CTIP2 Associates with the NuRD Complex on the Promoter of p57KIP2, a Newly Identified CTIP2 Target Gene*

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Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2 (CTIP2), also known as Bcl11b, is a transcriptional repressor that functions by direct, sequence-specific DNA binding activity or by recruitment to the promoter template by interaction with COUP-TF family members. CTIP2 is essential for both T cell development and axonal projections of corticospinal motor neurons in the central nervous system. However, little is known regarding the molecular promoter template by interaction with COUP-TF family members. CTIP2 complexes that were isolated from SK-N-MC neuroblas-toma cells were found to harbor substantial histone deacetylation activity, which was likely conferred by the nucleosome remodeling and deacetylation (NuRD) complex. CTIP2 was found to associate with the NuRD complex through direct interaction with both RbAp46 and RbAp48, and components of the NuRD complex were found to be recruited to an artificial promoter template in a CTIP2-dependent manner in transfected cells. Finally, the NuRD complex and CTIP2 were found to co-occupy the promoter template of p57KIP2, a gene encoding a cyclin-dependent kinase inhibitor, and identified herein as a novel transcriptional target of CTIP2 in SK-N-MC cells. Therefore, it seems likely that the NuRD complex may be involved in transcriptional repression of CTIP2 target genes and contribute to the function(s) of CTIP2 within a neuronal context.

Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2 (CTIP2, Bcl11b, Rit-1β) is a novel C2H2 zinc finger protein that was first isolated and identified as a COUP-TF-interacting protein (1). Within the immune system, CTIP2/Bcl11b is predominantly expressed in mouse thymocytes, and is required for development and survival of α/β T lymphocytes (2). Inactivation of CTIP2/Bcl11b by homozygous deletions and point mutations of CTIP2 gene are associated with γ-ray-induced thymic lymphomas in mouse (3, 4), and ectopic expression of CTIP2/Bcl11b in HeLa cells results in suppression of cell growth (3). Although these findings suggest that CTIP2 may function as a tumor suppressor, its involvement in human carcinogenesis remains unclear. However, several reports have described a link between chromosomal rearrangements of CTIP2 and human T cell acute lymphoblastic leukemia (T-ALL) (5–9).

In addition to substantial expression in thymocytes, CTIP2/Bcl11b (CTIP2 hereafter) is also expressed at a high level in the central nervous system (CNS) of pre- and postnatal mouse brain, more specifically in developing cerebral cortex primarily in layer V, the striatum, olfactory bulb, hippocampus, limbic system, basal ganglia, and also in the intermediate region of the spinal cord (10–13). Ctip2-null mice exhibit defective axonal projections of corticospinal motor neurons (CSMNs), indicating that CTIP2 plays a critical role in development of the CNS (11). Although several lines of evidence have shown that CTIP2 is required for T cell and CNS development, little is known concerning the mechanism(s) by which CTIP2 may function in these processes, or transcriptional targets of CTIP2 in any cell or tissue.

CTIP2, and the highly related protein CTIP1, are transcriptional repressors that are recruited to the template either by interaction with COUP-TFs (1) or by direct, sequence-specific DNA binding activity (14). In both cases, CTIPs mediate transcriptional repression that has been found to be largely insensitive to reversal by trichostatin A (TSA), an inhibitor of class I and II histone deacetylases (HDACs). SIRT1 (sirtuin 1), a class III HDAC, may underlie TSA-insensitive transcriptional repression mediated by CTIPs (15, 16), but it is not known if TSA-insensitive histone deacetylation entirely underlies CTIP2-mediated transcriptional repression in all cell types and/or promoter contexts.

The NuRD complex harbors ATP-dependent, nucleosome remodeling and histone deacetylase activities, and consists of several subunits, minimally including RbAp46, RbAp48,
HDAC1, HDAC2, MTA1, MTA2, MTA3, MBD3, and Mi-2α and β (17–20). The NuRD complex is considered to play a key role in transcriptional repression mediated by sequence-specific transcription factors including p53 (21), Ikaros (22), Hunchback (23), Tramtrack69 (24), KAP-1 (25), BCL-6 (20), and FOG-1 (26).

In the present report, we found that transcriptional repression mediated by CTIP2 was partially sensitive to inhibition by TSA in the context of a minimal promoter. Consistently, both ectopically expressed and endogenous CTIP2 complexes were found to harbor TSA-sensitive HDAC activity in vitro. We found that CTIP2 associated with the NuRD complex in both transfected HEK293T and in untransfected SK-N-MC neuroblastoma cells, and this appeared to be via direct interaction with RbAp46 and/or RbAp48. We also found that the NuRD complex was recruited to a CTIP2-responsive promoter template in a CTIP2-dependent manner in transfected HEK293T cells. Here we report a newly identified CTIP2 target gene, p57KIP2, which encodes a cyclin-dependent kinase (cdk) inhibitor. p57KIP2 plays important roles in control of cell cycle and neuronal differentiation, and was found herein to be repressed by CTIP2 in SK-N-MC cells. Subsequent ChIP and re-ChIP analyses of the p57KIP2 promoter demonstrated co-occupancy by CTIP2, MTA2, HDAC2, and RbAp46/48, suggesting that CTIP2-mediated repression of this target gene is likely to involve recruitment of the NuRD complex to the template. Together, these findings suggest that the NuRD complex may play a role in CTIP2-mediated transcriptional repression, at least on a subset of genes, and in a neuron-like context.

MATERIALS AND METHODS

Constructs—The Lex-Gal-LUC reporter and LexA-VP16 constructs were kind gifts from Dr. Malcolm G. Parker (Imperial College, London; Ref. 27). The Gal4-CTIP2, FLAG-CTIP2, and deletion mutants of the latter were previously described (15). FLAG-CTIP2-(129–350) was prepared by PCR amplification with primers containing appropriate restriction sites for insertion into pcDNA3.1/HisC (Invitrogen), and verified by complete DNA sequencing. Recombinant baculoviruses expressing Mi-2β, MTA2, HDAC1, HDAC2, RbAp46, and RbAp48 were kindly gifts from Dr. Danny Reinberg (University of Medical and Dentistry of New Jersey; Ref 17).

Cell Culture—HEK293T cells were cultured on 10-cm plates in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% (v/v) fetal bovine serum (Atlas Biologicals) and 1% (v/v) penicillin/streptomycin (Invitrogen). SK-N-MC neuroblastoma cells were grown under identical conditions except that 1% sodium pyruvate (Invitrogen) was added to the media. roblastoma cells were grown under identical conditions except 1% (v/v) penicillin/streptomycin (Invitrogen). SK-N-MC neuronal differentiation, and was found herein to be repressed by CTIP2 in SK-N-MC cells. Here we report a newly identified CTIP2 target gene, p57KIP2, which encodes a cyclin-dependent kinase (cdk) inhibitor. p57KIP2 plays important roles in control of cell cycle and neuronal differentiation, and was found herein to be repressed by CTIP2 in SK-N-MC cells. Subsequent ChIP and re-ChIP analyses of the p57KIP2 promoter demonstrated co-occupancy by CTIP2, MTA2, HDAC2, and RbAp46/48, suggesting that CTIP2-mediated repression of this target gene is likely to involve recruitment of the NuRD complex to the template. Together, these findings suggest that the NuRD complex may play a role in CTIP2-mediated transcriptional repression, at least on a subset of genes, and in a neuron-like context.

Transfection and Reporter Assays—At ~60% confluence, HEK293T cells were transiently transfected with 3 μg of the Lex-Gal-LUC reporter gene, 0.1 μg of an expression vector encoding the LexA-VP16 fusion protein, and 1 or 5 μg of either Gal4-DBD or Gal4-DBD-CTIP2 (Gal4-CTIP2), using the calcium phosphate method. Twenty-four hours after transfection, cells were treated with TSA (100 ng/ml) or vehicle, and harvested 24 h later. Whole cell lysates were subjected to a luciferase assay (Promega). Luciferase levels were measured using a LUMAT LB 9507 (EG&G Berthold) luminometer. Luciferase activities were normalized across all samples by protein concentration as determined using the Bradford assay.

Antibodies—Anti-acetylated-histone H3 and -H4 antibodies were purchased from Upstate. Anti-CTIP2 antisera was raised against CTIP2 peptide corresponding to amino acids 25–44 and purified on a peptide affinity column. Anti-Mi-2α/β and -FLAG M5 monoclonal antibodies were obtained from BD Biosciences and Sigma, respectively. Anti-RbAp46/RbAp48, -CTIP2 (25B6), and -β-actin monoclonal antibodies were obtained from Abcam. Anti-MTA2, -HDAC1, -HDAC2, and -HA polyclonal antibodies were purchased from Santa Cruz Biotechnology and Abcam, and anti-p57KIP2 was obtained from BD Pharmingen.

Immunoprecipitation Analyses—HEK293T cells were transiently transfected as described above with 10 μg of an expression vector encoding FLAG-CTIP2 or the corresponding empty vector (pcDNA3.1). Forty-eight hours after transfection, cells were lysed with NET-N buffer (150 mM NaCl, 0.5% Nonidet P-40) (Nonidet P-40) (Nonidet P-40, 10% glycerol, 1 mM EDTA, 20 mM Tris-HCl, pH 8, and a protease inhibitor mixture), and incubated on ice for 30 min with occasional vortexing prior to centrifugation (16,000 × g for 15 min). Cell lysates (800 μg of protein per immunoprecipitation reaction) were precleared with protein G-Sepharose (Amersham Biosciences) in Buffer IP (10 mM HEPES, pH 7.5, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40) at 4 °C for 60 min to reduce nonspecific protein binding. After centrifugation, the precleared samples were incubated with 2 μg of an anti-MTA2 or irrelevant (anti-HA) antibody on ice for 60 min, followed by addition of protein G-Sepharose. Samples were then incubated at 4 °C overnight with rocking. The Sepharose beads were collected by centrifugation, washed three times with buffer IP, and resuspended in denaturing sample buffer. Immune complexes were separated by SDS-PAGE and analyzed by Western blotting with appropriate antibodies. For SK-N-MC cells, the nuclear extract was made as described previously (28), except buffer C was modified to contain 0.72 mM NaCl, and the pellet remaining after nuclear lysis was re-extracted with an equal volume of this buffer followed by brief sonication and centrifugation. Final nuclear extracts were dialyzed against buffer D (20 mM HEPES, pH 8.0, 10% glycerol, 0.1 mM EDTA, 300 mM NaCl), aliquoted, and quickly frozen or used directly in IP assays, which were performed as described above using 300 μg of protein per cell lysate sample. In buffer D containing 0.05% Nonidet P-40. SF9 cells were infected with baculovirus individually directing expression of Mi-2β, MTA2, HDAC1, HDAC2, RbAp46, and RbAp48. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10% glycerol, 0.5 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol), and sonicated three times for 20 s to lyse cells, followed by centrifugation. Approximately 120–160 μg of total protein from cell lysates was used per IP reaction, which was performed in buffer IP using 1 μg of specific antibodies against individual NuRD complex proteins.

GST Pull-down Experiments—GST pull-down experiments were conducted as described previously (29).

HDAC Activity Assays—Immunoprecipitation assays were performed essentially as described above except that more protein was used (HEK293T, 2 mg/IP; SK-N-MC, 500 μg/IP).
Immunoprecipitates obtained from SK-N-MC cells or transiently transfected HEK293T cells were analyzed for HDAC activity using an HDAC Fluorescent Activity Assay kit (Biomol Research Laboratories, Inc.), in the absence or presence of TSA (0.25 μM). The fluorophore produced from the reactions was excited at 360-nm light and emission was followed at 460 nm on a Gemini XPS microplate spectrofluorometer (Molecular Devices).

**Superose 6 Size Exclusion Column Chromatography—SK-N-MC nuclear extract was dialyzed against buffer D. Approximately 6 mg of nuclear protein was concentrated to 1 ml using a Millipore Ultracel centrifugal filter apparatus (10 kDa nominal molecular mass limit), and then applied to an 850 × 20 mm Superose 6 size exclusion column (Amersham Biosciences) that had been equilibrated with buffer D containing 1 mM dithiothreitol, and calibrated with protein standards (blue dextran, 2000 kDa; thyroglobulin, 669 kDa; RNase A, 13.7 kDa, all from Amersham Biosciences). The column was eluted at a flow rate of 0.4 ml/min, and fractions were collected for 5 min (2 ml). The chromatographic elution profiles of CTIP2 and the NuRD complex proteins were determined by immunoblotting with appropriate antibodies and chemiluminescence detection.

**Chromatin Immunoprecipitation (ChIP) Assays—**ChIP assays were conducted as previously described (15, 16) with some modifications. Briefly, HEK293T cells were washed sequentially after cross-linking with phosphate-buffered saline, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), and then lysed followed by brief sonication. The sonicated lysates were diluted 3.5-fold with ChIP dilution buffer, and 10% of the diluted lysates was reserved as an input sample to determine the total amount of reporter plasmid in transfected cells for subsequent normalization procedures. The remaining lysates were aliquoted equally and used for IP with and without the addition of 5 μg of anti-MTA2 (Santa Cruz Biotechnology), -RbAp46/48 (Abcam), or -HDAC2 (Santa Cruz Biotechnology) antibody. Chromatin complexes were subjected to reversal of protein-DNA cross-links at 65 °C overnight and proteinase K treatment at 45 °C for 2 h. DNA was recovered by using a Qiaquick Spin kit (Qiagen) and amplified using a forward primer (5’-GTCGAGGGGATGATAATG-3’) upstream of the multimerized 17-mer, and a reverse primer (5’-ACAGTACCGGAGTCTCAG-3’) downstream of the promoter but upstream of a transcriptional start site of the luciferase gene. Amplification of GPDH promoter region (negative control) was performed with a forward primer (5’-TCTTCTGTTCATCTCCAACG-3’), and a reverse primer (5’-TAGTGCCGCCCCCTATCTTT-3’). Conditions of the amplification reactions were as follows; a predenaturation step of 2 min at 94 °C was followed by 23 (for the reporter) and 31 (for GPDH) cycles of 94 °C (denaturation) for 40 s, 56 °C (annealing) for 45 s, 70 °C (elongation) for 1 min, and a final elongation step of 5 min at 72 °C. The resulting 210-bp PCR product of the reporter gene promoter region and 218-bp PCR product of the GPDH promoter region were analyzed by agarose gel electrophoresis and ethidium bromide staining. Experiments were performed at least three times. ChIP assays in SK-N-MC cells were performed as described above except that the antibodies (anti-CTIP2 and RbAp46/48, respectively) were coupled to magnetic Dynabeads (Dyina/Invitrogen).

**Re-ChIP Assays—**The 1st ChIP was performed as described above using anti-CTIP2 antibody. Immune complexes were eluted from the beads with 20 mM dithiothreitol. Eluates were then diluted 30-fold with ChIP dilution buffer, and subjected to the 2nd immunoprecipitation reaction using either anti-MTA2, -HDAC2, or -RbAp46/48 antibody. The final elution step was performed using 1% SDS solution in Tris-EDTA buffer, pH 8.0. The enrichment of DNA template was analyzed by conventional PCR using primers specific for p57KIP2 proximal promoter (forward: 5’-GCCAATCGCCGTGGTTGTTGT-3’; reverse: 5’-GTGTTGGACCTCTTCTCGTCT-3’). Amplification of HMOX-1 proximal promoter was performed using a forward primer 5’-GCCAGACTTTGTCTTCCCAAG-3’, and a reverse primer 5’-GAGGAGGCGACGCTTCTGACTG-3’.

**Quantitative Real-Time PCR (qPCR)—**Purified immunoprecipitated promoter fragments were analyzed by quantitative real-time PCR (DNA Engine Opticon® 2 Thermal Cycler, MJ Research, Inc.) using SYBR Green I methodology. Amplification reactions were performed as follows; a predenaturation step of 10 min at 95 °C was followed by 35 cycles of 94 °C (denaturation) for 10 s, 56 °C (annealing) for 20 s, 70 °C (elongation) for 20 s, and a final elongation step of 5 min at 72 °C.

**siRNA Transfection—**Transfection of SK-N-MC cells were performed using Lipofectamine 2000 (Invitrogen) and a siRNA pool targeting CTIP2 (custom synthesized by Dharmacon). Lipofectamine (60 μl) as well as siRNA (60 μl of 15 μM SMART-POOL or nonspecific siRNA) were preincubated in Opti-MEM media for 5 min. After preincubation, the two reagents were mixed together and allowed to incubate for additional 20 min. Immediately before transfection, SK-N-MC cells were transferred from Dulbecco’s modified Eagle’s medium to Opti-MEM (without serum), and the transfection mix was added dropwise to the plates (siRNA final concentration 100 nM). Four independent plates were used for each condition (CTIP2 siRNA and nonspecific siRNA). After 24 h, the cells were transferred back to Dulbecco’s modified Eagle’s medium and allowed to incubate for another 24 h prior to harvesting for either RNA preparation or protein isolation (see below).

**RNA Preparation and Microarray Analysis—**Total RNA was prepared using Qiagen RNeasy Mini kit, labeled using the ENZO RNA Transcript Labeling kit, and used to probe the Affymetrix human microarray chip HG-U133. Results were analyzed using GeneSpring 7.2 (Silicon Genetics) software, and genes that differed from the control by at least 2-fold (p < 0.05 as determined by one-way analysis of variance), such as p57KIP2, were identified, and confirmed to be regulated by CTIP2 by immunoblotting and/or RT-PCR.

**Whole Cell Extract Preparation—**SK-N-MC cells (from a 100-mm plate at ~80% confluence) were lysed with 100 μl of lysis buffer (0.5 x NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM Tris-HCl, pH 7.8) containing a protease inhibitor mixture. The supernatant was clarified by centrifugation at 16,000 × g for 20 min, aliquoted, and stored at ~80 °C. Extracts were analyzed by Western blot.
RESULTS

The Class I and Class II Histone Deacetylase Inhibitor TSA Partially Reverses Transcriptional Repression Mediated by Gal4-CTIP2 on a Minimal Promoter—Gal4-CTIP2 has been previously shown to possess strong, and predominantly TSA-insensitive transcriptional repression activity in the context of the herpes simplex thymidine kinase (tk) promoter, which may be caused by CTIP2-mediated recruitment of the histone deacetylase SIRT1 to the template (15). To determine if the TSA insensitivity of CTIP2-mediated transcriptional repression generalizes to other promoter contexts, we assessed the ability of CTIP2 to mediate repression of a luciferase reporter gene driven by a minimal promoter (Lex-Gal-LUC; Ref 27 in transiently transfected HEK293T cells. Expression of the reporter gene was stimulated by co-expression with LexA-VP16 to facilitate evaluation of GAL4-CTIP2-mediated repression. TSA-stimulated expression of the reporter gene over 7-fold in the presence of GAL4-DBD (Fig. 1A, lane 2). Gal4-CTIP2 strongly repressed expression of the reporter gene (Fig. 1A, lane 3), as previously described within the tk promoter (15). However, in the context of this minimal promoter, Gal4-CTIP2-mediated repression was found to be partially reversed by TSA (Fig. 1A, compare lanes 3 and 4), indicating the possible involvement of class I and class II histone deacetylases (HDACs).

Deacetylation of Histone H3/H4 Associated with the Reporter Template in Cells Expressing Gal4-CTIP2—ChIP experiments were conducted to determine if transfection of Gal4-CTIP2 resulted in deacetylation of template-associated histones H3 and/or H4. Transfection of Gal4-DBD minimally reduced the level of acetylated H3/H4 associated with the template as determined by both conventional and quantitative PCR (qPCR; Fig. 1B, compare lanes 6 and 8). This effect was consistent with the lack of transcriptional repression activity of Gal4-DBD observed in reporter assays (data not shown). However, the level of acetylated H3/H4 associated with the template decreased by over 4-fold upon transfection with Gal4-CTIP2 as determined by both conventional and qPCR (Fig. 1B, compare lanes 6, 8, and 10). Together, these findings indicate that transfection of Gal4-CTIP2 resulted in deacetylation of H3/H4 on the template of the minimal promoter.

CTIP2 Complexes Harbor TSA-sensitive HDAC Activity—To determine if TSA-sensitive histone deacetylase activity is associated with CTIP2 complexes in mammalian cells, histone deacetylase activity assays were performed in vitro using whole cell lysates from HEK293T cells, which had been transiently transfected with an expression vector encoding FLAG-CTIP2 or the corresponding empty vector. Immunoprecipitates of FLAG-CTIP2 complexes indeed harbored HDAC activity that was nearly 10-fold greater than that observed with control immunoprecipitates (Fig. 2A, lanes 1 and 3). Inclusion of TSA in the in vitro assay completely abolished the in vitro HDAC activity of FLAG-CTIP2 immunoprecipitates (Fig. 2A, compare lanes 1, 2, and 3). To investigate if complexes containing CTIP2 harbor TSA-sensitive histone deacetylase activity in a more natural cellular context, the HDAC activity assays were performed similarly as above on nuclear extracts derived from SK-N-MC human neuroblastoma cells, which like Jurkat cells (15), endogenously express two splice variants of CTIP2 (see Fig. 3B). CTIP2 complexes from SK-N-MC cells possessed robust

FIGURE 1. CTIP2-mediated transcriptional repression of a minimal promoter is partially reversed by TSA. A, HEK293T cells were co-transfected with 3 μg of Lex-Gal-LUC reporter, 0.1 μg of LexA-VP16 and either 1 μg of Gal4-DBD or Gal4-CTIP2 using the calcium phosphate method. Twenty-four hours after transfection, cells were treated with TSA (100 ng/ml; open bars) or vehicle (solid bars) as indicated for 24 h prior to harvesting for luciferase reporter assays. Light units were normalized across all samples by protein concentration and expressed as fold-repression or fold-activation relative to lane 1 (Gal4-DBD). The data shown here represent the mean fold-repression or -activation ± S.E. derived from three independent experiments. Statistical significance is indicated by asterisk (p < 0.05; Student’s t test) when comparing lane 4 to lane 3. B, upper, schematic diagram of a Lex-Gal-LUC reporter illustrating LexA and Gal4 (17-mer) binding sites, which are upstream of LUC. Arrows represent positions of forward and reverse primers for PCR amplification shown in the lower panel. The size of PCR product (210 bp) present in the lower panel is indicated. Lower, ChIP assays were performed on HEK293T cells following transient transfection as described above. Lane 1 corresponds to a template control in which a reporter plasmid was used directly in the amplification reaction. Inputs in lanes 2–4 were 5% of total amount of template used in the reactions. Lanes 5–10 represent quantification of the template present, expressed as percent of input, as determined by qPCR. Results are representative of three independent experiments.
HDAC activity (Fig. 2B, lane 1). In contrast, the immunoprecipitation performed with an irrelevant antibody (anti-HA) exhibited only negligible amounts of HDAC activity (Fig. 2B, lane 3). As observed in transfected HEK293T cells, TSA completely inhibited HDAC activity in CTIP2 immunoprecipitates from SK-N-MC cells (Fig. 2B, compare lanes 1 and 2). These data suggest that class I and/or class II HDACs mediate the TSA-sensitive HDAC activity that is associated with CTIP2 complexes in vitro. It is important to note that the HDAC activity assays reported herein were performed without addition of NAD⁺, a cofactor that is required for deacetylation mediated by SIRT1 (30, 31), a class III HDAC that interacts with CTIP2 in HEK293 and Jurkat cells (15). Thus, the HDAC activity of CTIP2 immunoprecipitates that we observed in the present studies was most likely due to the catalytic activity of class I and/or class II HDACs. We have not observed either NAD⁺-
stimulated or nicotinamide-inhibited HDAC activity in CTIP2 immunoprecipitates from SK-N-MC cells under the conditions used herein (data not shown). However, we cannot exclude the possibility that SIRT1 may also contribute to HDAC activity of CTIP2 complexes in cells. Indeed, we note that TSA only reverses a fraction of transcriptional repression mediated by GAL4-CTIP2 in transiently transfected HEK293T cells (Fig. 1A, lanes 3 and 4).

CTIP2 Associates with the NuRD Complex in HEK293T Cells—CTIP2 has been recently demonstrated to interact with the NuRD complex in Jurkat cells (32). To investigate the physical association between CTIP2 and components of the NuRD complex in HEK293T cells, immunoprecipitation experiments were performed on whole cell extracts from cells overexpressing FLAG-CTIP2. As expected, Mi-2α/β and HDAC2 were co-immunoprecipitated with MTA2 by an anti-MTA2 antibody in the absence of FLAG-CTIP2 expression (Fig. 3A, lane 4). MTA1 was also detected in MTA2 immunoprecipitates (Fig. 3A, lanes 4 and 6), and this was caused by the cross-reactivity of the anti-MTA2 antibody used in immunoprecipitation and/or immunoblotting procedures. However, none of these proteins was co-immunoprecipitated by an irrelevant antibody (anti-HA, Fig. 3A, lane 3). Overexpressed FLAG-CTIP2 was co-immunoprecipitated with MTA2 and the other NuRD components by the anti-MTA2 antibody (Fig. 3A, lane 6), but not by anti-HA antibody (lane 5), suggesting that FLAG-CTIP2 associates with the NuRD complex in transiently transfected HEK293T cells. It is interesting to note that upon overexpression of FLAG-CTIP2 in HEK293T cells, we observed substantially decreased expression of MTA1, MTA2, and Mi-2α/β, but not of HDAC2 (Fig. 3A, compare lanes 1 and 2). In addition, we observed significantly less Mi-2α/β in the NuRD complexes when CTIP2 was overexpressed (Fig. 3A, compare lanes 4 and 6 of first panel). Although we do not know yet the mechanism for this effect, we speculate that Mi-2α/β, MTA1, and MTA2, but not HDAC2, may be direct or indirect targets of CTIP2-mediated transcriptional repression in HEK293T cells. It is also possible that CTIP2 may be a general repressor of transcription in HEK293T cells, although this is not consistent with the lack of effect of CTIP2 on expression of HDAC2. (Fig. 3A, compare lanes 1 and 2 of last panel). These data clearly demonstrate that NuRD complex proteins interact with CTIP2 either directly or indirectly in transiently transfected HEK293T cells.

CTIP2 Associates with the NuRD Complex in SK-N-MC Cells—The co-IP results shown above were performed using transiently transfected cells overexpressing CTIP2. However, it is important to verify association of CTIP2 with the NuRD complex when expressed at physiological levels, and in untransfected cells. To assess these possibilities, IP experiments were performed using nuclear extracts derived from SK-N-MC cells. Endogenous MTA2, HDAC2, RbAp46/48, and to some extent Mi-2α/β, were co-immunoprecipitated with CTIP2 by an anti-CTIP2 antisera (Fig. 3B, lane 3), but not by an irrelevant antibody (anti-HA, lane 7). The results from reciprocal experiments revealed that endogenous CTIP2 was efficiently co-immunoprecipitated by anti-MTA2, -Mi-2α/β, -HDAC2, and -RbAp46/48 antibodies (second panel of Fig. 3B, lanes 2, 4, 5, and 6, respectively), but not by an anti-HA antibody (lane 7).

These findings indicate that endogenous CTIP2 stably associates with the NuRD complex when expressed at physiological levels in SK-N-MC neuroblastoma cells.

To determine the mass of native CTIP2 complexes in SK-N-MC cells, nuclear extracts were fractionated on a Superose 6 size exclusion column. CTIP2 immunoreactivity eluted from the Superose 6 column as a relatively symmetrical peak centered between 669 and ~1000 kDa. (Fig. 4A and first panel of B, fractions 13–33). The elution pattern of CTIP2 appeared to overlap that of some NuRD complex proteins including MTA2, HDAC1, and HDAC2. (third, fourth, and fifth panels of Fig. 4B), but only partially overlapped that of Mi-2α/β (second panel). The partial co-elution of Mi-2α/β with CTIP2 and other NuRD proteins may explain, at least in part, why Mi-2α/β was weakly detected in anti-MTA2, -CTIP2, and -HDAC2 immunoprecipitates from SK-N-MC nuclear extracts (Fig. 3B, lanes 2, 3, and 5). These size exclusion chromatography results demonstrated that native CTIP2 in SK-N-MC cells eluted with an apparent mass greater than that of the monomeric protein for the two relevant CTIP2 splice variants (95.5 and 88.5 kDa, respectively), consistent with the possibility that CTIP2 exists within a large complex in SK-N-MC cells. Moreover, the partial co-elution of CTIP2 with most NuRD complex proteins further confirms the interaction of CTIP2 with this complex, which
Association of CTIP2 and NuRD Complex on p57KIP2 Promoter

A

| IP Antibodies | NuRD |
|---------------|------|
| INPUT         | IgG  |
|               | αMi-2/β |
|               | dMTA2 |
|               | cHDAC1 |
|               | HDAC2 |
|               | αRbAp48 |
|               | αRbAp46 |

B

| INPUT IgG | αNuRD |
|-----------|-------|
| Mi-2/β    |       |
| MTA2      |       |
| HDAC1     |       |
| HDAC2     |       |
| RbAp48    |       |
| RbAp46    |       |

C

| INPUT (5%) | [35S]CTIP2 AND MUTANTS |
|------------|------------------------|
|            | GST-RbAp46             |
|            | GST-RbAp48             |
| 1-813      | 1-136                  |
| 1-141      | 1-136                  |
| 1-229      | 1-136                  |
| 1-350      | 1-136                  |
| 1-356      | 1-136                  |
| 1-716      | 1-136                  |

FIGURE 5. CTIP2 interacts directly with RbAp46 and RbAp48 in vitro. A and B, in vitro translated and [35S]Met-labeled, full-length FLAG-CTIP2 was mixed with SF9 cell extracts containing individual recombinant NuRD complex proteins and these mixtures were immunoprecipitated with antibodies against the NuRD complex proteins, or nonspecific antibody (IgG), as indicated. A, immunoprecipitates were resolved by SDS-PAGE and the presence of [35S]Met-labeled FLAG-CTIP2 was determined by autoradiography (lanes 2–8). Input [35S]Met-labeled FLAG-CTIP2 is shown in lane 1. B, controls demonstrating efficiency of immunoprecipitation reactions. All NuRD complex proteins were efficiently immunoprecipitated by cognate antibodies. C, in vitro translated and [35S]Met-labeled, full-length CTIP2 and truncation mutants were incubated with equivalent amounts of bacterially expressed GST-RbAp46 (lanes 8–14) and GST-RbAp48 (lanes 15–21) fusion proteins or GST (data not shown). After extensive washing, [35S]Met-labeled CTIP2 associated with the affinity resin was resolved by SDS-PAGE and visualized by autoradiography. Arrows indicate strong interaction between [35S]Met-labeled CTIP2-(129–350) and GST-RbAp46, -RbAp48 fusion proteins.

Components of the NuRD complex tested (Fig. 5A, lanes 3–6). This interaction is most likely specific as FLAG-CTIP2 was not immunoprecipitated by nonspecific IgG (a negative control; Fig. 5A, lane 2). The efficiency of the IP reactions was confirmed by immunoblotting with cognate antibodies (Fig. 5B). These results indicate that CTIP2 interacts directly with RbAp46 and RbAp48 in vitro.

Next, GST pull-down assays were performed to map RbAp46- and RbAp48-interaction interfaces of CTIP2. CTIP2 deletion mutants containing amino acids 129–350 strongly interacted with both GST-RbAp46 (Fig. 5C, lanes 8, 9, and 11) and GST-RbAp48 (lane 15, 16, and 18), but not with GST alone (data not shown for simplicity). Deletion mutants of CTIP2 lacking this region weakly interacted (Fig. 5C, lanes 12–14 and 19–21, respectively), or did not interact at all (lanes 10 and 17, respectively) with GST-RbAp46 and GST-RbAp48. As CTIP2-(129–350) interacted strongly (Fig. 5C, lanes 11 and 18, respectively), whereas CTIP2-(171–350) interacted more weakly (Fig. 5C, lanes 12 and 19, respectively) with GST-RbAp46 and -RbAp48, amino acids 129–171 of CTIP2 are likely to be important for mediating interaction with GST-RbAp46 and -RbAp48. Accordingly, we tested the interaction between CTIP2-(129–171) and GST-RbAp46 and -RbAp48. To our surprise, we did not observe appreciable interaction of this CTIP2 fragment and RbAp proteins (data not shown). However, these results indicate that CTIP2-(129–350), a region including a C2H2 zinc finger motif and also a proline-rich domain (1), appears to be primarily responsible for interaction of CTIP2 with RbAp46 and RbAp48 in vitro.

GAL4-CTIP2 Recruits the NuRD Complex to the Promoter Template—CTIP2 represses transcription and is associated with TSA-sensitive histone deacetylase activity in vitro and in cells, which may be conferred by components of the NuRD complex, such as HDAC1 and/or HDAC2 (17, 18). However, the NuRD complex must be recruited to a CTIP2-responsive promoter in a CTIP2-dependent manner to play a role in transcriptional repression mediated by this repressor. To investigate this directly, ChIP experiments were performed in...
HEK293T cells that had been co-transfected with the Lex-Gal-LUC reporter (the same reporter construct used in Fig. 1), and either Gal4-DBD or Gal4-CTIP2. The presence of Gal4-CTIP2 on this template was confirmed by ChIP (data not shown). We found that Lex-Gal-LUC reporter template was immunoprecipitated by anti-MTA2, -RbAp46/48, and -HDAC2 antibodies in the manner that was stimulated by co-expression of Gal4-CTIP2 (Fig. 6A, compare lane 7 to lane 5 of all panels). In contrast, very little template was mock-immunoprecipitated (Fig. 6A, lanes 4 and 6 of all panels). These results suggest that MTA2, RbAp46/48, and HDAC2 are recruited to the promoter template of this transfected reporter in a CTIP2-dependent manner. This effect was specific to the promoter template, as expression of Gal4-CTIP2 did not affect the enrichment of MTA2, RbAp46/48, and HDAC2 on the GAPDH promoter (Fig. 6B). The results of these experiments demonstrate that CTIP2 recruits endogenous NuRD complex proteins to the promoter template of a transfected reporter gene in HEK293T cells, suggesting that the NuRD complex may potentially play a role in CTIP2-mediated transcriptional repression.

Identification of CTIP2 Target Genes in SK-N-MC Cells—SK-N-MC human neuroblastoma cells express high levels of two splice variants of CTIP2 (Fig. 3B), but undetectable levels of CTIP1 (data not shown). Therefore, these cells were chosen as a model system for the identification of CTIP2 target genes in a neuron-like context, without the potentially confounding effects of complementation by CTIP1. Transcriptome analyses were performed on SK-N-MC cells that had been transfected with CTIP2-specific (CTIP2KD) or mock (CTIP2Mock) siRNAs. The microarray analyses confirmed that CTIP2 knockdown was achieved in CTIP2KD cells (≈60% knockdown at the mRNA level; compare lanes 1 and 2 of Fig. 7A; see also immunoblot in top panel of Fig. 7A). The expression of a number of genes was increased in the CTIP2KD relative to CTIP2Mock cells, consistent with the previously described role of CTIP2 as a repressor of transcription (1, 14, 15). Four of these genes, which have been confirmed as CTIP2 target genes at the mRNA and/or protein levels (data not shown), are listed in Table 1. These genes are heme oxygenase-1 (HMOX-1), fibronectin-1 (FN-1), cadherin-10, and p57KIP2. In light of the neuronal phenotype of CTIP2^{-/-} mice, i.e. defective axonal projections of CSMN (11), it is of interest that two of these genes encode proteins involved in the function of the extracellular matrix. For the purposes of this article, we chose to focus on p57KIP2, which encodes a cyclin-dependent kinase inhibitor belonging to the CIP/KIP family (33). Affymetrix microarray analyses revealed that the expression of p57KIP2 was increased 4-fold in CTIP2KD cells (compare lanes 3 and 4 of Fig. 7A), and this, as well as knockdown of CTIP2 protein, was confirmed at the protein level (Fig. 7B, compare lanes 1 and 2 of middle panel).

Induction of p57KIP2 expression was also observed at the protein level in cells treated with the HDAC inhibitor, TSA (Fig. 7C, compare lanes 1 and 2), consistent with the hypothesis that TSA-sensitive HDACs, such as those present within the NuRD complex, may be involved in dictating the basal expression of p57KIP2 in SK-N-MC cells.

p57KIP2 Is a Direct Target of CTIP2 in SK-N-MC Cells—As the p57KIP2 proximal promoter region contains several puta-
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with p57KIP2 promoter, but not with that of heme oxygenase-1 (HMOX-1; Fig. 8B), even though the latter contains multiple consensus CTIP2 binding sites, and was similarly identified as an induced gene in our transcriptome analyses of CTIP2KD cells (Table 1 and data not shown). Although we found that CTIP2 did not associate with the promoter region of HMOX-1 we tested (see “Materials and Methods”), we cannot exclude the possibility that CTIP2 may associate with other upstream or downstream regulatory regions of HMOX-1, and/or CTIP2 may regulate HMOX-1 expression indirectly via a mechanism(s) that remains to be investigated. These findings demonstrate that endogenously expressed CTIP2 in SK-N-MC cells is associated with the p57KIP2 promoter either directly or indirectly. In light of the transcriptome analyses of CTIP2KD cells, we hypothesize that transcriptional repression is the functional outcome of the interaction of CTIP2 with this promoter template.

In transient transfection experiments with the Lex-Gal-LUC reporter gene, we showed that components of the NuRD complex were recruited to the promoter template upon co-expression with Gal4-CTIP2 (Fig. 6). Therefore, we looked for the presence of the NuRD complex on the promoter of p57KIP2 gene in untransfected SK-N-MC cells by ChIP/re-ChIP analyses. Soluble chromatin was immunoprecipitated with the anti-CTIP2 antibody; immune complexes were released and then subjected to a second immunoprecipitation with the antibodies against different components of the NuRD complex, namely MTA2, HDAC2 and RbAp46/48. Of these proteins, MTA2 appears to be specific for and define the presence of the NuRD complex (17), and our re-ChIP analyses demonstrated the association of MTA2 and CTIP2 with the same fragment of p57KIP2 promoter (upper panel of Fig. 8C), but not with the HMOX-1 promoter (lower panel of Fig. 8C). These findings demonstrate that CTIP2 and the NuRD complex co-occupy the p57KIP2 promoter. To establish this further, we performed re-ChIP analyses with CTIP2 and two additional NuRD complex proteins, HDAC2 and RbAp46/48. HDAC2 and RbAp46/48 were both found to co-occupy the promoter of p57KIP2 (upper panels of Fig. 8, D and E) but not that of HMOX-1 (lower panels of Fig. 8, D and E) with CTIP2. Given our findings that CTIP2 and several components of the NuRD complex (MTA2, HDAC2, RbAp46/48) co-occupy the proximal promoter region of p57KIP2, and that CTIP2 and RbAp46/48 interact directly (Fig. 5), we conclude that CTIP2 recruits the NuRD complex to the p57KIP2 template resulting in transcriptional repression of this gene under basal conditions.

**DISCUSSION**

Previously, CTIP1 and CTIP2 were demonstrated to repress transcription in a predominantly TSA-insensitive manner, possibly because of recruitment of SIRT1 to CTIP1/2-responsive promoters, at least in transiently transfected cells, and in the context of the tk promoter (1, 14–16). However, in the context of the minimal promoter, we found herein that transcriptional repression mediated by CTIP2 was partially sensitive to inhibition by TSA (see Fig. 1), sug-

**TABLE 1**

Up-regulated genes in CTIP2KD SK-N-MC cells

| GenBank ID | Identity                      | Fold up-regulation |
|------------|-------------------------------|--------------------|
| NM_006727  | Cadherin 10, type 2           | 6.5                |
| NM_002133  | Heme oxygenase 1 (HMOX-1)     | 5.1                |
| N33167     | CDK inhibitor 1C (p57KIP2)    | 4.3                |
| X02761     | Fibronectin-1 (FN-1)          | 3.8                |

**FIGURE 7.** CTIP2 knockdown in SK-N-MC cells results in increased expression of p57KIP2. SK-N-MC cells were transfected with 100 ng/ml CTIP2 siRNA (CTIP2KD) or nonspecific siRNA (CTIP2Mock). A, microarray analyses of mRNA levels of CTIP2, p57KIP2, and β-actin in CTIP2KD and CTIP2Mock cells. The results represent mean mRNA levels (shown as relative signal) ± S.E. derived from three independent experiments. The difference of mRNA levels between CTIP2KD and CTIP2Mock cells is statistically significant for CTIP2 and p57KIP2 (p < 0.05; indicated by an asterisk). B, protein expression of CTIP2, p57KIP2, and β-actin as analyzed by immunoblotting in CTIP2KD and CTIP2Mock cells. β-Actin was used as a negative and loading control (bottom panel). C, induction of p57KIP2 expression at the protein level by TSA treatment as demonstrated by immunoblot analysis. SK-N-MC cells were treated with 100 ng/ml TSA or vehicle for 7 h, prior to harvesting, whole cell extract preparation and immunoblotting. Again, β-actin serves a loading control.
gesting that TSA-insensitive, SIRT1-mediated histone deacetylation may not necessarily generalize to all CTIP2-responsive promoters and/or cell types. Other transcriptional repressors have been similarly found to function in TSA-sensitive and -insensitive manners, as well as in a cell- and promoter-dependent contexts. For example, the retinoblastoma tumor suppressor protein (Rb) represses expression of Cdc2, topoisomerase IIα, and thymidylate synthase in a TSA-sensitive manner, but Rb-mediated repression of cyclin A is not reversed by TSA, demonstrating that the mechanism of Rb-mediated transcriptional repression is promoter-specific (34). Similarly, RE-1 silencing transcription factor (REST)-mediated repression of connexin36 was found to be reversible by TSA, whereas that of the two other REST target genes, BDNF and GluR2, was not (35). Moreover, REST-mediated repression of connexin36 was TSA-sensitive only in pancreatic α and β cells, but not in neuronal cells, indicating that REST represses transcription in both promoter- and cell type-specific manners (35). The differential responses of promoters and cell types to TSA may be an important scheme for transcriptional repressors, perhaps including CTIP2, to function in transcriptional regulation.

In addition to TSA-sensitive and NAD+/H11001-dependent HDACs, histone and DNA methylation may also be involved in transcriptional repression (36, 37). As we did not observe complete inhibition of CTIP2-mediated repression by TSA in our present studies, we cannot exclude the possibility that CTIP2 may use other mechanism(s), in addition to TSA-sensitive histone deacetylation, to repress transcription in the context of the minimal promoter.

The CTIP2 complex in from SK-N-MC cells appeared to migrate with a peak centered between 669 and 1000 kDa (see Fig. 4). In contrast, the size of CTIP2 complex in Jurkat cells was found be up to 2000 kDa (15). Although we found that the NuRD complex proteins co-fractionated with CTIP2 in Jurkat cells (data not shown), the difference in apparent masses of the CTIP2 complexes in these two cell types possibly suggests differing compositions of CTIP2 complexes, which may be of functional significance.

The components of the NuRD complex were recruited to a CTIP2-targeted promoter in a CTIP2-dependent manner (see Fig. 6), and were found to co-occupy the promoter of an endogenous CTIP2-target gene in SK-N-MC neuroblastoma cells (see Fig. 8). This recruitment is likely mediated by the direct interaction of CTIP2 with the histone binding proteins, RbAp46 and/or RbAp48 (see Fig. 5). Additionally, many other transcription factors involved in transcriptional repression have been reported to interact with different subunits of the NuRD complex (20–26), suggesting the possibility that the potentially differential recruitment of the NuRD complex to a particular, nucleating transcription factor may result in the formation of a gene-specific repressor complex(es).

Extensive studies of the biological function of the NuRD complex have shown that the components of this complex are required for morphogenesis of Drosophila (23), embryonic patterning, vulva development and signaling in Caenorhabditis elegans (38, 39), and mouse embryogenesis (40). In combination with the data from RT-PCR analysis illustrating
the expression of MBD3, Mi2, HDAC1, and HDAC2 from a very early stage of embryonic development (41), transcriptional silencing by the NuRD complex may play a significant role in embryonic development in many species ranging from nematodes to mammals. In addition, recent reports revealed an important role of the NuRD complex in control cell fate determination during B and T cell development (20, 42).

The crucial function of CTIP2 in the context T cell development, as well as in the development of corticospinal motor neurons (CSMN), suggests that this protein may play a global role during mammalian development. As both CTIP2 and the NuRD complex appear to play significant, and possibly convergent, roles in cell fate determination and differentiation, association of CTIP2 with this complex raises the possibility that the histone deacetylase and chromosome remodeling activities of the NuRD complex may be implicated in regulation of both T cell and CSMN specification and development by CTIP2. This hypothesis may be tested in vivo by analysis of compound mutant mice.

The cdk inhibitor p57KIP2 was newly identified in this report as one of the putative CTIP2 target genes in SK-N-MC neuroblastoma cells. The cdk inhibitor p57KIP2 is a putative tumor suppressor, and has the ability to associate with and inhibit the catalytic activity of a number of cyclin-cdk complexes (33). The human p57KIP2 gene is paternally imprinted in both humans and mice, and the human p57KIP2 locus is on chromosome 11p15, a region that has been implicated in various sporadic human malignancies, and also in Beckwith-Wiedeman syndrome (43).

Several studies suggest that p57KIP2 plays a distinct role in neuronal differentiation, which may or may not be related to the function of this protein as a cdk inhibitor. During embryogenesis, p57KIP2 is expressed in mitotic progenitor cells that migrate away from retinal ventricular zone, and in this context p57KIP2 appears to be required for proper exit from cell cycle (44). Postnatally, however, p57KIP2 is expressed in a restricted population of amacrine neurons, and it has been proposed that p57KIP2 can influence cell fate specification and differentiation long after terminal mitosis (44). In addition, p57KIP2 is expressed in postmitotic differentiating midbrain dopaminergic neurons and is required for the maturation of these cells (45). Interestingly, the mechanism by which p57KIP2 promotes maturation of dopaminergic neuronal cells does not require cdk inhibitor activity but rather is achieved through the direct protein-protein interaction of p57KIP2 with orphan nuclear receptor Nurr1 (45).

The likely recruitment of the NuRD complex to the promoter of p57KIP2 via direct interaction with CTIP2 suggests that the NuRD complex plays a role in transcriptional repression mediated by CTIP2 in a neuron-like context. At present, we do not know if CTIP2 directly regulates expression of p57KIP2 within neuronal subpopulations in vivo, or the possible contribution of the NuRD complex to this and other CTIP2-mediated transcriptional repressive events. Further studies employing the power of Ctip2−/− mice are necessary to clarify role(s) of this protein and the corresponding transcriptional repression pathway(s) in vivo.

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