectivity for the observed mechanism. Likewise, the functions of Zeb1 may be selective, recruiting CtBP to the growth hormone (GH) gene in pituitary cells with repressed but not active GH expression (30). The basis for Zeb1 selectivity has yet to be examined.

The presence of CtBP and RREB1 at an actively transcribed promoter, as reported here, has not been previously described. Although a number of early studies suggested that CtBP might be involved in increasing gene expression, the observed effects in some cases may have been indirect (55–57). These older studies were completed prior to the discovery that CtBP was part of a large multiprotein complex involved in histone and chromatin modification. Recent ChIP-sequencing (ChIPseq) analysis of CtBP-occupied genes identified a small fraction that may be activated by CtBP (58). However, the mechanisms contributing to increased transcription by CtBP in mammalian cells have not been characterized.

LSD1, a component of the CtBP complex, functions in both transcriptional repression and activation. One study proposed that the effects of LSD1 depend on its association in a corepressor versus a coactivator complex. In the anterior pituitary, LSD1 repressed growth hormone expression in developing lactotrophs when associated with the CtBP complex while increasing growth hormone expression in somatotrophs as part of WDR5/MLL1 coactivator complexes (30). However, a switch to a coactivator complex cannot explain our findings, since the effects of LSD1 in stimulating transcription from NeuroD1-occupied promoters depend on its association with CtBP.

The effects of LSD1 enzymatic activity on gene transcription may depend on the substrate utilized. Demethylation of H3K4Me2 in association with the CtBP complex results in transcriptional repression (27, 28). LSD1 also catalyzes demethylation of H3K9Me2, with resultant transcriptional activation (29). Removal of repressive methylation marks at H3K9 by LSD1 has been implicated in ligand-dependent activation of androgen and estrogen receptor-dependent transcription (29, 47, 59, 60).

The opposing effects of demethylation at H3K4 versus that at H3K9 on transcription imply the existence of mechanisms that favor demethylation at H3K9 while inhibiting H3K4 demethylation. We previously showed that demethylation of H3K9 associated with androgen receptor-dependent transcriptional activation occurs without demethylation of H3K4 (29) and that H3T6 phosphorylation prevents H3K4 demethylation (60). Another mechanism for regulating LSD1 substrate specificity involves PELP1, an estrogen receptor (ER) coregulator that associates with LSD1 and ERα in the presence of ligand to switch its specificity from H3K4Me2 to H3K9Me2 in estrogen receptor-activated transcription (47). Finally, simultaneous demethylation of both H3K4 and H3K9 occurs at some actively transcribed genes, indicating that selection between H3K4 and H3K9 as LSD1 substrates does not occur in all cases (59).

The ability to interrogate the genome for occupancy by transcription-modifying proteins has identified a very large number of genes that associate with CtBP or LSD1. Depending on the methodology and cell types studied, LSD1 associates with >10,400 genes (59, 61), with CtBP occupying >6,600 binding sites across the genome (58). Although most biochemical studies have focused on the functional role of these two proteins as corepressors, chromatin occupancy studies indicate that LSD1 is predominantly associated with approximately 81% of actively expressed and bivalent genes, whereas less than one quarter of silenced genes associate with LSD1. The association of LSD1 with actively transcribed genes may indicate a more significant role for its H3K9 demethylase activity than previously appreciated. The relationship between CtBP binding and transcriptional activation is less
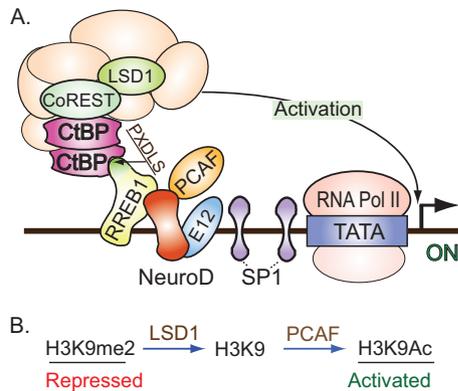
FIG 7 Model for transcriptional regulation by NeuroD1. NeuroD-dependent transcriptional activation relies on the presence of multiple cofactors assembling at the promoters of NeuroD target genes. (A) NeuroD and RREB1 bind to nearby DNA elements and physically associate. Through its PXDLS motifs, RREB1 binds to CtBP, recruiting other CtBP-associated proteins, like CoREST and LSD1, to the promoter. PCAF, which is not generally part of the CtBP complex, is recruited to the promoter by virtue of its association with NeuroD. (B) The demethylation of H3K9Me2 (repressive histone marks) by LSD1 facilitates subsequent acetylation of H3K9 to H3K9Ac (activation marks) by the HAT activity of PCAF.

well characterized, since CtBP appears to be more frequently associated with repression.

Both CtBP and LSD1 have been implicated as regulators of gene expression. The discovery that a very large number of genes are associated with LSD1 or CtBP implies that additional mechanisms are needed to account for their specificity in transcriptional regulation. The presence of either protein at core promoter and enhancer regions throughout the genome suggests that functional interactions with specific transcription factors may define how these proteins regulate gene expression and potentially play a role in their function as coactivators or corepressors.

The basis for increased gene expression associated with CtBP in mammalian cells has not been previously identified. We believe that the association of CtBP/LSD1 with NeuroD may represent an example of such a mechanism involving a tissue-specific transcription factor. A first level of selectivity results from the restriction of NeuroD expression to endocrine cells and their precursors. The presence of RREB1 binding sites close to NeuroD binding sites and their direct physical interaction with each other may facilitate recruitment of CtPB to specific genes expressed in endocrine cells as second means of selective gene activation. Finally, the recruitment of the histone acetyltransferase PCAF by NeuroD to endocrine gene promoters and its acetylation of H3K9 may contribute to CtBP/LSD1 functioning as coactivators in the specific context studied here (Fig. 7). Our observations may be an example of a broader context for how proteins like CtBP and LSD1 interact with transcription factors to regulate specific gene expression programs. The ability of LSD1 and/or CtBP to associate with different transcription factors also implies their involvement in many distinct biochemical mechanisms for regulating gene expression.

ACKNOWLEDGMENTS

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