Role of the PLDLS-Binding Cleft Region of CtBP1 in Recruitment of Core and Auxiliary Components of the Corepressor Complex[∇]

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C-terminal binding protein (CtBP) family proteins CtBP1 and CtBP2 are highly homologous transcriptional corepressors and are recruited by a large number of transcription factors to mediate sequence-specific transcriptional repression. In addition to DNA-binding repressors, the nuclear protein complex of CtBP1 consists of enzymatic constituents such as histone deacetylases (HDAC1/2), histone methyl transferases (HMTases; G9a and GLP), and the lysine-specific demethylase (LSD1). Additionally, CtBPs also recruit the components of the sumoylation machinery. The CtBPs contain two different unique structural elements, a hydrophobic cleft, with which factors that contain motifs related to the E1A PLDLS motif bind, and a surface groove that binds with factors containing motifs related to the sequence RRTGXPPXL (RRT motif). By structure-based functional dissection of CtBP1, we show that the PLDLS-binding cleft region functions as the primary recruitment center for DNA-binding factors and for the core and auxiliary enzymatic constituents of the CtBP1 corepressor complex. We identify HDAC1/2, CoREST/LSD1, and Ubc9 (E2) as the core constituents of the CtBP1 complex, and these components interact with the PLDLS cleft region through non-PLDLS interactions. Among the CtBP core constituents, HDACs contribute predominantly to the repression activity of CtBP1. The auxiliary components include an HMTase complex (G9a/Wiz/CDYL) and two SUMO E3 ligases, HPC2 and PIAS1. The interaction of auxiliary components with CtBP1 is excluded by PLDLS (E1A)-mediated interactions. Although monomeric CtBP1 is proficient in the recruiting of both core and auxiliary components, NAD(H)-dependent dimerization is required for transcriptional repression. We also provide evidence that CtBP1 functions as a platform for sumoylation of cofactors.

The C-terminal binding proteins (CtBPs) are highly conserved in animals and function as transcriptional corepressors and modulate the expression of genes that control development, oncogenesis, and apoptosis (4). The vertebrate genomes contain two *CtBP* genes that code for two highly homologous proteins (CtBP1 and CtBP2), while invertebrate genomes contain a single *CtBP* locus. The two mammalian *CtBP* genes exhibit redundant and unique functions during animal development (20). Although CtBPs predominantly function in transcriptional repression, both vertebrate CtBP (20) and *Drosophila melanogaster* CtBP (dCtBP) (8) have been reported to function as transcriptional activators in certain contexts. Although CtBPs share striking amino acid (47) and structural (27, 38) homologies to D-isomer-specific 2-hydroxy acid dehydrogenases (D2-HDH), it appears that the primary function of the HDH fold is to bind to NAD(H) dinucleotides and to facilitate the dimerization of CtBP.

More than 30 different transcription factors have been reported to recruit CtBPs to mediate the transcriptional repression of various target genes (5, 59). Most of these factors

interact with CtBPs through binding motifs that closely resemble the adenovirus E1A CtBP-binding motif, PLDLS (47). A few factors that do not contain obvious PLDLS-like motifs also have been reported to interact with CtBP to mediate transcriptional repression (36). Certain factors interact with CtBP through the PLDLS-like motifs as well as through a second redundant motif known as the RRT motif (42).

A proteomic analysis of the CtBP1 nuclear protein complex has revealed the presence of about two dozen CtBP cofactors (50). This complex contained sequence-specific DNA-binding repressors such as ZEB1/2 (41, 58), RREB-1 (57), and Znf217 (6). The CtBP1 complex also contained enzymatic constituents that catalyze three different modifications on histones. These enzymes included class I histone deacetylases (HDACs) (HDAC1/2), histone lysine methyl transferases (HMTases; G9a and GLP) (56), and a histone lysine-specific demethylase (LSD1) (49). The mode of recruitment of these key enzymatic constituents by CtBP is not known. Additionally, the CtBP1 complex also contained certain corepressors, such as CoREST (1) and LCoR (9). The possibility that these corepressors link the enzymatic constituents to CtBP is unresolved.

Two different structural elements of CtBP that serve as cofactor recruiting centers have been identified. The first is a hydrophobic cleft formed by the N-terminal region (amino acids [aa] 27 to 121 in the CtBP1 long isoform [CtBP1-L]), which is part of the substrate-binding domain (27, 32, 38). A C-terminal β strand (aa 327 to 352) also constitutes part of the substrate-binding domain and might contribute to the protein

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interaction with the N-terminal cleft region. Peptides modeled after the E1A PLDLS motif have been shown to interact with the N-terminal cleft (37, 38). It is widely believed that the primary function of the PLDLS cleft is to link CtBP with DNA-binding factors. The second is a surface groove (located on the dinucleotide-binding domain) with which the RRT-motif-containing proteins interact (43). The RRT binding groove is functionally redundant with the PLDLS cleft, and all RRT-motif-containing proteins also contain the PLDLS-like motifs. In addition to these two protein-binding sites, CtBPs also contain other potential protein interaction sites. CtBP1 contains a PDZ-binding domain (44) and a target site for SUMO modification (25, 30) in the C-terminal unstructured region (39). In certain transcription factors, the SUMO moiety has been shown to interact with HDACs (reviewed in references 12 and 19) and LSD1 (46). Since CtBPs form dimers, each of the CtBP cofactor would have two opportunities to bind with a CtBP dimer. Thus, the precise mode of interaction of various DNA-binding transcription factors and enzymatic cofactors with CtBP to mediate sequence-specific transcriptional repression is not known.

Here, we have used a high-density structure-based mutational analysis of CtBP1 and a CtBP1-E1A chimeric construct to elucidate the regulation of cofactor recruitment by CtBP1 and identify the core constituents that contribute to the transcriptional activity of CtBP1. We also provide evidence that CtBP1 functions as a platform for sumoylation of certain cofactors.

MATERIALS AND METHODS

Plasmids and mutants. Different versions of CtBP1 expression plasmids (pcDNA3-CtBP1, pCMV-Flag-CtBP1, and pcDNA3-T7-CtBP1) have been described previously (47, 55). Various base substitution mutations in CtBP1 were introduced using a commercial site-directed mutagenesis kit (QuikChange; Stratagene). Chimeric gene constructs were constructed by PCR-based approaches. Plasmids expressing Gal4-CtBP1 (35), Flag-Wiz (60), Flag-BCH110 (LSD1), dominant-negative LSD1 (DN-LSD1) (29), wild type G9a (wt-G9a), DN-G9a (17), wt-ZEB1 (41), Flag-HDAC2, glutathione *S*-transferase-HDAC2 (GST-HDAC2) (64), Znf217 (wt and Δ DLARRT) (42), and T7-Ubc9 (25) were gifts from various investigators.

Reporter assays. For luciferase reporter assays, MEF90 (*Ctbp*^{-/-}) cells were transfected with 0.2 μ g of reporter plasmid, 0.05 μ g of the promoterless phRL-0 (internal control), and 0.5 μ g of CtBP plasmids using the JetPEI reagent (Polyplus transfection) in 12-well plates in triplicates for 48 h. Cells were lysed, and dual-luciferase assays were performed with the dual-luciferase assay kit (Promega). For pE-Cad-Luc and pG5- κ B-MBP-Luc reporter assays, luciferase activity was normalized to that of the *Renilla* luciferase.

Cell lysis, immunoprecipitation, and Western blotting. To examine the CtBP1 protein complexes, HeLa or COS7 cells were transfected with various mutants and chimeric constructs using the Lipofectamine reagent in 100-mm dishes. The cells were collected and lysed by following previously described conditions (69). The cell lysates were precleared with protein A-agarose and then bound to the Flag-agarose antibody (Ab) beads (Sigma). The bound proteins were eluted with 2 $\times$ sodium dodecyl sulfate sample loading buffer without reducing agent and then combined with 0.1 M dithiothreitol before gel electrophoresis on a 4 to 15% polyacrylamide gel. The following commercial Abs were used for Western blotting: G9a (no. 07-551; Upstate Biotech), ZEB1 (no. SC-25388; Santa Cruz Biotechnology), CoREST (no. 612146; BD Biosciences), HDAC2 (no. SC-9959; Santa Cruz Biotechnology), HPC2 (no. SC-19299; Santa Cruz Biotechnology), Ubc9 (no. 10748; BD Biosciences), PIAS1 (no. SC 8152; Santa Cruz Biotechnology), CDYL (no. ab5188; AbCam), and RREB1 (no. 200-401-909; Rockland). The monoclonal Abs (MAbs) for the Flag (M2) and Myc epitopes were purchased from Sigma and AbCam, respectively. The Abs to LSD1 (49), Znf217 (6), and Wiz (60) were gifts from various investigators. A MAb specific for the C-terminal region (no. 612042) was purchased from BD Biosciences.

Subcellular localization of CtBP1. MEF90 cells cultured on coverslips were transfected with the indicated plasmids using the JetPEI reagent. Forty-two hours after transfection, cells were fixed with 3.7% formaldehyde-phosphate-buffered saline (PBS) for 10 min at room temperature, followed by permeabilization using methanol for 6 min at -20°C . Cells then were treated with 1% bovine serum albumin-PBS and incubated with CtBP1 MAb (1:50 dilution) for 90 min at 37°C , washed, and incubated with secondary anti-mouse horseradish peroxidase Ab (Santa Cruz Biotechnology) (1:2,500 dilution) for 60 min, followed by 10 min of incubation with fluorescein tyramide (1:50; TSA Fluorescence Systems; Perkin-Elmer Life Science Inc.). The cells were counterstained with propidium iodide and mounted on the Vectashield mounting medium (Vector Laboratories), and cells were visualized by using a Bio-Rad 1024 confocal scanning microscope. Images were merged using ImageJ software (NIH).

RESULTS

Functional analysis of CtBP1 mutants. To establish a link between the interaction of various cofactors with CtBP and transcriptional repression, we constructed a library of CtBP1-L mutants based on the structures of CtBP1 and CtBP1 complexed with a model peptide (PIDSKK) (27, 32, 38, 42). Specifically, several of these mutants were designed to obliterate the PLDLS-binding cleft, RRT-binding surface groove, NAD(H)-binding motif, the dimerization interface, and the catalytic residues for D2-HDH activity (Fig. 1A). Additionally, other sites that potentially could serve as direct factor-binding sites, such as the PDZ-binding domain and the sumoylation site as well as the phosphorylation sites, were mutagenized. Multiple mutants of major structural elements such as the PLDLS-binding cleft, the RRT-binding groove, and the dimerization interface were constructed, since they involve extensive sequences. Several mutants previously have been validated for targeted structural perturbations (Table 1). All mutants used here expressed stable mutant proteins as determined by Western blot analysis (not shown).

The transcriptional repression activity of various mutants was determined using a luciferase reporter (50) expressed from an established CtBP target (E-cadherin [E-Cad]) promoter (15, 16) in CtBP1/2 double-knockout mouse embryo fibroblasts (MEFs) (20). This analysis (Fig. 1B) identified three major determinants for the CtBP1-mediated repression of the E-Cad promoter: the PLDLS-binding cleft, the NAD(H)-binding motif, and the dimerization interface. Mutants in most other structural elements did not significantly impair the repression activity. It should be noted that mutants in the RRT-binding groove, which is redundant with the PLDLS cleft for the interaction of certain factors (42), did not impair the repression activity. Although a mutation of the sumoylation target site (K428A) previously has been reported to impair the CtBP1 activity due to defects in nuclear accumulation (25, 30), we did not find significant defects in subcellular localization (not shown) or in repression activity (Fig. 1B). Our results from the use of this mutant also rule out the potential role of the SUMO moiety in the recruitment of repression effectors such as class I and class II HDACs (12, 19) or LSD1 (46). Similarly, deletion of the entire C-terminal 86-aa (mutant D354stp) unstructured region (39) that includes the PDZ-binding domain also did not affect the repression function.

Interaction of cofactors with CtBP1 mutants. To associate transcriptional repression with various cofactors, we chose to determine the pattern of interaction of CtBP cofactors with selected functionally deficient mutants (Fig. 2A). Immunoflu-

FIG. 1. Transcriptional repression activities of CtBP1 mutants. (A) Linear domain structure map of CtBP1. The indicated coordinates correspond to positions on CtBP1-L. The splice variant, CtBP1-S, also is indicated. S369 indicates an extra Ser residue present in some splice variants of CtBP1-S. An insertion of the corresponding residue in CtBP1-L (ins380S) is analyzed in panel B. The core D2-HDH domain is indicated. (B) Relative repression activity of CtBP1 mutants. *Ctbp1/2* double-knockout cells were cotransfected with the reporter E-Cad-Luc and CtBP1, and the luciferase activity was determined 48 h after transfection.

orescence analysis revealed that three of the selected mutants (A52E, G183A/G186A, and R141A/R163L) were deficient in proper nuclear localization (Fig. 2B). To determine the patterns of interaction of various nuclear cofactors, all functionally deficient mutants were targeted to the nucleus by tagging them with the simian virus 40 nuclear localization signal (NLS) at the C terminus. As expected, all NLS-tagged mutants were localized in the nucleus (Fig. 2B, bottom panel). Additionally, we also used chimeric constructs that expressed CtBP1-E1A C-terminal (Cter) fusion proteins with the canonical PLDLS

motif or a mutant (PLDL→ASAS) motif. We postulated that the E1A sequences linked to the unstructured C terminus of CtBP1 would occupy the PLDLS-binding cleft of CtBP1 and hence would facilitate the identification of cofactors that specifically recognize the cleft region. Since the E1A C-terminal region contains an autonomous NLS (33), the CtBP1-E1A fusion proteins also were found to be localized in the nucleus (Fig. 2B). As CtBP1 mutants in the NAD(H)-binding motif (G183A/G186A) and in the dimerization interface (R141A/R163L) would be expected to be deficient in dimerization (27), we examined the dimerization properties of the Flag- and NLS-tagged versions of these mutants with T7-tagged wt CtBP1 by coimmunoprecipitation analysis (Fig. 2C). Both mutants were deficient in dimerization with wt CtBP1, while efficient dimerization was observed with a different mutant (V66R) or with the wt CtBP1 NLS (Fig. 2C).

In general, in the E-Cad luciferase assays the transcriptional repression activities of the NLS-tagged versions (Fig. 2A, insert) were comparable to those observed with non-NLS versions (Fig. 1B). The PLDLS cleft-occupied construct (CtBP1/E1ACter) was deficient in repression, while the CtBP1-Cter ΔPLDL mutant repressed in a manner similar to that of the wt CtBP1 NLS. The results from the analysis of amino acid substitution mutants and the cleft-occupied constructs reinforce the critical requirement of factors that are recruited by CtBP1 via the PLDLS-binding cleft. A point of interest was the observation that NLS-tagged versions of monomeric forms of

TABLE 1. Previously validated CtBP1 mutations

Mutation(s)	Function affected	Reference(s)
A52E, V66R	PLDLS binding	38
G183A ^a	NAD(H) binding	38, 66
E175A/D231A ^b	RRT binding	42
S158A	Phosphorylation by Pak1	3
H315Q	Catalysis	2
S422A	Phosphorylation by Hipk2 and JNK1	62, 68
K428A	Sumoylation	25, 30
R141A, R142A, R163A, R171A	Dimerization	27
D438A	PDZ binding	45

^a Although mutant G183A was reported to be deficient in NAD(H) binding, it may have partial activity.

^b Corresponding to CtBP2-L.

FIG. 2. Transcriptional activities of nuclear-targeted CtBP1 mutants and CtBP1-E1A chimeras. (A) Repression activity. The top is a diagram showing selected mutants in the PLDLS-binding cleft, RRT groove, NAD(H)-binding motif, and dimerization interface that are tagged with the simian virus 40 NLS (SV40-NLS). The CtBP1-E1A (Cter) chimeric construct is shown below. A mutation in the E1A PLDLS motif (Δ PLDL) also

CtBP1 mutants (G183A/G186A and R141A/R163L) showed detectable repression activity, albeit lower than that of wt CtBP1.

Core and auxiliary components of CtBP1 complex. To identify the core and auxiliary components of the CtBP1 corepressor complex, we determined the patterns of interaction of various cofactors with wt CtBP1 and several selected mutants. Here, we define the core components as chromatin-modifying enzymatic/adaptor components associated with CtBP1 when the PLDLS-binding cleft is occupied by the prototypical PLDLS motif factor E1A. The auxiliary constituents are defined as enzymatic/adaptor constituents that can be displaced by the PLDLS(E1A)-mediated interaction. HeLa cells were transfected with Flag-tagged versions of wt CtBP1 or various mutants, and cell lysates were immunoprecipitated with the Flag Ab and were analyzed by Western blotting. The blots were probed with Abs specific to 12 different proteins (Fig. 3A and B). Nine of these proteins previously have been reported to be the constituents of a nuclear protein complex of CtBP1 (50). Ubc9 has been reported to interact with CtBP1 by coimmunoprecipitation analysis of cells transfected with plasmids expressing these proteins (25) and yeast two-hybrid interaction screens with the CtBP2 bait (42). Our analysis included PIAS1 on the basis of expected functional relevance and the presence of a potential PLDLS-like motif in PIAS1. PIAS1 also has been reported to function as an E3 ligase for sumoylation of CtBP1 *in vitro* (30). Wiz was included, since it was reported to be a link between G9a and CtBP (60). The interaction of several key cofactors with CtBP1 also was observed when the endogenous CtBP1 was immunoprecipitated with a MAb specific to the C-terminal region of CtBP1 (not shown).

We first analyzed the interaction of different cofactors with mutants in the two protein-binding sites (i.e., the PLDLS-binding cleft and RRT-binding groove) of CtBP1 (Fig. 3A). Three mutants within the PLDLS-binding cleft [A52E, DGRD34-37(A)4, and V66R] were deficient in interaction with a number of cofactors, suggesting that they interact with the cleft region directly or indirectly. In contrast, the interaction of mutants A52E and DGRD34-37(A)4 with Ubc9 was enhanced, while the interaction of Ubc9 with V66R was not evident, suggesting that Ubc9 also interacted with the cleft region. The interaction of factors such as ZEB1, RREB-1, CoREST, LSD1, and HPC2 was not significantly affected for the mutant in the RRT-binding groove (E175A/D231A), suggesting that the RRT groove is not important for the recruitment of these factors. In contrast, the interactions of G9a/Wiz, CDYL, and Znf217 with the groove mutant were diminished, suggesting that these factors interact with the RRT-binding groove in addition to the PLDLS cleft (see Fig. 4).

Since multiple factors were found to be associated with the PLDLS cleft, we employed a CtBP1-E1A (Cter) chimeric construct to identify factors that interacted via the prototypical PLDLS contact sites. Additional mutants to define the PLDLS cleft also were employed. Since the C-terminal region of CtBP1 (aa 327 to 354) forms part of the substrate-binding domain that includes the PLDLS cleft, a truncation mutant that lacked the C-terminal region (mutant M327stp) as well as mutants at the N-terminal boundary region of the cleft (L29A and Δ 30-33) also were used. Further, the role of CtBP1 dimerization on factor recruitment was investigated using monomeric (NLS-tagged) mutants (G183A/G186A and R141A/R163L) (Fig. 3B). The immunoprecipitation and Western blot analyses indicated that all CtBP cofactors, including Znf217 (which contains both PLDLS-like and RRT motifs), interacted primarily through the PLDLS-binding cleft. Importantly, the cleft occupation by C-terminal E1A did not displace HDAC2, CoREST, LSD1, and Ubc9, suggesting that these factors constitute the core constituents of the CtBP corepressor complex and could mediate the transcriptional activities of CtBP when anchored to the promoter by DNA-binding factors that recruit CtBP through PLDLS-like motifs. Although cleft occupation by E1A did not displace the core constituents, the result that the cleft mutant V66R (Fig. 3A) did not interact with them suggests that this mutation affects non-PLDLS interactions at or near the cleft region due to the more drastic nature (V→R) of the mutation.

The results shown in Fig. 3B also revealed that the monomeric forms of CtBP1 (mutants G183A/G186A and R141A/R163L) interacted with cofactors at relatively high levels compared to those of wt CtBP1 (middle two lanes). These results suggest that dimerization is not critical for recruiting factors via the PLDLS cleft. Thus, results based on the analyses of several CtBP1 mutants also suggest that HDACs (HDAC1/2), CoREST/LSD1, and Ubc9 are the core constituents of the CtBP1 corepressor complex. Coimmunoprecipitation analysis of cells transfected with increasing concentrations of Myc-HDAC2, Myc-CoREST, and Flag-LSD1 with CtBP1 indicated a dose-dependent interaction of HDAC2 and CoREST, suggesting a more direct interaction of these proteins with CtBP1 (Fig. 3C). Pull-down assays using bacterially expressed GST-CoREST and GST-HDAC2 with bacterially expressed CtBP1 suggest that CtBP1 directly interacts with HDAC2 as well as CoREST (Fig. 3D). In contrast, LSD1 did not interact with GST-CtBP1, suggesting that it is recruited through other factors, such as CoREST (29, 51).

In addition to the core components, other enzymatic and adaptor components also may contribute to the overall transcriptional activity of CtBP. Among such constituents, the most prominent is the HMTase G9a. The results presented in Fig. 3A clearly indicated that the interaction of G9a with CtBP1

is shown. The repression activities of various constructs on the E-Cad-Luc reporter are shown in the boxed area. (B) Subcellular localization of CtBP1 mutants. Mutants that were excluded from proper nuclear localization and the NLS-tagged versions are shown. Only the merged images of NLS-tagged mutants are shown. The propidium-iodide-stained images and CtBP1 Ab-stained images are not shown. The enlarged images of the monomeric mutant (R141A/R163L) are shown in the lower right corner. (C) Dimerization of CtBP1 and mutants. Flag (Fg)- and NLS-tagged CtBP1 mutants were cotransfected with T7-tagged wt CtBP1 and immunoprecipitated (IP) with the Flag Ab, and the Western blot was probed with the T7 Ab. The Western blots of lysates probed with the T7 Ab or Flag Ab are shown at the bottom.

FIG. 3. Protein complexes associated with CtBP1 mutants. (A and B) HeLa cells were transfected with Flag-CtBP1 (Fg-CtBP1) or mutants and immunoprecipitated (IP) with the Flag Ab, and the Western blots (W) were probed with the indicated Abs. Flag-CtBPs were detected by chemiluminescence. (C) The interaction of core constituents with CtBP1. HeLa cells were transfected with Flag-CtBP1 or T7-CtBP1 and increasing concentrations (1, 5, and 10 μ g) of Myc-HDAC2, Myc-CoREST, or Flag-LSD1, immunoprecipitated, and analyzed by Western blotting with the indicated Abs. (D) GST pull-down assays. Bacterially expressed His(6)-CtBP1 was incubated with GST, GST-HDAC2, or GST-CoREST, and the bound fractions were analyzed by Western blotting using CtBP1 Ab. Baculovirus-expressed Flag-LSD1 was incubated with GST or GST-CtBP1, and the bound and unbound fractions were analyzed by Western blotting using the Flag Ab.

was impaired by mutations to both the PLDLS cleft and the RRT groove. The interaction pattern of G9a was similar to that of Znf217, a known cleft- and groove-binding protein (42). In contrast, the interaction of the SUMO E3 ligase HPC2 was impaired only by the cleft mutation. Coimmunoprecipitation analysis of cells transfected with the PIAS1 expression vector and various cleft mutants also revealed that PIAS1 interacted with the cleft (not shown). It should be noted that PIAS1 contains a prototypical PLDLS motif (PADLS). Unlike other cleft-interacting proteins such as ZEB1 and RREB-1 (Fig. 3B),

the interaction of both E3 ligases was enhanced by the deletion of the C-terminal β strand of the substrate-binding domain (mutant M327stp). These results suggest that, in addition to the N-terminal PLDLS cleft region of the substrate-binding domain, the C-terminal region also contributes to the recruitment of some, but not all, PLDLS motif factors.

Recruitment of Znf217 and WIZ complexes. Znf217 has been reported to interact with CtBP1/2 through both PLDLS-like and RRT motifs (42). The pattern of the interaction of Znf217 with CtBP1 mutants (Fig. 3A) was consistent with that re-

FIG. 4. Interaction of Znf217 and Wiz complexes with CtBP1/2. (A) Interaction of the Znf217 complex. COS cells were transfected with Flag-wt Znf217 (Fg-wt Znf217) or a Flag-Znf217 Δ DL Δ RRT mutant, immunoprecipitated (IP), and analyzed by Western blotting (W) using the indicated Abs. (B) Interaction of CDYL and Wiz. HeLa cells were transfected with Flag-CDYL or Flag-Wiz. The respective immunoprecipitates were analyzed by Western blotting using the CDYL or Wiz Ab.

ported previously. Znf217 also has been reported to interact with the CoREST complex (CoREST/HDAC1/HDAC2/LSD1) (6). To determine whether CtBPs play a role in linking CoREST and associated factors with Znf217, we carried out a coimmunoprecipitation and Western blot analysis of cells transfected with wt Flag-Znf217 or a mutant (Flag-Znf217 Δ DL Δ RRT) (Fig. 4A). While wt Znf217 interacted well with CoREST/LSD1/HDAC2 and CtBP1/2, the mutant interacted well only with CoREST/LSD1/HDAC2 and not with CtBP1/2 (Fig. 4A). These results suggest that Znf217 interacts with CoREST/LSD1/HDACs independently of CtBP.

The results presented in Fig. 3A also indicated that the interactions of G9a, Wiz, and CDYL with mutants were impaired in the PLDLS cleft as well as in the RRT motif of CtBP1. Previously, Wiz was reported to link G9a with CtBPs through PLDLS-like motifs (60). Since the pattern of the interaction of CDYL paralleled the patterns of the interaction of G9a and Wiz in several different studies, we reasoned that CDYL might also be associated with Wiz. We carried out an immunoprecipitation and Western blot analysis of cells transfected with Flag-Wiz (Fig. 4B). This analysis detected the association of endogenous CDYL with Wiz. A reciprocal analysis using Flag-CDYL also revealed an interaction with endogenous Wiz. Thus, CDYL and G9a appear to be linked to CtBP via Wiz. Although Wiz has been reported to interact with CtBPs through two PLDLS-like motifs (60), it is possible that Wiz also interacts via an RRT-like motif as well, particularly when the PLDLS cleft is occupied (see Discussion). An inspection of the sequences of human and mouse Wiz suggest potential RRT motifs in these proteins.

Role of core enzymatic components in CtBP1 repression activity. The above results identified three enzymatic components, HDACs, LSD1, and Ubc9, as the core constituents of the CtBP1 complex. We then investigated the role of these constituents in the repression activity of CtBP1. Since auxiliary constituents might also contribute to the repression activity, we investigated the role of G9a. The role of class I HDACs was investigated using the inhibitor trichostatin A (TSA). The roles of other enzymatic constituents were investigated by transfection of vectors that express the catalytically active enzymes or the DN mutants. Treatment of CtBP-null MEF cells transfected with a pE-Cad-Luc reporter and CtBP1 with TSA relieved the repression activity significantly, albeit partially (Fig. 5A). Expression of wt G9a inhibited the basal E-Cad-Luc activity. Coexpression of CtBP1 and wt G9a had an additive repression activity, while that of DN-G9a (17) did not (Fig. 5B). It is possible that the enhanced repression activities seen in cells that coexpressed G9a and CtBP1 are the result of independent effects of CtBP1 and G9a. Expression of wt LSD1 resulted in an appreciable activation of the E-Cad promoter (Fig. 5C). Coexpression of wt LSD1 and CtBP1 reduced the level of overall repression, while that of DN-LSD1 (29) did not affect the level of CtBP1-mediated repression, suggesting that LSD1 does not significantly influence the activity of CtBP1 in the transient assay conditions. Similarly, expression of Ubc9 resulted in a modest enhancement of E-Cad promoter activity (Fig. 5D). Coexpression of either catalytically active or inactive DN-Ubc9 (13) did not have a significant effect on CtBP1-mediated repression. From these results, we conclude that among the enzymatic constituents associated with CtBP1, HDACs contribute more significantly to the repression activity under the assay conditions and the promoter contexts used here. However, these results do not rule out the role of enzymatic constituents such as Ubc9 in modulating the assembly and dissociation of individual components of the CtBP1 corepressor complex (see below).

Role of CtBP1 in cofactor sumoylation and function. Although the above transient transfection studies did not reveal a significant effect of ectopic overexpression of Ubc9 on CtBP1-mediated repression of the E-Cad promoter, it is possible that CtBP1 serves as a platform for SUMO modification of cofactors. Such modifications may influence the dynamics of cofactor association depending on the promoter context. Since a previous study (31) reported the sumoylation of ZEB1/2 proteins, we examined the effect of CtBP1 on SUMO modification of ZEB1 (Fig. 6). For these studies, we used a method known as Ubc9-fusion-directed sumoylation (23). HeLa cells were transfected with a Flag-tagged CtBP1-Ubc9 fusion construct with or without plasmids expressing hemagglutinin-SUMO1 (HA-SUMO1) or HA-SUMO2. The CtBP1-Ubc9 protein complex was immunoprecipitated from transfected HeLa cells and examined for the presence of CtBP1-associated factors (ZEB1 and LSD1) by Western blot analysis (Fig. 6). As seen in lane 1, ZEB1 associated with CtBP1-Ubc9 fusion protein exhibited slower mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ZEB1 from cells transfected with HA-SUMO1 (lane 2) or HA-SUMO2 (lane 4) had a higher quantity of the slow-mobility ZEB1 species, suggesting that they represent sumoylated forms of ZEB1. As expected, a mutation within the PLDLS cleft (A52E) of the CtBP1 moiety of the

FIG. 5. Effect of core enzymatic components on CtBP1 repression activity. (A) Effect of class I HDACs. MEF90 cells were transfected with plasmids expressing the E-Cad reporter and CtBP1 and treated with 0.1 μ g of TSA 24 h after transfection, and the luciferase activity was determined 24 h later. The luciferase activity for both vector and vector plus TSA was normalized to 1.0. (B) Effect of G9a. The reporter assays were carried out in the presence of wt G9a or DN-G9a (N903L/H904E). (C) Reporter assays were carried out in the presence of wt LSD1 or DN-LSD1 (K661A). (D) Reporter assays were carried out in the presence of wt Ubc9 or DN-Ubc9(C93S).

fusion protein dramatically reduced the levels of sumoylated ZEB1 in the presence of SUMO1 (lane 3) or SUMO2 (lane 5). Exposure of the Western blot for a shorter time revealed that two major bands corresponded to sumoylated ZEB1. This is consistent with an earlier report that ZEB1 was sumoylated at two different sites (31). The sumoylation of ZEB1 by CtBP1-Ubc9 fusion protein also was detectable in the crude lysate (lanes 1, 2, and 4), whereas cell lysates from CtBP1(A52E)-Ubc9-transfected cells (lanes 3 and 5) or from the untransfected control (lane 6) did not contain significant amounts of sumoylated ZEB1. Western blot analysis of LSD1 suggested that CtBP1-Ubc9 fusion protein did not result in the sumoylation of LSD1 (panels 3 and 4 from the top). These results suggest that CtBP serves as a platform for sumoylation of certain interacting cofactors, such as ZEB1.

Among the CtBP1 mutants that we have analyzed, mutant DGRD34-37(A)4 was interesting, since it interacted with en-

dogenous Ubc9 more proficiently than other cleft mutants. This mutant also interacted with PIAS1 in cotransfection experiments and with endogenous PIAS1 (not shown). In certain experiments, we observed an unstable association of a higher-molecular-mass form of HDAC2 (~80 kDa) with the mutant. We reasoned that the efficient interaction of the SUMO E2 (Ubc9) and an E3 ligase (PIAS1) with the mutant might enhance SUMO modification of CtBP1 (which share a conserved sumoylation target site [7]), resulting in an unstable association with HDAC1/2. We rationalized that mutant DGRD34-37(A)4 would be suited to investigate the potential effect of sumoylation on CtBP1-mediated transcriptional repression. Since this mutant was found to be defective in binding to DNA-binding factors such as ZEB and RREB-1, we used a Gal4-DNA-binding domain chimeric construct. Cotransfection of Gal4-CtBP1 (35) with an MLP-based reporter (G5- κ B-MLP-Luc) in MEF90 cells resulted in the efficient repression of reporter

FIG. 6. Effect of CtBP1 on sumoylation of ZEB1. HeLa cells were transfected with Flag (Fg)-CtBP1-Ubc9 (lane 1) or together with HA-SUMO1 (lane 2) or HA-SUMO2 (lane 4). As controls, Flag-CtBP1(A52E)-Ubc9 was cotransfected with HA-SUMO1 (lane 3) or HA-SUMO2 (lane 5). Cell lysates were prepared (in the presence of 30 mM of *N*-ethylmaleimide), immunoprecipitated (IP) with the Flag Ab, and examined by Western blotting with the following Abs: panels 1 and 2, ZEB1 Ab; panels 3 and 4, LSD1 Ab; panel 5, Flag Ab.

expression (Fig. 7). Interestingly, Gal4-DGRD34-37(A)4 was more efficient in repression than Gal4-wt CtBP1. The repression mediated by the mutant was completely relieved by treatment with TSA (Fig. 7, right), suggesting that an HDAC primarily contributes to the repression activity. The repression activity of Gal4-DGRD34-37(A)4 was significantly relieved by cotransfection with Ubc9 (wt) compared to that of cotransfec-

tion with DN-Ubc9. These results suggest that Ubc9 regulates the dynamics of certain factor interactions with CtBP1, resulting in diminished transcriptional repression by the CtBP1 mutant.

DISCUSSION

An analysis of a large panel of structure-based CtBP1 mutants identified three critical structural elements: the PLDLS-binding cleft (part of the substrate-binding domain), the NAD(H)-binding motif, and the dimerization interface for transcriptional repression. Our results have suggested that the effect of NAD(H) binding and dimerization are related to their requirement of nuclear localization of CtBP1 rather than co-factor binding. Thus, the PLDLS-binding cleft region appears to be the most critical determinant that is directly related to transcriptional repression. This conclusion was strengthened by the analysis of the CtBP1-E1A chimeric construct that was designed to occupy the cleft in *cis*. Analyses of protein complexes associated with CtBP1 mutants and CtBP1-E1A chimeric constructs indicated that the hydrophobic cleft of CtBP1 serves as the center for the interaction of factors that possess PLDLS-like motifs as well as for key enzymatic constituents that associate with CtBP1 through non-PLDLS interactions. These enzymatic constituents include class I HDACs, the CoREST/LSD1 complex, and the SUMO E2 Ubc9. These factors remained associated with CtBP1 even though the PLDLS cleft was occupied by E1A in *cis*. Thus, our results have illuminated how the recruitment of CtBP1 by a prototypical PLDLS-containing transcription factor could target histone-modifying enzymes, such as HDAC1/2, to the chromatin. We have shown the efficient interaction of monomeric CtBP1 with factors that contain the PLDLS-like motifs as well as non-PLDLS factors. Previous studies have reported both positive (66, 67) and neg-

FIG. 7. Effect of Ubc9 on HDAC-dependent repression. MEF90 cells were cotransfected with Flag (Fg)-Gal4-CtBP1 chimeric constructs and wt Ubc9 or DN-Ubc9 along with the G5- κ B-MLP-Luc reporter, and the luciferase activity was determined. In the right panel, transfected cells were treated with TSA as described in the legend to Fig. 5, and the luciferase activity was determined.

ative (11, 36) effects of NAD(H) dinucleotide on factor recruitment and transcriptional activities of CtBP1/2. Our results have clearly indicated that the effect of NAD(H) dinucleotides on the transcriptional activity of CtBP1 might be indirect through dimerization and nuclear localization of CtBP1. Our results also have revealed the differential interaction of factors containing PLDLS-related motifs. For example, two SUMO E3 ligases, HPC2 and PIAS1 (Fig. 3B), interacted normally with the mutant M327stp, which lacked the C-terminal β strand of the substrate-binding domain, while the interaction of DNA-binding factors such as ZEB1/2 and RREB-1 was impaired. These results suggest that the interaction of different PLDLS motif factors with the PLDLS-binding cleft is regulated differently depending on the context.

Our results have suggested that the obliteration of the RRT groove did not significantly impair the repression activity of CtBP1 (Fig. 1B and 2A). A similar result was reported for CtBP2 (42). Our results have suggested that a key enzymatic constituent, G9a, implicated in transcriptional repression and in long-term chromatin silencing (56), and its adapter protein, Wiz (60), interact with CtBP1 through the PLDLS cleft and the RRT groove. However, mutations that affected only the PLDLS cleft as well as the occupation of the cleft by the E1A C terminus prevented the interaction of both Wiz and G9a. A similar result also was observed with Znf217 (Fig. 2A and B). These results suggest that the PLDLS cleft is the primary anchoring site with CtBP1 for cofactors that also carry the RRT motif, employing the two-site interaction mechanism. However, previous yeast two-hybrid studies have identified interaction proteins such as Hipk2 (65) and Znf217 (42) that contain both PLDLS and RRT motifs under conditions in which the PLDLS cleft was occupied. It is possible that the RRT-binding groove is recognized by the bi-motif factors only when the PLDLS cleft is occupied. In a previous study, we observed that the cotransfection of E1A 13S and CtBP2 did not significantly displace G9a from CtBP2 (69). Thus, it is possible that, during the interaction of certain sole PLDLS motif factors, the RRT groove is used as a hinge to retain bi-site-binding factors bound to the RRT groove. Such a mechanism could explain coordinated histone modification by deacetylation of histone H3-K9 by HDAC1/2 (see below) and methylation by a G9a/GLP heterodimer, as reported by Shi and colleagues (50). Such a scenario also could be operational during CtBP-mediated long-term silencing of chromosomal genes.

We have identified HDAC1/2, CoREST/LSD1, and Ubc9 as the core constituents of the CtBP1 complex. Since LSD1 did not interact with CtBP1 in cotransfection experiments and in GST pull-down assays, we believe that it was recruited to the CtBP1 complex through CoREST. The interaction between LSD1 and CoREST previously has been reported by others (29, 51). The association of HDAC1/2 with CoREST has been reported in several studies (18, 22, 63). Cotransfection studies have suggested a more direct association between HDAC1 and CoREST (28). Our results have suggested that HDACs and CoREST interact directly with CtBP1 through non-PLDLS-dependent interactions. Although cleft occupation by E1A did not interfere with the association of factors such as HDAC, CoREST, and Ubc9, certain cleft mutations (e.g., V66R) that affected PLDLS interactions also abolished the interaction of

the non-PLDLS factors. A previous two-hybrid analysis indicated that a deletion mutant within the N-terminal cleft region abolished the interaction of Ubc9 with CtBP2 (42). It remains to be determined whether the interaction between CtBP1 and Ubc9 is direct or indirect. The molecular basis of the interaction of non-PLDLS factors with the PLDLS-binding cleft region remains to be investigated in detail.

Our result showing that CoREST is a core constituent of the CtBP complex suggests that there might be a close functional link between the two corepressors. A recent transcriptional study has reported the derepression of several CoREST target genes in *Ctbp*^{-/-} MEFs (11). The results shown in Fig. 5 suggested that CtBP1-mediated transcriptional repression was dependent largely on HDACs. Although LSD1 was found to be one of the core components, we did not detect any appreciable effect on CtBP1-mediated repression. Instead, a modest *trans* activation was observed. LSD1 has been reported to form both repression and activation complexes, depending on the context (61). Since invertebrate and vertebrate CtBPs have been shown to activate transcription under certain contexts (8, 20), it is possible that LSD1 functions in an activation mode to regulate certain CtBP-responsive genes in specific contexts. We have observed that CtBP1-mediated repression was not relieved fully by treatment with TSA. This observation was in contrast to that for CtBP2, the repressive activity of which was fully relieved by TSA (not shown). It is possible that CtBP1, unlike CtBP2, uses additional HDAC-independent mechanisms (see below). Previous reports have suggested that CtBP1 antagonizes the activities of global *trans* activators such as p300/CBP and P/CAF (26, 34, 48).

We have shown that Ubc9 is a core component of the CtBP1 complex. Previous studies have shown that CtBP1 is a SUMO target (24, 30). Although these reports have shown that SUMO modification of CtBP1 was required for nuclear localization of CtBP1, we did not observe a major defect in transcriptional repression (Fig. 1B) or in subcellular localization by a CtBP1 mutant deficient in SUMO modification (not shown). The observation that Ubc9 was the core constituent and that at least two different SUMO E3 ligases (HPC2 and PIAS1) were recruited by CtBP1 suggest that the sumoylation machinery plays an important role in CtBP1 activity. Sumoylation of histone H4 has been linked to transcriptional repression (52). The potential role of CtBP1 in targeting the sumoylation machinery to the chromatin is an attractive possibility and remains to be explored. CtBP1 has been reported to antagonize the activity of positive transcription factors such as p300/CBP (26, 34, 48). Since sumoylation of p300 has been reported to reverse its transcriptional function (14), the possibility that CtBP1 inhibits the activity of p300/CBP through sumoylation also remains to be investigated.

Several CtBP-interacting proteins have been reported to be SUMO targets. These include DNA-binding repressors such as ZEB1/2 (31) and BKLf (40). Certain enzymes, such as Hipk2 (21) and HDAC1 (7), that are associated with CtBP1 have been reported to be SUMO targets. It is possible that the CtBP-associated SUMO E2 and E3 influence SUMO modification of these factors. A recent report suggested that the SUMO modification of ZEB1 enhanced its DNA-binding activity (61). Although SUMO modification of ZEB1/2 was reported to interfere with the interaction of these factors with

FIG. 8. CtBP1 corepressor complex. The CtBP1 dimer is depicted as interacting with a chromatin-anchoring transcriptional repressor through a PLDLS-dependent interaction with each PLDLS-binding cleft. The PLDLS-binding cleft also is depicted as recruiting HDAC1/2, CoREST/LSD1 complex, and Ubc9 through non-PLDLS-dependent interactions. The PLDLS-dependent interaction with CtBP1 is shown to displace the protein complexes associated with Wiz (G9a-GLP and CDYL) and Znf217 (CoREST/LSD1/HDAC1/2) that interact with the PLDLS cleft and RRT groove. Although two E3 ligases (HPC2 and PIAS1) associate with CtBP1 through PLDLS-related motifs, they are postulated to bind with the cleft in a manner different from that of other DNA-binding factors, such as ZEB and RREB-1 (indicated in gray). The mode of the interaction of Ubc9 with the CtBP1 cleft (direct or indirect) is unknown (indicated by the question mark).

CtBP (31), we have detected copious amounts of sumoylated ZEB1 in the CtBP1-Ubc9 complex. The result presented in Fig. 6 are consistent with a model in which CtBP1 serves as the platform for SUMO modification of certain associated cofactors to regulate interaction or functions.

In summary, we have demonstrated that the PLDLS-binding cleft region serves as the primary recruitment center for enzymatic constituents that mediate the modification of histones and nonhistone proteins. Our results provide an understanding of how the constituents of the CtBP1 corepressor complex might function to repress transcription by recruiting PLDLS-containing transcription factors and non-PLDLS factors (Fig. 8). Our observation that the CtBP1 transcriptional repression activity could be relieved *in cis* by a powerful viral PLDLS motif suggests a therapeutic strategy to inhibit the transcriptional activity of CtBPs. This is particularly important considering the accumulating evidence that suggests that CtBPs play a crucial role in tumor progression by controlling the epithelial-mesenchymal transition (reviewed in reference 10). Our observation that the interaction of ZEB with CtBP1 could be strongly inhibited by the E1A C terminus may be useful in reversing the ZEB activity in many high-grade tumors in which the expression of ZEB proteins is activated (53, 54).

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REFERENCES

- Andrés, M. E., C. Burger, M. J. Peral-Rubio, E. Battaglioli, M. E. Anderson, J. Grimes, J. Dallman, N. Ballas, and G. Mandel. 1999. CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc. Natl. Acad. Sci. USA* **96**:9873–9878.
- Balasubramanian, P., L. J. Zhao, and G. Chinnadurai. 2003. Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. *FEBS Lett.* **537**:157–160.
- Barnes, C. J., R. K. Vadlamudi, S. K. Mishra, R. H. Jacobson, F. Li, and R. Kumar. 2003. Functional inactivation of a transcriptional corepressor by a signaling kinase. *Nat. Struct. Biol.* **10**:622–628.
- Chinnadurai, G. 2006. CtBP family proteins: unique transcriptional regulators in the nucleus with diverse cytosolic functions, p. 1–17. *In* G. Chinnadurai (ed.), CtBP family proteins. Landes Bioscience and Springer Science-Business Media, New York, NY.
- Chinnadurai, G. 2002. CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell* **9**:213–224.

6. Cowger, J. J., Q. Zhao, M. Iovic, and J. Torchia. 2007. Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: identification of a ZNF217 consensus recognition sequence. *Oncogene* **26**: 3378–3386.
7. David, G., M. A. Neptune, and R. A. DePinho. 2002. SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. *J. Biol. Chem.* **277**:23658–23663.
8. Fang, M., J. Li, T. Blauwkamp, C. Bhambhani, N. Campbell, and K. M. Cadigan. 2006. C-terminal-binding protein directly activates and represses Wnt transcriptional targets in *Drosophila*. *EMBO J.* **25**:2735–2745.
9. Fernandes, I., Y. Bastien, T. Wai, K. Nygard, R. Lin, O. Cormier, H. S. Lee, F. Eng, N. R. Bertos, N. Pelletier, S. Mader, V. K. Han, X. J. Yang, and J. H. White. 2003. Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. *Mol. Cell* **11**:139–150.
10. Frisch, S. M. 2006. CtBP: a link between apoptosis and the epithelial-mesenchymal transition, p. 39–44. *In* G. Chinnadurai (ed.), CtBP family proteins. Landes Bioscience and Springer Science-Business Media, New York, NY.
11. Garriga-Canut, M., B. Schoenike, R. Qazi, K. Bergendahl, T. J. Daley, R. M. Pfender, J. F. Morrison, J. Ockuly, C. Stafstrom, T. Sutula, and A. Roopra. 2006. 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat. Neurosci.* **9**:1382–1387.
12. Gill, G. 2004. SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev.* **18**:2046–2059.
13. Giorgino, F., O. de Robertis, L. Laviola, C. Montrone, S. Perrini, K. C. McCowen, and R. J. Smith. 2000. The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. *Proc. Natl. Acad. Sci. USA* **97**:1125–1130.
14. Girdwood, D., D. Bumpass, O. A. Vaughan, A. Thain, L. A. Anderson, A. W. Snowden, E. Garcia-Wilson, N. D. Perkins, and R. T. Hay. 2003. P300 transcriptional repression is mediated by SUMO modification. *Mol. Cell* **11**:1043–1054.
15. Grooteclaes, M., Q. Deveraux, J. Hildebrand, Q. Zhang, R. H. Goodman, and S. M. Frisch. 2003. C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs. *Proc. Natl. Acad. Sci. USA* **100**:4568–4573.
16. Grooteclaes, M. L., and S. M. Frisch. 2000. Evidence for a function of CtBP in epithelial gene regulation and anoikis. *Oncogene* **19**:3823–3828.
17. Gyory, I., J. Wu, G. Fejer, E. Seto, and K. L. Wright. 2004. PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. *Nat. Immunol.* **5**:299–308.
18. Hakimi, M. A., D. A. Bochar, J. Chenoweth, W. S. Lane, G. Mandel, and R. Shiekhattar. 2002. A core-BRAF35 complex containing histone deacetylase mediates repression of neuronal-specific genes. *Proc. Natl. Acad. Sci. USA* **99**:7420–7425.
19. Hay, R. T. 2005. SUMO: a history of modification. *Mol. Cell* **18**:1–12.
20. Hildebrand, J. D., and P. Soriano. 2002. Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. *Mol. Cell. Biol.* **22**:5296–5307.
21. Hofmann, T. G., E. Jaffray, N. Stollberg, R. T. Hay, and H. Will. 2005. Regulation of homeodomain-interacting protein kinase 2 (HIPK2) effector function through dynamic small ubiquitin-related modifier-1 (SUMO-1) modification. *J. Biol. Chem.* **280**:29224–29232.
22. Humphrey, G. W., Y. Wang, V. R. Russanova, T. Hirai, J. Qin, Y. Nakatani, and B. H. Howard. 2001. Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. *J. Biol. Chem.* **276**:6817–6824.
23. Jakobs, A., J. Koehnke, F. Himstedt, M. Funk, B. Korn, M. Gaestel, and R. Niedenthal. 2007. Ubc9 fusion-directed sumoylation (UFDS): a method to analyze function of protein sumoylation. *Nat. Methods* **4**:245–250.
24. Kagey, M. H., T. A. Melhuish, S. E. Powers, and D. Wotton. 2005. Multiple activities contribute to Pc2 E3 function. *EMBO J.* **24**:108–119.
25. Kagey, M. H., T. A. Melhuish, and D. Wotton. 2003. The polycomb protein Pc2 is a SUMO E3. *Cell* **113**:127–137.
26. Kim, J. H., E. J. Cho, S. T. Kim, and H. D. Youn. 2005. CtBP represses p300-mediated transcriptional activation by direct association with its bromodomain. *Nat. Struct. Mol. Biol.* **12**:423–428.
27. Kumar, V., J. E. Carlson, K. A. Ohgi, T. A. Edwards, D. W. Rose, C. R. Escalante, M. G. Rosenfeld, and A. K. Aggarwal. 2002. Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol. Cell* **10**:857–869.
28. Lee, M. G., C. Wynder, D. A. Bochar, M. A. Hakimi, N. Cooch, and R. Shiekhattar. 2006. Functional interplay between histone demethylase and deacetylase enzymes. *Mol. Cell. Biol.* **26**:6395–6402.
29. Lee, M. G., C. Wynder, N. Cooch, and R. Shiekhattar. 2005. An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature* **437**: 432–435.
30. Lin, X., B. Sun, M. Liang, Y. Y. Liang, A. Gast, J. Hildebrand, F. C. Brunicardi, F. Melchior, and X. H. Feng. 2003. Opposed regulation of corepressor CtBP by sumoylation and PDZ binding. *Mol. Cell* **11**:1389–1396.
31. Long, J., D. Zuo, and M. Park. 2005. Pc2-mediated sumoylation of Smad-interacting protein 1 attenuates transcriptional repression of E-cadherin. *J. Biol. Chem.* **280**:35477–35489.
32. Lundblad, J. R. 2006. Structural determinants of CtBP function, p. 83–92. *In* G. Chinnadurai (ed.), CtBP family proteins. Landes Bioscience and Springer Science-Business Media, New York, NY.
33. Lyons, R. H., B. Q. Ferguson, and M. Rosenberg. 1987. Pentapeptide nuclear localization signal in adenovirus E1a. *Mol. Cell. Biol.* **7**:2451–2456.
34. Meloni, A. R., C. H. Lai, T. P. Yao, and J. R. Nevins. 2005. A mechanism of COOH-terminal binding protein-mediated repression. *Mol. Cancer Res.* **3**:575–583.
35. Meloni, A. R., E. J. Smith, and J. R. Nevins. 1999. A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. *Proc. Natl. Acad. Sci. USA* **96**:9574–9579.
36. Mirnezami, A. H., S. J. Campbell, M. Darley, J. N. Primrose, P. W. Johnson, and J. P. Blaydes. 2003. Hdm2 recruits a hypoxia-sensitive corepressor to negatively regulate p53-dependent transcription. *Curr. Biol.* **13**:1234–1239.
37. Molloy, D. P., A. E. Milner, I. K. Yakub, G. Chinnadurai, P. H. Gallimore, and R. J. Grand. 1998. Structural determinants present in the C-terminal binding protein binding site of adenovirus early region 1A proteins. *J. Biol. Chem.* **273**:20867–20876.
38. Nardini, M., S. Spano, C. Cericola, A. Pesce, A. Massaro, E. Mollo, A. Luini, D. Corda, and M. Bolognesi. 2003. CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. *EMBO J.* **22**:3122–3130.
39. Nardini, M., D. Svergun, P. V. Konarev, S. Spano, M. Fasano, C. Bracco, A. Pesce, A. Donadini, C. Cericola, F. Secundo, A. Luini, D. Corda, and M. Bolognesi. 2006. The C-terminal domain of the transcriptional corepressor CtBP is intrinsically unstructured. *Protein Sci.* **15**:1042–1050.
40. Perdomo, J., A. Verger, J. Turner, and M. Crossley. 2005. Role for SUMO modification in facilitating transcriptional repression by BKLf. *Mol. Cell. Biol.* **25**:1549–1559.
41. Postigo, A. A., and D. C. Dean. 1999. ZEB represses transcription through interaction with the corepressor CtBP. *Proc. Natl. Acad. Sci. USA* **96**:6683–6688.
42. Quinlan, K. G., M. Nardini, A. Verger, P. Francescato, P. Yaswen, D. Corda, M. Bolognesi, and M. Crossley. 2006. Specific recognition of ZNF217 and other zinc finger proteins at a surface groove of C-terminal binding proteins. *Mol. Cell. Biol.* **26**:8159–8172.
43. Quinlan, K. G., A. Verger, A. Kwok, S. H. Lee, J. Perdomo, M. Nardini, M. Bolognesi, and M. Crossley. 2006. Role of the C-terminal binding protein PXDLS motif binding cleft in protein interactions and transcriptional repression. *Mol. Cell. Biol.* **26**:8202–8213.
44. Riefler, G. M., and B. L. Firestein. 2001. Binding of neuronal nitric oxide synthase to carboxy-terminal binding protein changes the localization of carboxy-terminal binding protein from the nucleus to the cytosol. A novel function for targeting by the PDZ domain of neuronal nitric oxide synthase. *J. Biol. Chem.* **276**:5–5.
45. Riefler, G. M., and B. L. Firestein. 2001. Binding of neuronal nitric-oxide synthase (nNOS) to carboxyl-terminal-binding protein (CtBP) changes the localization of CtBP from the nucleus to the cytosol: a novel function for targeting by the PDZ domain of nNOS. *J. Biol. Chem.* **276**:48262–48268.
46. Rosendorff, A., S. Sakakibara, S. Lu, E. Kieff, Y. Xuan, A. DiBacco, Y. Shi, and G. Gill. 2006. NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression. *Proc. Natl. Acad. Sci. USA* **103**: 5308–5313.
47. Schaeper, U., J. M. Boyd, S. Verma, E. Uhlmann, T. Subramanian, and G. Chinnadurai. 1995. Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl. Acad. Sci. USA* **92**:10467–10471.
48. Senyuk, V., K. K. Sinha, and G. Nucifora. 2005. Corepressor CtBP1 interacts with and specifically inhibits CBP activity. *Arch. Biochem. Biophys.* **441**:168–173.
49. Shi, Y., F. Lan, C. Matson, P. Mulligan, J. R. Whetstone, P. A. Cole, and R. A. Casero. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**:941–953.
50. Shi, Y., J. Sawada, G. Sui, B. Affar, J. R. Whetstone, F. Lan, H. Ogawa, M. P. Luke, and Y. Nakatani. 2003. Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**:735–738.
51. Shi, Y. J., C. Matson, F. Lan, S. Iwase, T. Baba, and Y. Shi. 2005. Regulation of LSD1 histone demethylase activity by its associated factors. *Mol. Cell* **19**:857–864.
52. Shio, Y., and R. N. Eisenman. 2003. Histone sumoylation is associated with transcriptional repression. *Proc. Natl. Acad. Sci. USA* **100**:13225–13230.
53. Spaderna, S., O. Schmalhofer, F. Hlubek, G. Bex, A. Eger, S. Merkel, A. Jung, T. Kirchner, and T. Brabletz. 2006. A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. *Gastroenterology* **131**:830–840.
54. Spoelstra, N. S., N. G. Manning, Y. Higashi, D. Darling, M. Singh, K. R.

- Shroyer, R. R. Broaddus, K. B. Horwitz, and J. K. Richer. 2006. The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res.* **66**:3893–3902.
55. Subramanian, T., and G. Chinnadurai. 2003. Association of class I histone deacetylases with transcriptional corepressor CtBP. *FEBS Lett.* **540**:255–258.
56. Tachibana, M., J. Ueda, M. Fukuda, N. Takeda, T. Ohta, H. Iwanari, T. Sakihama, T. Kodama, T. Hamakubo, and Y. Shinkai. 2005. Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. *Genes Dev.* **19**:815–826.
57. Thiagalingam, A., A. De Bustros, M. Borges, R. Jasti, D. Compton, L. Diamond, M. Mabry, D. W. Ball, S. B. Baylin, and B. D. Nelkin. 1996. RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. *Mol. Cell. Biol.* **16**:5335–5345.
58. Turner, J., and M. Crossley. 1998. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *EMBO J.* **17**:5129–5140.
59. Turner, J., and M. Crossley. 2001. The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *Bioessays* **23**:683–690.
60. Ueda, J., M. Tachibana, T. Ikura, and Y. Shinkai. 2006. Zinc finger protein Wiz links G9a/GLP Histone methyltransferases to the co-repressor molecule CtBP. *J. Biol. Chem.* **281**:20120–20128.
61. Wang, J., K. Scully, X. Zhu, L. Cai, J. Zhang, G. G. Prefontaine, A. Krones, K. A. Ohgi, P. Zhu, I. Garcia-Bassets, F. Liu, H. Taylor, J. Lozach, F. L. Jayes, K. S. Korach, C. K. Glass, X. D. Fu, and M. G. Rosenfeld. 2007. Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature* **446**:882–887.
62. Wang, S. Y., M. Iordanov, and Q. Zhang. 2006. c-Jun NH2-terminal kinase promotes apoptosis by down-regulating the transcriptional co-repressor CtBP. *J. Biol. Chem.* **281**:34810–34815.
63. You, A., J. K. Tong, C. M. Grozinger, and S. L. Schreiber. 2001. CoREST is an integral component of the CoREST-human histone deacetylase complex. *Proc. Natl. Acad. Sci. USA* **98**:1454–1458.
64. Zeng, Y., C. M. Tang, Y. L. Yao, W. M. Yang, and E. Seto. 1998. Cloning and characterization of the mouse histone deacetylase-2 gene. *J. Biol. Chem.* **273**:28921–28930.
65. Zhang, Q., A. Nottke, and R. H. Goodman. 2005. Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis. *Proc. Natl. Acad. Sci. USA* **102**:2802–2807.
66. Zhang, Q., D. W. Piston, and R. H. Goodman. 2002. Regulation of corepressor function by nuclear NADH. *Science* **295**:1895–1897.
67. Zhang, Q., S. Y. Wang, C. Fleuriel, D. Leprince, J. V. Rocheleau, D. W. Piston, and R. H. Goodman. 2007. Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex. *Proc. Natl. Acad. Sci. USA* **104**:829–833.
68. Zhang, Q., Y. Yoshimatsu, J. Hildebrand, S. M. Frisch, and R. H. Goodman. 2003. Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP. *Cell* **115**:177–186.
69. Zhao, L. J., T. Subramanian, and G. Chinnadurai. 2006. Changes in C-terminal binding protein 2 (CtBP2) corepressor complex induced by E1A and modulation of E1A transcriptional activity by CtBP2. *J. Biol. Chem.* **281**:36613–36623.