A Novel Corepressor, BCoR-L1, Represses Transcription through an Interaction with CtBP*

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Corepressors play a crucial role in negative gene regulation and are defective in several diseases. BCoR is a corepressor for the BCL6 repressor protein. Here we describe and functionally characterize BCoR-L1, a homolog of BCoR. When tethered to a heterologous promoter, BCoR-L1 is capable of strong repression. Like other corepressors, BCoR-L1 associates with histone deacetylase (HDAC) activity. Specifically, BCoR-L1 coprecipitates with the Class II HDACs, HDAC4, HDAC5, and HDAC7, suggesting that they are involved in its role as a transcriptional repressor. BCoR-L1 also interacts with the CtBP corepressor through a CtBP-interacting motif in its amino terminus. Abrogation of the CtBP binding site within BCoR-L1 partially relieves BCoR-L1-mediated transcriptional repression. Furthermore, BCoR-L1 is located on the E-cadherin promoter, a known CtBP-regulated promoter, and represses the E-cadherin promoter activity in a reporter assay. The inhibition of BCoR-L1 expression by RNA-mediated interference results in derepression of E-cadherin in cells that do not normally express E-cadherin, indicating that BCoR-L1 contributes to the repression of an authentic endogenous CtBP target.

Although the role of coactivator proteins in transcriptional regulation is well established, the equally important role of corepressor proteins in gene regulation has become apparent only relatively recently. There are many corepressor proteins, recruited to a wide range of transcriptional silencers. They regulate many processes, including differentiation, proliferation, apoptosis, and the cell cycle (2). The aberrant function of corepressors can lead to developmental defects and disease, since genes that should be turned off are instead aberrantly expressed, or “derepressed” (3). Alternately, overactive repression leads to enhanced silencing and has been reported in several types of leukemia, involving gene fusions to transcriptional silencers (4). Increased corepressor binding to these fusion proteins leads to inappropriate repression of target genes important for normal cellular differentiation.

Corepressors act within multiprotein complexes containing DNA-binding proteins, histone deacetylases, methyl-CpG-binding proteins, nucleosomal histones, and the basal transcriptional machinery. The same corepressor can be found in different corepression complexes, and multiple corepressors can be used by individual silencers. Corepressors are thought to bridge the interaction between DNA-bound transcriptional repressors and the chromatin-modifying enzymes that mediate repression. They typically act in multiple ways, including via the targeted modification of chromatin structure, nucleosomal remodeling, and sequestration of the basal transcription machinery as well as by inhibiting trans-activation (2).

Lysine acetylation of the histone tails is a major modification associated with transcriptional activation, and in contrast, deacetylation is associated with transcriptional repression. It is thought that the acetylation of the lysine residues of the core histone tails neutralizes the positive charge on the lysine residue, thereby resulting in a more open structure of chromatin because of the decreased affinity between the histone and the DNA (5, 6). Chromatin is therefore more accessible to transcription factors and other proteins. Conversely, the deacetylation of histone substrates results in chromatin condensation and inhibits the accessibility of transcription factors and other proteins to the DNA.
The acetylation reaction is a reversible process, catalyzed by the opposing activity of histone acetyltransferases and deacetylases (HDACs)3 in vivo. There are several classes of histone acetyltransferases, each employing different mechanisms of catalysis (7). Likewise, there are three main classes of HDACs grouped according to homology to their counterparts in yeast, their subcellular localization, and enzymatic activities (8, 9). Class I (HDAC1, -2, -3, and -8; homologous to yeast Rpd3) are ubiquitously expressed and are primarily localized in the nucleus. Class II (HDAC4, -5, -6, -7, and -9; homologous to Hda1) are expressed in a tissue-specific manner and shuttle between the nucleus and cytoplasm (10). Class III (Sirt1, -2, -3, -4, -5, -6, and -7; similar to Sir2) use a different mode of catalysis, relying on NAD+ (11). Most targets are histones, although other proteins are deacetylated as well, including α-tubulin (12). All known repression complexes employ HDACs to mediate their repression.

CtBP is also a critical component of many transcriptional repression complexes (reviewed in Ref. 13). Microarray studies from CtBP knock-out mice implicate CtBP in the repression of genes involved in apoptosis and in the epithelial-to-mesenchymal transition (18, 19). To mediate its repression, it recruits enzymes involved in transcription repression, such as methylases and deacetylases, to the sequence-specific DNA-binding proteins via a conserved PDLS CtBP interaction motif in the CtBP-interacting proteins (14, 15). Recently, it has been shown that many histone acetyltransferases contain the PDLS motif and that CtBP inhibits histone acetylation by blocking access of nuclear histone acetyltransferases to their target (16, 17).

Interestingly, CtBP structure is similar to a subfamily of NAD+-dependent dehydrogenases, and biochemical and structural studies have recently demonstrated that CtBP possesses dehydrogenase activity (20–22). CtBP binds NADH with a higher affinity than NAD+ (22), although this has been controversial (20). CtBP function is regulated by the relative levels of NAD+ and NADH within the nucleus, suggesting a role for CtBP in sensing the redox state of the cell and regulating transcription accordingly (22, 23). Current models suggest that increases in NADH levels promote CtBP dimerization, which in turn increases the interaction between CtBP and proteins containing the PDLS CtBP-recruitment motif.

We have identified a novel PDLS-containing protein designated as BCoR-L1 in the GenBankTM data base. BCoR-L1 is related to the BCoR, a transcriptional corepressor that potentiates BCL6 repression in reporter assays (24). BCL6 is a transcription factor that is required for germinal center formation and is linked to lymphomagenesis (25). We find that, like BCoR, BCoR-L1 functions as a corepressor when tethered to DNA. BCoR-L1 interacts with Class II HDACs, HDAC4, HDAC5, and HDAC7, suggesting that they are involved in its function as transcriptional corepressor. It mediates its repression through recruitment of the CtBP corepressor protein and affects the repression of at least one CtBP target, the tumor suppressor protein, E-cadherin.

**MATERIALS AND METHODS**

Cloning of Full-length BCoR-L1 and Other Plasmids—Full-length BCoR-L1 (BCoR-L1a) and ΔExon9 BCoR-L1 (BCoR-L1) cDNAs were produced in two stages. The C terminus of BCoR-L1 was PCR-cloned using Pfu polymerase from cDNA derived from the normal ovarian epithelial cell line (HOSE 17.1). Primers used for this PCR were designed from the in silico predictions of the mRNA and later from known sequence. The cDNA was amplified in several overlapping fragments, which were cloned into the pPCR-Script vector (Stratagene) and later assembled into a single vector using appropriate restriction enzyme sites. The N terminus of BCoR-L1 was provided by a partial cDNA clone (bp 60–4449, FLJ00190), which was a generous gift of Kasuza Institute (Chiba, Japan). The first 60 bp of BCoR-L1 were incorporated into primers, which were used to amplify the N terminus of BCoR-L1 from the cDNA clone and then subcloned into the existing C terminus of BCoR-L1. The entire coding sequences for BCoR-L1 were then subcloned into pEGFP-C1 (Clontech) and pFLAG-CMV-2 (Sigma) parent plasmids using Sall and BamHI sites.

GST-BCoR-L1a-(1328–1785) vector for antibody production was constructed by cloning BCoR-L1 sequence (amino acids 1328–1785) in frame with pGEX-5X-1. The pSUPER vector system (26) (a gift of Dr. Reuven Agami; Netherlands Cancer Institute, Amsterdam) was used to create the siRNA construct to knock-down BCoR-L1 levels “pSUPER-BCoR-L1.” The following sequence was cloned into the pSUPER vector to produce pSUPER-BCoR-L1: forward (FWD), 5’-GATCCCCGGCGCGACTGAGGCTAGGGGCCCTTTCAAGAGAGCCCTCAGCCTCTGGCCGCTTGGAAA-3’; reverse (RVS), 5’-AGCTTTTCGAAAAACGTGGCAGAGGCTAGGGCTCTCTTGAAAGCCCTACGCTCTCTGCCACGTTTTTGGAAA-3’. This construct generated siRNAs targeting the following 21-nucleotide sequence: 5’-CTGTTGCCAGAGGCTAGGGCTCTCTTGAAAGCCCTACGCTCTCTGCCACGTTTTTGGAAA-3’. This construct was cloned into the pSUPER vector system (26) and was used to produce the pSUPER-BCoR-L1i vector (27). The pSUPER-GFP plasmid has been described previously (27). Gal4 DNA-binding domain (Gal4-DBD) vectors were constructed by replacing GFP from pEGFP vectors (Clontech) with the Gal4-DBD from pGBT9 (Clontech). BCoR-L1 was cloned as a Gal4-DBD fusion protein using Sall and BamHI sites. BCoR-L1 fragments were cloned as Gal4-DDB fusion proteins using BamHI and EcoRI sites incorporated into primers designed to amplify the fragments from plasmid. The Gal4-DDB-BCoR-L1 ΔCtBP binding site mutant (PLDLS to PLASS) was created using the QuickChangeTM site-directed mutagenesis method (Stratagene). FLAG-tagged expression vectors for HDAC4 to -7 (28, 29), the pGL3-hE-cad (E-Cad-Luc) (15), and Myc-tagged CtBP1 expression vectors (30) have been described previously (31, 32). The GFP-BCoR plasmid was subcloned from Myc- BCoR (kindly provided by Vivian Bardwell, University of Minnesota, Minneapolis, MN).

**Northern Analysis**—The probe for Northern analysis was prepared from RT-PCR products derived from the amino terminus of BCoR-L1 amplified from the HOSE 17.1 ovarian carcinoma cell line using the following primer sequences: forward, 5’-
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GTGCAACACTGGACCAGTCTCGGACCG-3'; reverse, 5’-GAGTCAGAGATGAGCCTGTCGGGACTG-3’. Probes were [α-32P]dCTP-labeled using the MegaPrime kit (Amersham Biosciences). Hybridization to a Clontech human Multiple Tissue Northern blot II membrane was carried out for 2 h in ExpressHyb solution (Clontech) at 65 °C, followed by a standard washing procedure.

Cell Culture and Transfections—Cells were maintained at 37 °C in a 5% CO2 incubator and grown in RPMI 1640 medium supplemented with 10% Serum Supreme, 1% l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Transfections of cells were carried out using either electroporation or lipofection. For electroporation, 293T cells in exponential growth were pelleted and resuspended at 10⁶ cells/300 μl in complete media and then electroporated at room temperature with 7 μg of DNA in a 0.4-cm cuvette (Interpath). Electroporation was performed with a BTX™820 electroporator (General Electronics Inc.) at 260 V with a time constant of 10 ms. HeLa cells growing in dishes were transfected using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Luciferase Assays—293T cells were grown in 24-well dishes and were transfected using Lipofectamine™ 2000. The pRL-TK plasmid (Promega) was cotransfected as a control for transfection efficiency. The amount of DNA per well was kept constant using an empty expression vector (pcMV; Stratagene). Cells were grown for 48 h prior to harvesting using Passive Lysis Buffer (Promega, Anannadale, Australia). The lysate was assayed for luciferase activity using the Dual-Luciferase™ reporter assay system (Promega), according to the manufacturer’s recommendations. Readings were taken using a TD-20/20 Luminometer (Turner Designs). Results shown are from representative experiments. The error bars represent one S.D.

Antibodies and BCoR-L1 Antibody Production—Polyclonal antibodies were produced in rabbits following standard injection protocols. Rabbits were immunized with GST-BCoR-L1a (1328–1785) fusion protein. An α-BCoR-L1 peptide antibody was raised against the sequence EERRAPLSDEESTGD by Open Biosystems. The following antibodies were used: rabbit anti-GFP (Molecular Probes), mouse monoclonal anti-FLAG (Sigma), mouse monoclonal anti-Myc (Cell Signaling Technology), ImmunoPure Recomb Protein A/G HRP conjugate (Molecular Probes), and rabbit anti-Gal4-DBD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

ChIP Analysis and RT-PCR—ChIP analysis of the E-cadherin promoter was performed according to Shi et al. (15). The PCR primers used to amplify the E-cadherin promoter were as follows: forward, 5’-TACCCTGGCAGTTGTTGTTGACCTG-3’; reverse, 5’-GTGGCTGTGCTGACCCAGGTGGAC-3’. The first primers were used for quantitative PCR detection of BCoR-L1 levels: forward, 5’-GACCAGATCTGGAAGAGTT-3; reverse, 5’-ATAAGCCACAGGAGGAGCCACAG-3’. The primers used to detect E-cadherin levels were as follows: forward, 5’-GAAAAATCCTGGAAGCCGCTGAT-3; reverse, 5’-GCCCTATTGGTCAAGG-3’.

Lysate Preparation, Coimmunoprecipitation, Immunoblotting, and Antibodies—Cell extracts were prepared by lysis in universal immunoprecipitation buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 0.2% Triton X-100, and 0.3% Nonidet P-40), supplemented with 25 mM sodium fluoride, 25 mM sodium orthovanadate, 0.1 mM phenylmethysulfonfluride, and a mixture of protease inhibitors (Roche Applied Science). For coimmunoprecipitation experiments, protein samples were preclared by incubation with protein G-Sepharose beads (Sigma) for 30 min at 4 °C. Supernatants (2 mg) were incubated with 2 μg of antibody overnight. Immune complexes were collected with protein G-Sepharose beads. Beads were washed four times with lysis buffer, eluted using Laemmlı sample buffer, and analyzed by immunoblotting with specific antibodies.

Immunofluorescence Staining—Cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline and permeabilized in 0.2% Triton X-100 in phosphate-buffered saline. Coverslips were blocked with 0.5% bovine serum albumin in phosphate-buffered saline and then incubated with primary antibodies diluted in blocking solution for 1 h at room temperature, followed by AlexaFluor-546-conjugated secondary antibodies (Molecular Probes). Cells were counterstained with 4’,6-diamidino-2-phenylindole (Molecular Probes). Coverslips were mounted on glass slides with Mowiol (Calbiochem) containing 0.6% diazobicyclo-octane (Sigma). Fluorescence was visualized using a Zeiss Axioskop 20 microscope, Zeiss AxioCam MRc digital camera, and MRGrab 1.0 software. Dual confocal images were collected sequentially using a 635 objective on Leica TCS SP2 confocal fluorescent microscope system using a 488-nm argon laser for GFP and 543-nm helium-neon laser to excite the AlexaFluor-546. Confocal hardware was driven by a work station running Leica confocal imaging software. Figures were processed using Adobe Photoshop 6.0.
A Novel BCoR-related Protein—BCoR-L1 (BCoR-like 1) was isolated as a partial cDNA using full-length BRCA1 as bait in a yeast two-hybrid screen with a testis cDNA library. The basis and significance of the interaction with BRCA1 is currently under investigation and is not discussed here. One set of positive clones encoded the partial cDNA of a hypothetical protein (GenBank™ number NM_019294, BCL6 corepressor-like 1; function unknown), expressed from the X chromosome (Xq25–26.1). The original BCoR-L1 transcript contains 14 exons spliced from ~75 kb of genomic DNA. The original yeast two-hybrid clone contained an additional exon (exon 9) not present in the composite sequence on GenBank™ and represents an alternatively spliced form of BCoR-L1 mRNA, which we call BCoR-L1a. The complete cDNA sequence of full-length BCoR-L1 (BCoR-L1a) and BCoR-L1 ΔExon 9 (BCoR-L1; NM_019294) was generated in mammalian expression vectors (see “Materials and Methods”).

The open reading frame of BCoR-L1 cDNA encodes a protein of 1711 amino acids containing a putative bipartite nuclear localization signal (NLS) and tandem ankyrin repeats (ANK) (Fig. 1). BCoR-L1a is 1785 amino acids long. Neither BCoR-L1 nor BCoR has identifiable DNA-binding domains. BCoR-L1 contains a PXDLS motif, involved in binding the CtBP corepressor (33, 34). BCoR-L1 also contains two LXXLL nuclear receptor recruitment motifs found in coregulator proteins (35). The amino acid sequence shares homology with several proteins involved in chromatin remodeling, transcription, and repair of DNA damage. More specifically, BCoR-L1 is homologous to BCoR, a transcriptional corepressor for the BCL6 transcriptional repressor (24). Additionally, part of the BCoR-L1 sequence shares homology with the predominant BRCA1-interacting protein, BARD1 (36). A region of BCoR-L1 is related to the Drosophila recombination repair protein (dRRP1), a repair endonuclease. Although BCoR-L1 lacks the RRP1 nuclease domain, it shares homology with the RRP1 domain that catalyzes strand transfer during homologous recombination (37, 38). A high degree of sequence conservation exists between human BCoR-L1 and mouse and rat orthologues (80 and 77% amino acid identity, respectively). We have not been able to identify any BCoR-L1 orthologues in lower eukaryotes.

Tissue Expression of BCoR-L1—Northern blot analysis using the multiple-tissue Northern II filter (Clontech) demonstrated that BCoR-L1 is expressed at low levels in many tissues. The
The highest level of expression was observed in testis and prostate. Medium levels of expression were also seen in peripheral blood lymphocytes and spleen (Fig. 2A).

A rabbit polyclonal antibody was raised against recombinant GST-BCoR-L1a (aa 1328–1785). The specificity of the antibody for immunoblot analysis was tested against recombinant FLAG-tagged BCoR-L1 and BCoR-L1 in protein extracts prepared from HeLa cells. The antibody recognized a band migrating through SDS-polyacrylamide gels at a size of ~200 kDa and co-migrating with exogenously expressed FLAG-tagged BCoR-L1 (Fig. 2B). This result provides evidence that endogenous BCoR-L1 is predominantly encoded from the BCoR-L1 ΔExon 9 isoform, which was also the predominant isoform detected by RT-PCR analysis (results not shown). Furthermore, this band was significantly diminished in intensity when cells were depleted of BCoR-L1 by siRNA, further demonstrating the specificity of BCoR-L1 antibody (Fig. 2C).

BCoR-L1 Is a Nuclear Protein—Since the amino acid sequence of BCoR-L1 contains a classical bipartite nuclear localization signal and its homolog BCoR is a nuclear protein, we expected BCoR-L1 to be nuclear. To confirm this, BCoR-L1 was cloned as a GFP fusion protein and transfected into HeLa cells, and its localization was examined by epifluorescence microscopy. GFP-BCoR-L1 localized exclusively in the nucleus and was distributed in a heterogeneous subnuclear pattern of dots (Fig. 3). Between 5 and 30 bright dots were scattered throughout the nucleus of each cell. Interestingly, its pattern of localization differed from that of GFP-BCoR. Although a substantial proportion of GFP-BCoR-L1 was present in the nucleosol, BCoR was exclusively found in many speckle-like dots of a consistent size. On the other hand, GFP-BCoR-L1 localization was highly heterogeneous, with cells displaying dots of various dimensions and number, suggesting that BCoR-L1 localization could be regulated through the cell cycle.

BCoR-L1 Is a Strong Transcriptional Repressor—We next examined whether BCoR-L1 is able to regulate transcription, given its high homology to the BCoR corepressor. We used a standard luciferase reporter system in transient transfection assays. We cloned BCoR-L1 as a GAL4-DBD fusion protein, which recognizes the GAL4 binding sites upstream of a luciferase reporter gene (Luc) driven by the herpes simplex virus (HSV) thymidine kinase (TK) promoter (TK-Luc). The activity exhibited in cells transfected with the Luc reporter construct alone was considered to be the basal transcriptional activity. When expressed in fusion with
the Gal4-DNA binding domain (Gal4-DBD), BCoR-L1 repressed expression of the Luc reporter gene in a dose-dependent manner. Comparable expression of FLAG-tagged BCoR-L1, which is not tethered to the Gal4 sites, did not repress luciferase activity, demonstrating that BCoR-L1 is able to repress basal transcription only when physically tethered to a heterologous promoter (Fig. 4A).

To determine whether BCoR-L1 was able to repress activated transcription, Gal4-DBD-BCoR-L1 was cotransfected with VP16-LexA, a strong transcriptional activator. In this assay, the LexA-VP16 transcriptional activator drives a LexA-responsive promoter, and the reporter construct encodes the luciferase gene with the Gal4-binding sites upstream of two LexA binding sites (39). LexA-VP16 activated the LexA-luciferase reporter gene ~300 fold. Gal4-DBD-BCoR-L1 was able to reduce VP16-mediated transactivation significantly, in a dose-dependent manner (Fig. 4B), thereby demonstrating that BCoR-L1 can repress activated transcription.

To identify the regions in BCoR-L1 that mediate repression, we divided BCoR-L1 cDNA into five overlapping fragments and expressed them as Gal4-DBD fusion proteins. Each fragment expressed a protein of the expected size. When these fragments were tested for repression in the luciferase assay, Fragment 2 (amino acids 360–675) of BCoR-L1 was sufficient for maximum repression, indicating that the repressive activity of BCoR-L1 is mediated through this domain. The amino acid sequence of Fragment 2 (amino acids 360–675) of BCoR-L1 contains a classical CtBP1/2 binding motif, PXLDS. Therefore, to test the hypothesis that CtBP is involved in repression through this domain, the CtBP recruitment motif was mutated from PXLDS to PLASS, and its effect on repressor function was compared with that of wild-type Fragment 2. The mutated fragment was no longer capable of efficient repression (Fig. 4C).

CtBP Is Required for BCoR-L1 Repression—We next studied the interaction of the full-length BCoR-L1 with CtBP1 using coimmunoprecipitation experiments. Myc-CtBP1 specifically coprecipitated with GFP-BCoR-L1. Similarly, GFP-BCoR-L1 coprecipitated with Myc-CtBP1 (Fig. 5A). After the consensus CtBP-binding site in BCoR-L1 was mutated from PLDLS (WT-BCoR-L1) to PLASS (ΔCtBP-BCoR-L1), ΔCtBP-BCoR-L1 was no longer capable of interacting with CtBP in reciprocal coimmunoprecipitation experiments, demonstrating that BCoR-L1 recruits CtBP via a classical CtBP-binding motif. In addition, mutation of the CtBP consensus binding motif in full-length Gal4-DBD-BCoR-L1 (from PLDLS to PLASS) significantly impaired repression at the Gal4-responsive promoter in a dose-dependent manner (Fig. 5B). Our results indicate that recruitment of CtBP is one mechanism employed by BCoR-L1 to achieve its repression.

BCoR-L1 Associates with Class II HDACs—The mechanism most commonly employed by repressors involves the recruitment of HDACs that remove acetyl groups from the terminal tails of histones. To investigate whether BCoR-L1 is associated with specific HDACs, we coexpressed GFP-BCoR-L1 and the FLAG-tagged mammalian expression constructs encoding the human Class I and Class II HDACs (HDAC1 to -7). Apart from HDAC6, which was exclusively cytoplasmic, as has been previously reported (40), each HDAC was observed in the nucleus, with various levels also present in the cytoplasm. GFP-BCoR-L1 coprecipitated with Myc-CtBP1 (Fig. 5B), indicating that BCoR-L1 recruits CtBP via a classical CtBP-binding site in BCoR-L1 was mutated from PXLDS to PLASS, and its effect on repressor function was compared with that of wild-type Fragment 2. The mutated fragment was no longer capable of efficient repression (Fig. 4C).

BCoR-L1 Resides on the E-cadherin Promoter—E-cadherin is a well characterized target of CtBP in vivo. CtBP associates
with the E-cadherin promoter to promote its repression, and accordingly, down-regulation of CtBP results in derepression of E-cadherin transcription in cells that do not normally express E-cadherin (15, 18, 22, 41). We performed chromatin immunoprecipitation assays to evaluate whether BCoR-L1 associates with the same region of the E-cadherin promoter as CtBP. As previously shown, Myc-CtBP physically associated with the endogenous E-cadherin promoter. GFP-BCoR-L1 also associated with the E-cadherin promoter, indicating that BCoR-L1 and CtBP share E-cadherin as a common target (Fig. 7A). Next, we used an E-cadherin promoter luciferase reporter gene (E-Cad-Luc) to determine if BCoR-L1 was able to repress transcription from the E-cadherin promoter. 293T cells were cotransfected with E-Cad-Luc and either WT-BCoR-L1 or ΔCtBP-BCoR-L1, and luciferase activity was measured. WT-BCoR-L1 was able to significantly suppress luciferase activity driven by the E-Cad promoter. ΔCtBP-BCoR-L1 was not able to reduce E-Cad promoter activity to the same extent as WT-BCoR-L1, suggesting that CtBP is partially required for BCoR-L1-mediated repression of E-cadherin (Fig. 7B). Next, we used siRNA to reduce BCoR-L1 levels and tested whether the reduction of BCoR-L1 decreases the repression of E-cadherin transcription in E-Cad-negative U2OS cells. We used the pSUPER vector system to direct the synthesis of siRNAs to specifically knock down BcoR-L1 levels. We measured E-cadherin transcript levels by RT-PCR in U2OS cells transfected with pSUPER-BCoR-L1 (depleted of BCoR-L1) or pSUPER-GFP (as control). In these experiments, BCoR-L1 levels were reduced to ~20%, as determined by RT-PCR (data not shown). A reduction of expression of BCoR-L1 resulted in an ~2.5-fold increase in the transcript levels of E-cadherin (Fig. 7C). Taken together, these data indicate that BCoR-L1 is directly involved in the repression of E-cadherin, an authentic CtBP target gene.

**DISCUSSION**

BCoR-L1 fits the definition of a corepressor protein in that, although it lacks a DNA-binding domain, it has a portable repression domain and is capable of repression when recruited to promoters. It exhibits a number of similarities with BCoR. They are both large proteins and in fact only differ in size by 10 amino acids (BCoR is 1721 amino acids) containing ankyrin repeats of unknown function, although presumably they are protein-protein interaction modules (42). BCoR was identified as a corepressor of the BCL6 transcriptional repressor (24). Unlike BCoR, BCoR-L1 does not interact with BCL6 or poten-
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Inappropriate BCL6-mediated repression (data not shown), providing evidence that BCoR-L1 and BCoR may have distinct functions in human cells. However, BCoR is likely to have roles independent of BCL6, given its ubiquitous expression in tissues where BCL6 is not expressed. Furthermore, we found that BCoR-L1 is expressed at much higher levels in the hormone-responsive tissues, prostate and testis, than in other tissues.

Typically, corepressors recruit multiple cofactors involved in chromatin remodeling, histone deacetylation, or basal transcription to mediate concerted transcriptional silencing through multiple repression pathways. We have shown that BCoR-L1 achieves its repression in at least two ways, via an interaction with the CtBP corepressor and possibly via Class II HDACs. Elimination of the CtBP-binding site within BCoR-L1 partially relieves BCoR-L1-mediated transcriptional repression, demonstrating that BCoR-L1 repression is mediated through CtBP. Since CtBP has been shown to be regulated by fluctuating levels of NADH in the cell, this raises the possibility that BCoR-L1 is regulated similarly (20, 22, 23).

Recently, the BCoR complex was shown to contain Polycomb group (PcG) proteins (NSPC1, RING1, RNF2, and RYBP) and a JmJC domain histone H3 K36 demethylase, which is able to remove methyl groups from lysine (43). Another complex associated with CtBP is the LSD1-CoREST complex, capable of demethylating H3-K4 within nucleosomes (15, 44, 45). It will be interesting to address whether BCoR-L1 interacts with histone-demethylating enzymes or Polycomb group proteins in the same fashion as BCoR and CtBP.

Here, we have shown that BCoR-L1 is involved in the repression of E-cadherin, a known CtBP target. This repression partially requires CtBP. A number of transcriptional repressors are known to regulate E-cadherin expression, including Snail (46), Slug (47, 48), Twist (49), and ZEB/δEF1 (50, 51), and it is possible that BCoR-L1 might function together with these repressors, or as part of a separate as yet unknown complex. Furthermore, it might be of great value to determine if BCoR-L1 represses the expression of other CtBP-regulated genes and possibly those involved in promoting apoptosis (18, 19).

E-cadherin is critical to maintain normal epithelial cell contact (52), and down-regulation of E-cadherin is seen in a large percentage of carcinomas or borderline tumors (53, 54). Since BCoR-L1/CtBP represses E-cadherin, interfering with BCoR-L1/CtBP function might prevent loss of the epithelial state. This would be the reverse of the phenotype induced by pinin/DRS, which binds CtBP and relieves its repression of the E-cadherin promoter (55, 56). Interestingly, CtBP binding to the E-cadherin promoter is induced by elevations in free NADH. This redox-regulated repression of E-cadherin has been postulated to be involved in increasing tumor cell migration (41). It is conceivable, therefore, that interfering with CtBP and/or BCoR-L1 might inhibit tumor metastasis.

CtBP recruits Class II HDACs via consensus CtBP-binding motifs within their amino termini (30). However, CtBP is not required for the interaction between BCoR-L1 and Class II HDACs, since our ΔCtBP-BCoR-L1 was able to interact with the Class II HDACs. It is possible that the class II HDACs interact with the ankyrin repeats of BCoR-L1 directly, since they interact with the ankyrin repeat of certain proteins, ANKRA1 (ankyrin-repeat family A protein) and ANKRA2 (57, 58). BCoR-L1 is able to partially repress transcription independently of CtBP, suggesting a contribution from HDACs or some other mechanism. This observation also fits with the principle that generally, the contributions of all of the separate components of any repressive complex act in an additive fashion to achieve full repression.

Only a relatively small number of transcription factors are known to interact with Class II HDACs, although interactions between Class I HDACs and many transcription factors have been reported between Class I HDACs and many transcription factors. The colocalization and coprecipitation of BCoR-L1 with Class II HDACs strongly, although indirectly, suggest that BCoR-L1 function is linked to that of Class II HDACs. Most research has focused on the association between Class II HDACs and members of the MEF2 (myocyte enhancer factor-2) family of MADS-box transcription factors via their amino-terminal extensions, which results in transcriptional repression (59–61). The MEF2 family regulate genes involved in myogenesis and accordingly class II HDACs inhibit pathologic cardiac hypertrophy by modulating MEF2 transcription factor activity (62, 63). Class II HDACs are highly expressed in heart, skeletal muscle, and brain, in contrast to the Class I HDACs, which have a more ubiquitous expression (64). At present, we do not know the relative expression of BCoR-L1 in heart, muscle, and brain, since these tissues were not represented on our Northern blot. In the future, it will be of interest to elucidate if BCoR-L1 plays a role in myogenesis or generally in Class II HDAC function.

We have not been able to identify of the nature of the subnuclear foci containing BCoR-L1. Notably, BCoR-L1 and BCoR localize in distinct compartments, suggesting that they have divergent functions. Many proteins involved in transcriptional regulation localize to specific compartments within the nucleus. It is unknown whether these subnuclear foci are sites of active gene silencing or are required to recruit repressors away from sites of active transcription. Class II HDACs localize to distinct nuclear bodies within the cell nucleus, although as yet the function and biological significance of these dots is unknown (10). Corepressors, such as SMRT and NCoR, are concentrated in these nuclear foci, sometimes called deacetylase bodies, with the HDACs (65). One remarkable observation is the redistribution of BCoR-L1 to large HDAC5 “patches” by overexpression of HDAC5. Interestingly, expression of the corepressor, NCoR, leads to recruitment of HDAC5 into intranuclear bodies (65). The BCoR-L1-HDAC5 relocation works in the opposite direction; rather than BCoR-L1 recruiting HDAC5, our data demonstrate that HDAC5 is responsible for the relocalization of BCoR-L1. Another similar example is seen in the redistribution of the NCoR by the PIT-1 transcription factor (67).

It is possible that BCoR-L1 is a substrate of the HDACs, since there is evidence of nonhistone substrates, including p53 (68, 69) and α-tubulin (40). Acetylation of a lysine residue adjacent to the CtBP recruitment motif in E1A protein has been shown to modulate its interaction with CtBP (34). Interestingly, BCoR-L1 sequence also contains this flanking lysine residue, raising the possibility that its interaction with CtBP is similarly regulated by acetylation.
A Novel CtBP-interacting Corepressor

The importance of active transcriptional repression is highlighted by the fact that aberrant gene silencing is linked to a range of diseases, including developmental diseases and cancers (70, 71). Recently, mutations in BCoR have been linked with the developmental abnormality oculoauciardiodental syndrome (66). This developmental syndrome has been linked to two loci, MAA1 (Xq27) and MAA2 (Xp11, the BCoR locus). Intriguingly, mutations in BCoR were found in MAA2 families. Coincidentally, MAA1 maps extraordinarily close to the BCoR-L1 locus. There is thus the possibility that aberrant BCoR-L1 function may contribute to this disease.

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A Novel Corepressor, BCoR-L1, Represses Transcription through an Interaction with CtBP

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