AN ABSTRACT OF THE DISSERTATION OF

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Title: Transcriptional Repression by CTIP2, a C_2H_2 Zinc Finger Protein.

Abstract approved: _____

Mark E. Leid

CTIP2, a novel C_2H_2 zinc finger protein, is a transcriptional repressor that functions by at least two mechanisms. CTIP2 interacts with and stimulates transcriptional repression mediated by COUP-TF family members. CTIP2 also represses transcription independently of COUP-TF proteins by direct, sequence-specific DNA binding activity. CTIP2 has been implicated in lymphoid malignancies and development of T lymphocytes and the central nervous system (CNS). However, very little is known concerning the molecular mechanism(s) by which CTIP2 functions in these processes. The goal of the studies described herein was to contribute towards a greater understanding of cellular functions of CTIP2 through the characterization of domains of CTIP2 required for transcriptional regulatory activity, identification of CTIP2 target genes, and elucidation of molecular mechanisms underlying the transcriptional repression mediated by CTIP2.

CTIP2 was found to repress transcription by recruiting at least three different histone deacetylases (HDAC) to the promoter template of target genes. The three

HDACs that were identified as being involved in CTIP2-mediated transcriptional repression were SIRT1, HDAC1, and HDAC2. The latter two were found in the same complex, which we identified as the **Nu**cleosome **R**emodeling and **D**eacetylation (NuRD) complex. The SIRT1 and NuRD complexes appeared to be differentially recruited to CTIP2 target genes as a function of promoter, and possibly cellular, context. CTIP2 was found to recruit the NuRD complex, but not the SIRT1 complex, to the promoter of the cyclin-dependent kinase inhibitor *p57KIP2* gene, a new transcriptional target of CTIP2 in neuroblastoma cells.

By analyses of regions of CTIP2 required for functionality, we found that the C-terminal zinc finger (ZnF) 5-7 module conferred self-associative activity, which appeared to be obligatory for high-affinity DNA binding and transcriptional repression of the protein. In contrast, the centrally located ZnF3-4 module of CTIP2 may confer sequence-specific DNA binding activity.

The results described herein provide a framework for understanding the mechanisms underlying the transcriptional regulatory activity of CTIP2, which may contribute to a better understanding of molecular and cellular basis for the activity of CTIP2 *in vivo.*

Transcriptional Repression by CTIP2, a C_2H_2 Zinc Finger Protein

by
Acharawan Khamsiritrakul Topark-Ngarm

A DISSERTATION

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APPROVED:

Major Professor, representing Pharmacy

Dean of the College of Pharmacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Acharawan Khamsiritrakul Topark-Ngarm, Author

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CONTRIBUTION OF AUTHORS

Chapter 2 is reproduced with the permission of the American Society for Biochemistry and Molecular Biology and *The journal of Biological Chemistry.* The experiments described in Fig. 2.1, 2.2, 2.3*A*, and 2.6 were performed by myself in the laboratory of Mark Leid. Brigetta Martinez provided technical assistance in HDAC activity assays. The experiments described in Fig. 2.7 and Fig. 2.8 were performed by Olga Golonzhka with assistance from Brian Barrett Jr. in RNA preparation. Olga Golonzhka was also involved with manuscript preparation. Valerie Peterson performed protein fractionation using column chromatography described in Fig. 2.4, immunoprecipitation and GST pull down experiments described in Fig. 2.3*B* and Fig. 2.5. Kristi Crofoot assisted in preparation of SF9 lysates in the laboratory of Theresa Filtz for the immunoprecipitation experiment described in Fig. 2.5*A-B.*

All experiments described in Chapter 3 were performed by myself in the Laboratory of Mark Leid.

All experiments described in Chapter 4 were performed by myself in the Laboratory of Mark Leid with following exception: Philippe Kastner at the Institut de Génétique et de Biologie Moléculaire et Cellulaire in Illkirch France performed microarray analyses as described in Fig. 4.1. Alignment of mouse and human *Id2* promoter was performed by Mark Leid. ChIP assays on mouse thymocytes and human Jurkat cells described in Fig. 4.2*C* were performed by Olga Golonzhka and Valerie Peterson.

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Chapter 1

Introduction

Eukaryotic DNA is packaged with histones and non-histone proteins to form highly structured entity termed chromatin. The basic repeating unit of chromatin, the nucleosome, includes an octamer of the core histones: two copies of each of the four core histones H2A, H2B, H3, and H4 wrapped nearly twice by 147 bp of DNA. The amino termini of core histones (histone tails) are relatively accessible, unstructured domains that protrude out of the nucleosome. Each nucleosome is separated by 10-60 bp of linker DNA, and the resulting nucleosomal array constitutes a chromatin fiber of approximately 10 nm in diameter (Luger et al., 1997). With the aid of linker proteins, including histone H1, this simple “bead-on-a-string” arrangement is further packaged, into 30-nm fibers with six nucleosomes per turn in a spiral or solenoid arrangement (Kornberg and Lorch, 1999; Hayes and Hansen, 2001). The 30-nm fibers are then further condensed to form more compacted interphase and metaphase chromosomal structures. Such a highly compacted structure of chromatin hinders DNA accessibility of protein factors to regulate cellular processes, such as transcription, replication, repair, and recombination (Peterson and Laniel, 2004).

1.1 Transcriptional regulation by chromatin modifications

In the past several years, studies have revealed two types of enzymatic activities that are capable of altering the chromatin structure (Becker and Horz, 2002; Peterson and Laniel, 2004). The first type of enzymes removes, displaces, or destabilizes nucleosome positioning utilizing the energy derived from ATP hydrolysis (Becker and Horz, 2002; Peterson and Laniel, 2004). The second class of enzymes modifies the core histone tails covalently by a variety of processes (Peterson and Laniel, 2004; Cosgrove and Wolberger, 2005).

1.1.1 ATP-dependent chromatin remodeling

It is well documented that the ATP-dependent nucleosome remodeling enzymes utilize the energy of ATP hydrolysis to overcome the repressive nature of the nucleosome and permit gene transactivation (Becker and Horz, 2002). All eukaryotes contain at least five families of ATP-dependent remodeling complexes: SWI/SNF, ISWI/SNF, NuRD/Mi-2/CHD, INO80 and SWR1, all of which consist of between two and twelve various subunits and a conserved ATPase subunit (Saha et al., 2006). The SWI/SNF and Mi2-/NuRD complexes are two of the most widely studied families. In humans, SWI/SNF-related complexes contain either of two ATPase subunits, BRG1 and BRM, and additional BRM- and BRG-associated factors (BAFs) (Becker and Horz, 2002; Martens and Winston, 2003). Both BRG and BRM appear to be involved in development (Elfring et al., 1998) and have specific roles in gene activation (Kadam and Emerson, 2003). The Mi-2/NuRD complexes consist of the Mi-2α or Mi-2β ATPase subunit and several associated proteins that include histone deacetylases HDAC1 and HDAC2,

RbAp46, RbAp48, MTA1, MTA2, MTA3, MBD3, indicating that post-translational modification by histone deacetylation and ATP-dependent chromatin remodeling are physically linked (Xue et al., 1998; Zhang et al., 1999; Yao and Yang, 2003; Fujita et al., 2004). The NuRD complexes are primarily implicated in gene silencing mediated by sequence-specific transcriptional factors. For example, The NuRD complex has been found to associate with tumor suppressor p53 and repress p53-mediated cell growth and apoptosis (Luo et al., 2000). FOG-1 recruits the NuRD complex to mediate transcriptional repression of its targeted genes in erythroid cells *in vivo* (Hong et al., 2005). Studies using RNAi or targeted gene deletion approaches have shown the requirement of the NuRD complex for embryonic development. Inactivation of *egl-27* and *egr-1* (homologues of the NuRD component MTA1) in *Caenorrhabditis elegans* resulted in abnormal embryonic patterning (Solari et al., 1999). Genetic analyses revealed that Mi-2 in Drosophila participated in Polycomb-mediated repression of *HOX* genes, the process required throughout development (Kehle et al., 1998). The data from RT-PCR analysis illustrating the expression of MBD3, Mi-2, HDAC1 and HDAC2 from a very early stage of embryonic development (Kantor et al., 2003) are consistent with the finding that MBD3 is essential for mouse embyogenesis (Hendrich et al., 2001). Moreover, the NuRD complexes have been shown to play crucial roles for controlling cell fate determination during B and T cell developments. The histone deacetylase activity from the MTA3-containing NuRD complex is required for a function of the transcriptional repressor BCL-6 to regulate cell fate of B lymphocytes during the germinal center reaction by preventing terminal differentiation of B lymphocytes into plasma cells (Fujita et al., 2004). By conditionally inactivating the *Mi-2β* gene in mice, Mi-2β activity has been shown to be required in at least three distinct stages of T cell development: in late

double negative cells to support their transition to the double positive (DP) stage, in DP cells for normal expression of the cell surface marker CD4, and finally in mature T cells for their proliferative expansion (Williams et al., 2004). Besides involving in deacetylation of histone proteins, a portion of the NuRD complexes that contains a methyl CpG-binding protein MBD2 suggest that the NuRD complexes might be recruited to methylated DNA by MBD2 and implicated in a stable transcriptional silencing by DNA methylation (Zhang et al., 1999; Humphrey et al., 2001).

1.1.2 Post-translational modifications

In addition to a class of the ATP-dependent chromatin remodeling enzymes, post-translational modifications of histones can alter chromatin structure. The amino termini of histones, especially histone H3 and H4, and the amino termini and carboxyl termini of histones H2A, H2B and H1, are susceptible to a variety of post-translational modifications: phosphorylation, acetylation, methylation, ubiquitination, sumoylation, ADP ribosylation, glycosylation, biotinylation and carbonylation (Margueron et al., 2005) as shown in Fig.1.1. A given modification on a specific histone residue can be determinant to subsequent modifications of the same histone or another histone molecule. For example, H3-S10 phosphorylation facilitates H3-K9 and H3-K14 acetylation; thereby inhibiting H3-K9 methylation (Margueron et al., 2005). It is now apparent that certain combinations of these modifications can serve as the “histone code” and have profound impacts on transcriptional regulation. Among these modifications, acetylation has been intensively studied in the context of transcriptional regulation. In general, acetylation reduces DNA-nucleosomal interactions to facilitate

transcription, and deacetylation reverses this effect (Wu and Grunstein, 2000; Peterson and Laniel, 2004). However, deacetylation has been shown to be involved in the late process of transcriptional activation, possibly by resetting the basal state of the promoter (Metivier et al., 2003). To modulate transcription, histone acetyltransferases (HATs) and deacetylases (HDACs) are recruited to targeted promoters by interacting with sequence-specific, DNA-binding transcription factors (Wu and Grunstein, 2000; Peterson and Laniel, 2004).

In addition to the modifications that occur on the histone tails, recent evidence provides information that post-translational modifications occurs in the histone core domains as well. While modifications in the histone tails regulate the binding of non-histone proteins to chromatin, core domain modifications may function through distinct mechanisms that affect high-order chromatin structure (Mersfelder and Parthun, 2006). However, it is presently unknown whether the same enzymes that act on the histone tails will also be able to accommodate the very different substrate topology found in the histone core domains.

1.1.2.1 Histone acetylation

Histone acetylation is carried out by histone acetyltransferases (HATs) which catalize the transfer of the acetyl group from acetyl-CoA to the lysine ε-amino groups on the N-terminal tails of histones (Verdone et al., 2005). HATs are quite diverse in terms of their enzymatic activity and regulation. The p300/CBP family proteins are ubiquitously expressed global transcriptional coactivators. CBP (CREB-binding protein) was originally cloned as a coactivator for the promoter-binding transcription

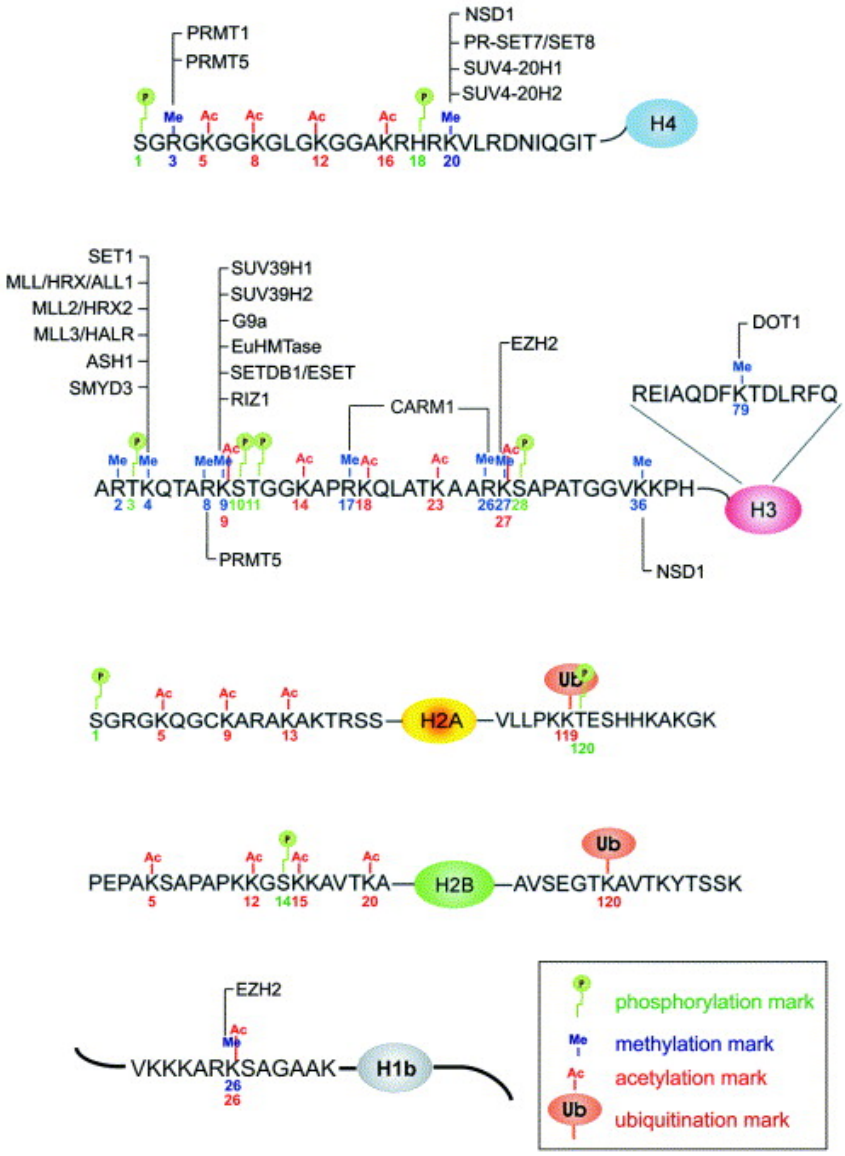

Figure 1.1. **Post-translational modification sites on the histone tails.** Histones are subjected to a variety of post-translational modifications. The modifications on human histones include acetylation (Ac, red), methylation (Me, blue), phosphorylation (P, green) and ubiquitination (Ub, brown). The enzymes responsible for methylation of mammalian histones are listed above or below their target sites. Note that there are several redundant enzymes specific for methylation of histone H3-K4 and H3-K9 (Reprinted from Curr Opin Genet Dev., (2005) 15(2), Margueron R, Trojer P, and Reinberg D., The key to development: interpreting the histone code?, 163-176, Copyright (2006), with permission from Elsevier).

factor CREB (cyclic adenosine monophosphate response-element-binding protein) (Lundblad et al., 1995). p300 was initially cloned by its association with the viral oncoprotein E1A, which is required for the full transforming phenotype of adenovirus (Eckner et al., 1994). Besides CREB and E1A, a large number of proteins such as nuclear hormone receptors, and oncoprotein-related activators such as c-Fos, c-Jun, and c-Myb, have been shown to recruit p300/CBP to activate transcription of specific genes (Luo et al., 1999; Verdone et al., 2005).

p300 and CBP not only acetylate histone, they also use the non-histone proteins as substrates, which include p53, GATA-1, nuclear receptor factor SRC-1, ACTR, and TIF2, and general factors TFIIE and TFIIF (Verdone et al., 2005). The p/CAF (a p300/CBP-interacting protein) belongs to a GNAT (GCN5-related N-acetyltransferase) family of HATs. p/CAF is structurally similar to GCN5 protein from yeast and tetrahymena, and has been shown to interact with p300/CBP (Yang et al., 1996). Studies investigating the role of p/CAF in transcription have shown its recruitment as a HAT and as a coactivator in several processes such as myogenesis, nuclear-receptor-mediated activation, and growth-factor-signaled activation (Verdone et al., 2005).

1.1.2.2 Histone deacetylation

There are three classes of histone deacetylases (HDACs) have been identified to date based on their homology to yeast histone deacetylases.

- Class I HDACs are homologous to the yeast RPD3 gene. Members of class I HDACs are HDAC1, HDAC2, HDAC3, and HDAC8 (Yang et al., 1997; Dangond et al., 1998; Emiliani et al., 1998; Hu et al., 2000; Van den Wyngaert et al., 2000).

All four members have a deacetylase catalytic domain, and HDAC1 and HDAC2 have the C-terminal RB binding motif adjacent to a basic region. Class I HDACs generally localize to the nucleus, and are ubiquitously expressed in many human cell lines and tissues (Thiagalingam et al., 2003).

Class I HDACs have been demonstrated to associate with the silencing CoREST complex (Fischle et al., 1999; Grozinger et al., 1999; Zhou et al., 2000; Zhou et al., 2001; Fischer et al., 2002; Gao et al., 2002), as well as the Sin3 and Mi-2/NuRD corepressor complexes (Zhang et al., 1997; Kao et al., 1998; Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998; Zhang et al., 1998; Li et al., 2000; You et al., 2001; Yao and Yang, 2003). HDAC3 has been shown to form a large complex with SMRT and nuclear receptor corepressor (NCoR) (Li et al., 2000; Yoon et al., 2003).

- Class II HDACs have high homology to the yeast Hda1, consisting of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10 and possibly HDAC11 (Fischle et al., 1999; Grozinger et al., 1999; Zhou et al., 2000; Zhou et al., 2001; Fischer et al., 2002; Gao et al., 2002). These HDACs are twice as large (approximately 1000 amino acids) as the class I HDACs, and are defined by a C-terminal catalytic domain, except for HDAC6, which has a second catalytic domain in an N-terminus. HDAC10 has an N-terminal catalytic domain and a C-terminal pseudorepeat that shares homology with the catalytic domain. Expression of class II HDACs is relatively more restricted, with the highest levels in the heart, brain, and skeletal muscle (Cress and Seto, 2000; Verdel et al., 2000; Hubbert et al., 2002; Zhang et al., 2002). The most recent cloned and characterized

HDAC11 shares homology in the core catalytic domains to both class I and class II HDACs. The size of the protein is in line with class I HDACs, but HDAC11 is differentially expressed in heart, brain, skeletal muscle, kidney, which is typical of class II HDACs (Gao et al., 2002).

All eleven members of class I and Class II HDACs have been shown to be sensitive to histone deacetylase-specific inhibitors that include trichostatin A (TSA). These HDACs are components of large protein complexes *in vivo* that direct gene-specific regulation of transcription, hormone signaling, the cell cycle and differentiation, and DNA repair (Thiagalingam et al., 2003).

- Class III HDACs, sirtuins, form a structurally distinct class of TSA-insensitive, but NAD^+-dependent enzymes that are related to yeast SIR2 proteins (Frye, 1999). In humans, the sirtuin protein family is comprised of seven members, SIRT1 through 7 (Blander and Guarente, 2004). Of these, SIRT1 (sirtuin 1) possesses the highest homology to yeast SIR2, and has been shown to play pivotal roles in apoptosis, cell survival, aging, transcriptional regulation, and metabolism (Denu, 2005). SIRTs not only deacetylate histone proteins (Vaquero et al., 2004; Michishita et al., 2005), they also deacetylate non-histone substrates such as NF-kappaB (Yeung et al., 2004), p300 (Bouras et al., 2005), MEF2 (Zhao et al., 2005), and FOXO1 (Yang et al., 2005). SIRT1 also deacetylates and antagonizes p53-dependent apoptosis and growth inhibition in response to DNA damage and oxidative stress (Luo et al., 2000; Vaziri et al., 2001; Langley et al., 2002).

1.2 C_2H_2 zinc finger proteins

Zinc-finger-containing proteins constitute the most abundant protein superfamily in the mammalian genome, and are best known as transcriptional regulators (Ravasi et al., 2003). Zinc finger proteins can bind to DNA, RNA, other proteins, or lipids as a modular domain in combination with other conserved structures. Association of many zinc finger proteins with DNA- and/or protein-binding domains allows the formation of multi-protein complexes in which DNA-binding motifs recognize a target sequence in a specific manner or protein-protein interaction domains allow the assembly of multiprotein regulatory complexes, commonly involved in chromatin remodeling (Aasland et al., 1995; David et al., 1998).

Among one of the most frequent zinc finger domains that has been characterized is the C_2H_2 zinc finger, which was discovered as repetitive motifs in transcription factor TFIIIA of *Xenopus laevis* (Miller et al., 1985). The structural motif which defines the C_2H_2 domain is the coordination of a central zinc ion by cysteine and histidine residues by forming three-dimensional structure---an alpha helix and two beta strands with antiparallel orientation forming a fingerlike bundle held together by a zinc ion (Lee et al., 1989; Suzuki and Yagi, 1994). An individual zinc binding domain contains approximately 30 amino acids conforming the following consensus sequence: (Tyr,Phe)-X-Cys-$X_{2,4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His-X_{2-6}, where as X is a variable amino acid (Lee et al., 1989). The individual zinc finger units are joined into arrays of two or more by relatively short linker sequences. One finger recognizes 3 or 4 bp along the major groove of double-stranded DNA, and the minimum numbers of fingers required for specific DNA binding is two (Choo et al., 1997). The C_2H_2 Zinc-finger proteins are further divided into

subfamilies, based on their structures (spacing and nature of their zinc-chelating residues; cysteine-histidine or cysteine-cysteine) and functions (Krishna et al., 2003; Ravasi et al., 2003). The most characterized domains commonly found in these zinc finger proteins include the E3 ubiquitin-ligase RING-H2 finger (Lorick et al., 1999), the Kruppel-associated box (KRAB) (Bellefroid et al., 1995; Losson, 1997), the poxvirus and zinc finger (POZ) domain (Bardwell and Treisman, 1994).

1.3 A novel family of C_2H_2 zinc finger proteins

CTIP1 (BCL11A, Evi9) and CTIP2 (BCL11B, Rit-1β) were first identified and characterized as two novel and highly related C_2H_2 zinc finger proteins (Avram et al., 2000). CTIP1 protein contains 776 amino acids with four zinc finger motifs, whereas CTIP2 consists of 813 amino acids with seven zinc finger motifs. Both CTIP1 and CTIP2 share high sequence identity throughout the proteins (61% Identity), especially within zinc finger motifs. Two centrally located C_2H_2 zinc fingers of these two proteins are nearly identical (93% identity). Also, the first two of three additional C_2H_2 zinc fingers at the carboxyl terminus of CTIP2 are related (71% identity) to the central zinc fingers of both CTIP1 and CTIP2 (Avram et al., 2000). However, these novel zinc finger proteins display distinct expressions in various tissues and differential implication in diseases (Avram et al., 2000; Nakamura et al., 2000; Saiki et al., 2000; Bernard et al., 2001; Satterwhite et al., 2001; Wakabayashi et al., 2003a; Leid et al., 2004; Przybylski et al., 2005). In this manuscript, we mainly focus our interest on CTIP2.

1.3.1 Role of CTIP2 in the T cell development and malignancies

From bone marrow, the precursors that give rise to T cells migrate to the thymus for their differentiation (see Fig. 1.2). The earliest T cell progenitors are present as the CD4 and CD8 double negative 1 (DN1) population and proliferate extensively. These cells are characterized as being $CD4^-$ and $CD25^-$ and still have the ability to mature into natural killer cells (NK). DN1 cells can also still differentiate to B cells, DCs and macrophages. T cell commitment begins upon activation of CD25 expression at the DN2 stage and is followed by initiation of gene rearrangements at TCR γ, δ, and β loci. At the DN2 stage, cells can still differentiate to DCs and NK cells. At the DN3 stage, the cells downregulate expression of CD 44 while retaining CD25 expression. This stage is critical for the transition to DN4 stage. If the gene rearrangements of TCR genes fail to generate an in-frame TCRβ, TCRδ, and TCRγ, then the cells do not survive. There are two choices available for cells at this stage. Cells that succeed in expressing TCRγ and TCRδ proteins by the DN3 stage develop into mature TCR $\gamma\delta$ cells. If they are successful at TCRβ arrangement and the express pre-TCR complex, the cells then undergo proliferation and differentiation into $CD4^+$ $CD8^+$ (DP cells), in a process called β-selection. DP cells then exit the cell-cycle and begin TCRα gene rearrangement. The expression of $\alpha\beta$ TCR allows DP cells to undergo major histocompatibility complex (MHC)-mediated positive or negative selection for self/non-self discrimination. Positively -selected DP thymocytes downregulate either CD4 or CD8 expression. Now the cells become single positive (SP) mature T lineage cells.

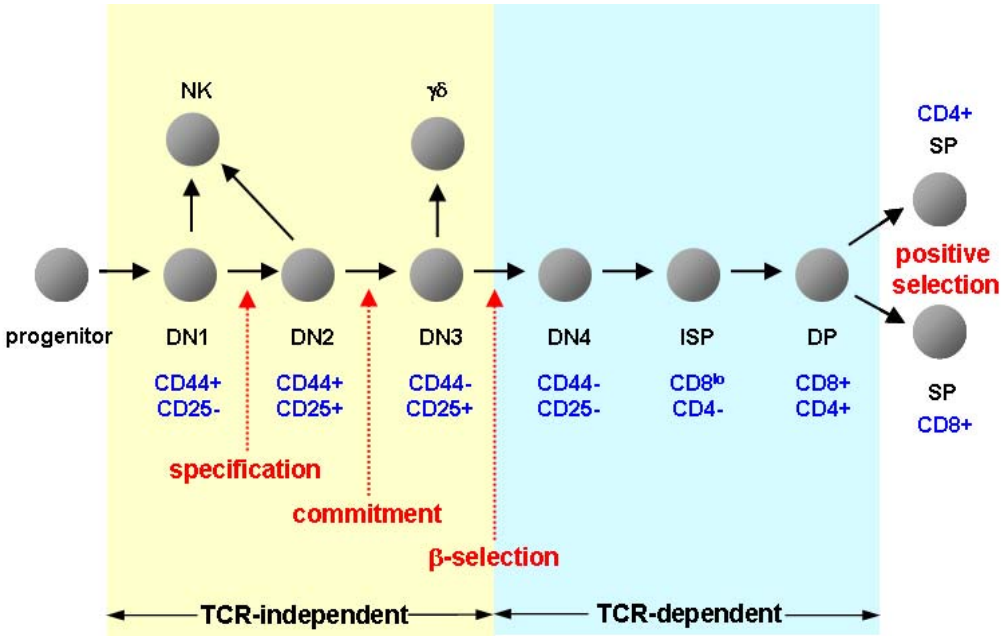

Figure 1.2. **Schematic represent diagram of T cell development.** The developmental stages are shown together with their characteristic cell surface markers. TCR arrangements at the appropriate stage are indicated by dotted arrow lines. (Adapted from Rothenberg and Taghon, 2005)

Finally, the mature T cells exit from the thymus to take up their roles in the immune system (Quong et al., 2002; Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005).

T cells are distinct among hematopoietic cells by their potentially infinite proliferative life spans. Environmental signals from antigen recognition and/or other ligand/receptor interactions can trigger differentiative specialization even after their mature features are in place. Thus, T cell development is a complex process with multiple stepwise stages in which different choices are available to cells. A number of transcription factors have been identified as regulatory proteins for controlling T cell development. However, many of these factors are recurrently used at different stages (Quong et al., 2002; Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005). Analyses of function of transcription factor GATA-3 by conditional knockout experiments have demonstrated that it is needed for β-selection and proliferation in the DN to the DP transition (Pai et al., 2003). It also has a prominent role at positive selection and promoting the development of CD4 SP cells (Nawijn et al., 2001; Hernandez-Hoyos et al., 2003; Pai et al., 2003). Type I basic helix-loop-helix (bHLH) E proteins are known to regulate T cell development at early stage prior to the DP stage (Engel et al., 2001; Staal et al., 2001; Greenbaum and Zhuang, 2002; Pan et al., 2002).

A number of T cell target genes are identified to be positively regulated by this class of proteins (Petersson et al., 2002; Tremblay et al., 2003). Forced expression of Id2, an inhibitor of DNA binding and/or differentiation 2, which heterodimerizes with E2A-class bHLH factors to block their DNA binding, completely aborts early T cell specification (Heemskerk et al., 1997; Morrow et al., 1999). While Id2 protein contributes to proliferation and other activation responses (Morrow et al., 1999), E proteins actively

promote G1 arrest at developmental checkpoints (Engel et al., 2001). Therefore, the balance between E protein and Id activity is essential for T-lineage development. Another key regulatory protein during T cell development is the membrane signaling receptor Notch1. Without Notch1, pluripotent precursors cannot develop into T cells at all, and B cells develop in the thymus instead (Pear and Radtke, 2003; Radtke et al., 2004; Robey and Bluestone, 2004). Notch family proteins also play more complex roles later in TCR-dependent selection events (Wolfer et al., 2002; Huang et al., 2003; Ciofani et al., 2004).

CTIP2 is predominantly expressed in mouse thymocytes, and is required for development of $\alpha\beta$ T lymphocytes but not for development of cells in B or $\gamma\delta$ T cell lineages (Wakabayashi et al., 2003a). The *CTIP2*-deficient thymocytes displayed unsuccessful recombination of Vβ to Dβ and lacked the pre-T cell receptor (TCR) complex on the surface, resulting in a failure in the transition of thymocytes from the DN3 stage to the DN4 stage and apoptosis (Wakabayashi et al., 2003a; Inoue et al., 2006). These results suggest that CTIP2 may be a key regulator of both differentiation and survival during thymocyte development. Besides its requirement for TCR expression, CTIP2 may control survival of $\alpha\beta$ T lymphocytes through other mechanisms as transgenic expression of TCR$\alpha\beta$ only partly rescues this apoptosis effect of *CTIP2*-deficient mice (Inoue et al., 2006). Inactivation of CTIP2 by homozygous deletions and point mutations of CTIP2 gene are associated with thymic lymphomagenesis in mouse (Wakabayashi et al., 2003b; Sakata et al., 2004), and ectopic expression of CTIP2 in HeLa cells resulted in suppression of cell growth (Wakabayashi et al., 2003b), indicating a potential role of CTIP2 as a tumor suppressor. In humans, a [t(15;14)(q35;q32)] translocation event involving the genomic locus of CTIP2 appeared to result in acute T

lymphoblastic leukemia (T-ALL) (Bernard et al., 2001). In addition, disruption of *CTIP2* gene through inv (14)(q11.2q32.31) that disturbed the expression of normal CTIP2 transcripts resulted in T-cell acute lymphoblastic leukemia (T-ALL) (Przybylski et al., 2005). However, the role of CTIP2 in human T cell carcinogenesis is not completely understood.

1.3.2 Role of CTIP2 in the central nervous system

Neurons constitute the most diverse cell population of any organ. During the development of the central nervous system, neuronal progenitors undergo precise stepwise differentiation to become neuronal subtypes with defined morphological, physiological and molecular characteristics. Considerable progress has been made in identifying the genes that determine cell fate and subtype specification (Bertrand, 2002).

CTIP2 has recently been shown to be among the genes that control neuronal subtype specification (Arlotta et al., 2005). CTIP2 is expressed diffusely in the mouse embryo at 10.5 days post-coitum (d.p.c), but becomes increasing restricted to the central nervous system and the thymus as the fetus approaches birth (Leid et al., 2004). Within the central nervous system, CTIP2 is expressed with high level in developing cerebral cortex primarily in layer V and VI, the striatum, olfactory bulb, hippocampus, limbic system, basal ganglia, and also in the intermediate region of the spinal cord (Leid et al., 2004; Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005). The specific domains of expression are maintained into adulthood (Leid et al., 2004). Loss of function experiments has demonstrated that CTIP2 is necessary for corticospinal motor neurons (CSMN) development *in vivo* (Arlotta et al., 2005). Specifically, CSMNs in *CTIP2*-null

mice exhibit defective axonal projections to the spinal cord, pathfinding, and fasciculation during the formation of the corticospinal tract. This phenotype is similar to that observed in mice lacking *Fezl*, a gene encoding a forebrain zinc-finger-like transcriptional repressor, which is also expressed in cortical layer V and VI, and required for specification of corticospinal projections. In addition, CTIP2 was found to be dysregulated in the cerebral cortex of *Fezl*-null mice (Chen et al., 2005; Molyneaux et al., 2005). It is possible that these *CTIP2* and *Fezl* may control CSMN development by acting in a common pathway.

1.3.3 Transcriptional repression mediated by CTIP2.

CTIP2 is a transcriptional repressor that has been demonstrated to modulate transcription by at least two mechanisms. CTIP2 is recruited to the template either by interaction with COUP-TFs (Avram et al., unpublished data), resulting in transcriptional repression of a reporter gene harboring a COUP-TF binding site. Later, CTIP2 was found to bind directly and specifically to GC-rich binding sites (5'-GGCCGG-3' of the upper strand; Avram et al., 2002). By direct, sequence-specific DNA binding activity, CTIP2 was found to repress transcription of a reporter gene harboring this binding site in its promoter region in a manner that was unaffected by COUP-TF proteins, suggesting that CTIP2 may function independently of COUP-TF proteins in some cell types and promoter contexts (Avram et al., 2002). This alternative mechanism may be of physiological relevance in cells that are devoid of COUP-TF expression, i.e. lymphoid-derived cells, or some neuronal cells, notably, the Purkinje cell layer of the cerebellum and the basal ganglia (Leid et al., 2004; Shepherd and Leid, manuscript in preparation).

In either a COUP-TF-dependent or –independent context, CTIP2 appears to repress transcription involving the recruitment of a class III TSA-insenstive HDAC, SIRT1 (Avram et al., unpublished data; Avram et al., 2002; Senawong et al., 2003). However, it is not known if CTIP2 mediates transcriptional repression via other mechanism(s) in addition to TSA-insensitive histone deacetylation.

Although several reports have demonstrated that CTIP2 is required for T cell and the central nervous system development, little is known concerning the molecular mechanism(s) by which CTIP2 may function in these processes. Therefore, more information regarding the properties, the molecular mechanism(s), and target genes of this C_2H_2 zinc finger protein are necessary for a thorough understanding of the biological function(s) of the protein.

1.4 Research objectives

A variety of biochemical and molecular biological techniques were employed herein to study CTIP2 toward the goal of elucidating the molecular basis for this transcriptional regulatory activity of the protein in mammalian cells. The specific aims are detailed in the following paragraphs.

Chapter 2 describes studies that examined the involvement of the nucleosome remodeling and deacetylation complex (NuRD) in transcriptional repression mediated by CTIP2. Reporter gene assays were conducted to assess the role of the TSA-sensitive histone deacetylation in transcriptional repression mediated by CTIP2. The alterations in levels of acetylated histone H3 and H4 at the promoter region of a reporter gene were

also determined by chromatin immonuprecipitation (ChIP) assays. Histone deacetylase (HDAC) activity assays were conducted to examine the HDAC activity associated with CTIP2 complex(es). The interaction between CTIP2 and components of the NuRD complex was evaluated in *in vitro* and in mammalian cells. CTIP2 truncation mutants were generated to identify the amino acid regions that are necessary for binding to RbAp46 and RbAp48, core components of the NuRD complex. The recruitment of the NuRD complex to CTIP2-targeted promoter was examined by ChIP assays. CTIP2 target genes in neuroblastoma cells were identified using Affymetrix microarray analyses, comparing cells in which CTIP2 had been knocked down using specific siRNA to cells treated with non-specific siRNAs.

Chapter 3 describes a series of studies that were designed to understand the molecular basis for self-association and DNA binding by CTIP2. Truncation and point mutants of CTIP2 were generated to identify the amino acid regions or the zinc fingers that are required for these activities. The DNA binding domain(s) and sequence-specific DNA binding activity of CTIP2 were examined using the Avidin (Streptavidin)-Biotin Complex with DNA (ABCD) binding assay. Self-associative properties of CTIP2 were studied by GST pull-down assays. The requirement of self-interaction and/or DNA binding domain (s) for CTIP2-mediated transcriptional repression was determined by the reporter gene assays in mammalian cells.

Chapter 4 describes studies that examined the transcriptional targets of CTIP2 during T cell developmental pathway. *Id2* was identified as a CTIP2 target gene in pooled DN and $\gamma\delta$ thymocytes of *CTIP2*-null mice by Affymetrix microarray analyses. The association of

CTIP2 with the *Id2* promoter was determined by ChIP analyses of mouse thymocytes and a human T cell leukemia cell line. Direct interaction between CTIP2 and the *Id2* promoter was determined by the ABCD binding assays. The responsiveness of *Id2* promoter to CTIP2 was examined by reporter gene assays in mammalian cells.

1.5 References

Aasland, R., Gibson, T. J., et al. (1995). "The PHD finger: implications for chromatin-mediated transcriptional regulation." Trends Biochem Sci **20**(2): 56-9.

Arlotta, P., Molyneaux, B. J., et al. (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron **45**(2): 207-21.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Bardwell, V. J. and Treisman, R. (1994). "The POZ domain: a conserved protein-protein interaction motif." Genes Dev **8**(14): 1664-77.

Becker, P. B. and Horz, W. (2002). "ATP-dependent nucleosome remodeling." Annu Rev Biochem **71**: 247-73.

Bellefroid, E. J., Marine, J. C., et al. (1995). "Emergence of the ZNF91 Kruppel-associated box-containing zinc finger gene family in the last common ancestor of anthropoidea." Proc Natl Acad Sci U S A **92**(23): 10757-61.

Bernard, O. A., Busson-LeConiat, M., et al. (2001). "A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia." Leukemia **15**(10): 1495-504.

Bertrand, D. (2002). "Neuronal Nicotinic Acetylcholine Receptors and Epilepsy." Epilepsy Curr **2**(6): 191-193.

Blander, G. and Guarente, L. (2004). "The Sir2 family of protein deacetylases." Annu Rev Biochem **73**: 417-35.

Bouras, T., Fu, M., et al. (2005). "SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1." J Biol Chem **280**(11): 10264-76.

Chen, B., Schaevitz, L. R., et al. (2005). "Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex." Proc Natl Acad Sci U S A **102**(47): 17184-9.

Choo, Y., Castellanos, A., et al. (1997). "Promoter-specific activation of gene expression directed by bacteriophage-selected zinc fingers." J Mol Biol **273**(3): 525-32.

Ciofani, M., Schmitt, T. M., et al. (2004). "Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation." J Immunol **172**(9): 5230-9.

Cosgrove, M. S. and Wolberger, C. (2005). "How does the histone code work?" Biochem Cell Biol **83**(4): 468-76.

Cress, W. D. and Seto, E. (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

Dangond, F., Hafler, D. A., et al. (1998). "Differential display cloning of a novel human histone deacetylase (HDAC3) cDNA from PHA-activated immune cells." Biochem Biophys Res Commun **242**(3): 648-52.

David, G., Alland, L., et al. (1998). "Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein." Oncogene **16**(19): 2549-56.

Denu, J. M. (2005). "The Sir 2 family of protein deacetylases." Curr Opin Chem Biol **9**(5): 431-40.

Eckner, R., Arany, Z., et al. (1994). "The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family." Cold Spring Harb Symp Quant Biol **59**: 85-95.

Elfring, L. K., Daniel, C., et al. (1998). "Genetic analysis of brahma: the Drosophila homolog of the yeast chromatin remodeling factor SWI2/SNF2." Genetics **148**(1): 251-65.

Emiliani, S., Fischle, W., et al. (1998). "Characterization of a human RPD3 ortholog, HDAC3." Proc Natl Acad Sci U S A **95**(6): 2795-800.

Engel, I., Johns, C., et al. (2001). "Early thymocyte development is regulated by modulation of E2A protein activity." J Exp Med **194**(6): 733-45.

Fischer, D. D., Cai, R., et al. (2002). "Isolation and characterization of a novel class II histone deacetylase, HDAC10." J Biol Chem **277**(8): 6656-66.

Fischle, W., Emiliani, S., et al. (1999). "A new family of human histone deacetylases related to Saccharomyces cerevisiae HDA1p." J Biol Chem **274**(17): 11713-20.

Frye, R. A. (1999). "Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity." Biochem Biophys Res Commun **260**(1): 273-9.

Fujita, N., Jaye, D. L., et al. (2004). "MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation." Cell **119**(1): 75-86.

Gao, L., Cueto, M. A., et al. (2002). "Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family." J Biol Chem **277**(28): 25748-55.

Greenbaum, S. and Zhuang, Y. (2002). "Regulation of early lymphocyte development by E2A family proteins." Semin Immunol **14**(6): 405-14.

Grozinger, C. M., Hassig, C. A., et al. (1999). "Three proteins define a class of human histone deacetylases related to yeast Hda1p." Proc Natl Acad Sci U S A **96**(9): 4868-73.

Hayes, J. J. and Hansen, J. C. (2001). "Nucleosomes and the chromatin fiber." Curr Opin Genet Dev **11**(2): 124-9.

Heemskerk, M. H., Blom, B., et al. (1997). "Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3." J Exp Med **186**(9): 1597-602.

Hendrich, B., Guy, J., et al. (2001). "Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development." Genes Dev **15**(6): 710-23.

Hernandez-Hoyos, G., Anderson, M. K., et al. (2003). "GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation." Immunity **19**(1): 83-94.

Hong, W., Nakazawa, M., et al. (2005). "FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1." Embo J **24**(13): 2367-78.

Hu, E., Chen, Z., et al. (2000). "Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor." J Biol Chem **275**(20): 15254-64.

Huang, T. T., Zong, Y., et al. (2003). "TCR-mediated hyper-responsiveness of autoimmune Galphai2(-/-) mice is an intrinsic naive CD4(+) T cell disorder selective for the Galphai2 subunit." Int Immunol **15**(11): 1359-67.

Hubbert, C., Guardiola, A., et al. (2002). "HDAC6 is a microtubule-associated deacetylase." Nature **417**(6887): 455-8.

Humphrey, G. W., Wang, Y., et al. (2001). "Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1." J Biol Chem **276**(9): 6817-24.

Inoue, J., Kanefuji, T., et al. (2006). "Expression of TCR alpha beta partly rescues developmental arrest and apoptosis of alpha beta T cells in Bcl11b-/- mice." J Immunol **176**(10): 5871-9.

Kadam, S. and Emerson, B. M. (2003). "Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes." Mol Cell **11**(2): 377-89.

Kantor, B., Makedonski, K., et al. (2003). "Expression and localization of components of the histone deacetylases multiprotein repressory complexes in the mouse preimplantation embryo." Gene Expr Patterns **3**(6): 697-702.

Kao, H. Y., Ordentlich, P., et al. (1998). "A histone deacetylase corepressor complex regulates the Notch signal transduction pathway." Genes Dev **12**(15): 2269-77.

Kehle, J., Beuchle, D., et al. (1998). "dMi-2, a hunchback-interacting protein that functions in polycomb repression." Science **282**(5395): 1897-900.

Kornberg, R. D. and Lorch, Y. (1999). "Chromatin-modifying and -remodeling complexes." Curr Opin Genet Dev **9**(2): 148-51.

Krishna, S. S., Majumdar, I., et al. (2003). "Structural classification of zinc fingers: survey and summary." Nucleic Acids Res **31**(2): 532-50.

Langley, E., Pearson, M., et al. (2002). "Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence." Embo J **21**(10): 2383-96.

Lee, M. S., Gippert, G. P., et al. (1989). "Three-dimensional solution structure of a single zinc finger DNA-binding domain." Science **245**(4918): 635-7.

Leid, M., Ishmael, J. E., et al. (2004). "CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis." Gene Expr Patterns **4**(6): 733-9.

Li, J., Wang, J., et al. (2000). "Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3." Embo J **19**(16): 4342-50.

Lorick, K. L., Jensen, J. P., et al. (1999). "RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination." Proc Natl Acad Sci U S A **96**(20): 11364-9.

Losson, R. (1997). "KRAB zinc finger proteins and nuclear receptors: a possible cross-talk." Biol Chem **378**(7): 579-81.

Luger, K., Rechsteiner, T. J., et al. (1997). "Characterization of nucleosome core particles containing histone proteins made in bacteria." J Mol Biol **272**(3): 301-11.

Lundblad, J. R., Kwok, R. P., et al. (1995). "Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP." Nature **374**(6517): 85-8.

Luo, J., Su, F., et al. (2000). "Deacetylation of p53 modulates its effect on cell growth and apoptosis." Nature **408**(6810): 377-81.

Luo, K., Stroschein, S. L., et al. (1999). "The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling." Genes Dev **13**(17): 2196-206.

Margueron, R., Trojer, P., et al. (2005). "The key to development: interpreting the histone code?" Curr Opin Genet Dev **15**(2): 163-76.

Martens, J. A. and Winston, F. (2003). "Recent advances in understanding chromatin remodeling by Swi/Snf complexes." Curr Opin Genet Dev **13**(2): 136-42.

Mersfelder, E. L. and Parthun, M. R. (2006). "The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure." Nucleic Acids Res **34**(9): 2653-62.

Metivier, R., Penot, G., et al. (2003). "Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter." Cell **115**(6): 751-63.

Michishita, E., Park, J. Y., et al. (2005). "Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins." Mol Biol Cell **16**(10): 4623-35.

Miller, K. G., Tower, J., et al. (1985). "A complex control region of the mouse rRNA gene directs accurate initiation by RNA polymerase I." Mol Cell Biol **5**(3): 554-62.

Molyneaux, B. J., Arlotta, P., et al. (2005). "Fezl is required for the birth and specification of corticospinal motor neurons." Neuron **47**(6): 817-31.

Morrow, M. A., Mayer, E. W., et al. (1999). "Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages." Mol Immunol **36**(8): 491-503.

Nakamura, T., Yamazaki, Y., et al. (2000). "Evi9 encodes a novel zinc finger protein that physically interacts with BCL6, a known human B-cell proto-oncogene product." Mol Cell Biol **20**(9): 3178-86.

Nawijn, M. C., Ferreira, R., et al. (2001). "Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice." J Immunol **167**(2): 715-23.

Pai, S. Y., Truitt, M. L., et al. (2003). "Critical roles for transcription factor GATA-3 in thymocyte development." Immunity **19**(6): 863-75.

Pan, L., Hanrahan, J., et al. (2002). "An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus." J Immunol **168**(8): 3923-32.

Parker, M. H., Perry, R. L., et al. (2006). "MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation." Mol Cell Biol **26**(15): 5771-83.

Pear, W. S. and Radtke, F. (2003). "Notch signaling in lymphopoiesis." Semin Immunol **15**(2): 69-79.

Peterson, C. L. and Laniel, M. A. (2004). "Histones and histone modifications." Curr Biol **14**(14): R546-51.

Petersson, K., Ivars, F., et al. (2002). "The pT alpha promoter and enhancer are direct targets for transactivation by E box-binding proteins." Eur J Immunol **32**(3): 911-20.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Quong, M. W., Romanow, W. J., et al. (2002). "E protein function in lymphocyte development." Annu Rev Immunol **20**: 301-22.

Radtke, F., Wilson, A., et al. (2004). "Notch signaling in T- and B-cell development." Curr Opin Immunol **16**(2): 174-9.

Ravasi, T., Huber, T., et al. (2003). "Systematic characterization of the zinc-finger-containing proteins in the mouse transcriptome." Genome Res **13**(6B): 1430-42.

Robey, E. A. and Bluestone, J. A. (2004). "Notch signaling in lymphocyte development and function." Curr Opin Immunol **16**(3): 360-6.

Rothenberg, E. V. and Dionne, C. J. (2002). "Lineage plasticity and commitment in T-cell development." Immunol Rev **187**: 96-115.

Rothenberg, E. V. and Taghon, T. (2005). "Molecular genetics of T cell development." Annu Rev Immunol **23**: 601-49.

Saha, A., Wittmeyer, J., et al. (2006). "Chromatin remodelling: the industrial revolution of DNA around histones." Nat Rev Mol Cell Biol **7**(6): 437-47.

Saiki, Y., Yamazaki, Y., et al. (2000). "Human EVI9, a homologue of the mouse myeloid leukemia gene, is expressed in the hematopoietic progenitors and down-regulated during myeloid differentiation of HL60 cells." Genomics **70**(3): 387-91.

Sakata, J., Inoue, J., et al. (2004). "Involvement of V(D)J recombinase in the generation of intragenic deletions in the Rit1/Bcl11b tumor suppressor gene in gamma-ray-induced thymic lymphomas and in normal thymus of the mouse." Carcinogenesis **25**(6): 1069-75.

Satterwhite, E., Sonoki, T., et al. (2001). "The BCL11 gene family: involvement of BCL11A in lymphoid malignancies." Blood **98**(12): 3413-20.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Solari, F., Bateman, A., et al. (1999). "The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning." Development **126**(11): 2483-94.

Staal, F. J., Weerkamp, F., et al. (2001). "Transcriptional control of t lymphocyte differentiation." Stem Cells **19**(3): 165-79.

Suzuki, M. and Yagi, N. (1994). "DNA recognition code of transcription factors in the helix-turn-helix, probe helix, hormone receptor, and zinc finger families." Proc Natl Acad Sci U S A **91**(26): 12357-61.

Thiagalingam, S., Cheng, K. H., et al. (2003). "Histone deacetylases: unique players in shaping the epigenetic histone code." Ann N Y Acad Sci **983**: 84-100.

Tong, J. K., Hassig, C. A., et al. (1998). "Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex." Nature **395**(6705): 917-21.

Tremblay, M., Herblot, S., et al. (2003). "Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL." J Biol Chem **278**(15): 12680-7.

Van den Wyngaert, I., de Vries, W., et al. (2000). "Cloning and characterization of human histone deacetylase 8." FEBS Lett **478**(1-2): 77-83.

Vaquero, A., Scher, M., et al. (2004). "Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin." Mol Cell **16**(1): 93-105.

Vaziri, H., Dessain, S. K., et al. (2001). "hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase." Cell **107**(2): 149-59.

Verdel, A., Curtet, S., et al. (2000). "Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm." Curr Biol **10**(12): 747-9.

Verdone, L., Caserta, M., et al. (2005). "Role of histone acetylation in the control of gene expression." Biochem Cell Biol **83**(3): 344-53.

Wakabayashi, Y., Watanabe, H., et al. (2003a). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Wakabayashi, Y., Inoue, J., et al. (2003b). "Homozygous deletions and point mutations of the Rit1/Bcl11b gene in gamma-ray induced mouse thymic lymphomas." Biochem Biophys Res Commun **301**(2): 598-603.

Williams, C. J., Naito, T., et al. (2004). "The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development." Immunity **20**(6): 719-33.

Wolfer, A., Wilson, A., et al. (2002). "Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes." Immunity **16**(6): 869-79.

Wu, J. and Grunstein, M. (2000). "25 years after the nucleosome model: chromatin modifications." Trends Biochem Sci **25**(12): 619-23.

Xue, Y., Wong, J., et al. (1998). "NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities." Mol Cell **2**(6): 851-61.

Yang, W. M., Yao, Y. L., et al. (1997). "Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family." J Biol Chem **272**(44): 28001-7.

Yang, X. J., Ogryzko, V. V., et al. (1996). "A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A." Nature **382**(6589): 319-24.

Yang, Y., Hou, H., et al. (2005). "Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation." Embo J **24**(5): 1021-32.

Yao, Y. L. and Yang, W. M. (2003). "The metastasis-associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity." J Biol Chem **278**(43): 42560-8.

Yeung, F., Hoberg, J. E., et al. (2004). "Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase." Embo J **23**(12): 2369-80.

Yoon, H. G., Chan, D. W., et al. (2003). "Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1." Embo J **22**(6): 1336-46.

You, A., Tong, J. K., et al. (2001). "CoREST is an integral component of the CoREST-human histone deacetylase complex." Proc Natl Acad Sci U S A **98**(4): 1454-8.

Zhang, C. L., McKinsey, T. A., et al. (2002). "Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation." Mol Cell Biol **22**(20): 7302-12.

Zhang, Y., Iratni, R., et al. (1997). "Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex." Cell **89**(3): 357-64.

Zhang, Y., LeRoy, G., et al. (1998). "The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities." Cell **95**(2): 279-89.

Zhang, Y., Ng, H. H., et al. (1999). "Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation." Genes Dev **13**(15): 1924-35.

Zhang, Y., Sun, Z. W., et al. (1998). "SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex." Mol Cell **1**(7): 1021-31.

Zhao, X., Sternsdorf, T., et al. (2005). "Regulation of MEF2 by histone deacetylase 4-and SIRT1 deacetylase-mediated lysine modifications." Mol Cell Biol **25**(19): 8456-64.

Zhou, X., Marks, P. A., et al. (2001). "Cloning and characterization of a histone deacetylase, HDAC9." Proc Natl Acad Sci U S A **98**(19): 10572-7.

Zhou, X., Richon, V. M., et al. (2000). "Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras." Proc Natl Acad Sci U S A **97**(26): 14329-33.

Chapter 2

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

Acharawan Topark-Ngarm[‡**], Olga Golonzhka[¶**], Valerie J. Peterson[‡], Brian Barrett Jr[‡], Brigetta Martinez[‡], Kristi Crofoot[‡], Theresa M. Filtz[‡], and Mark Leid[‡¶¶]

[‡]Departments of Pharmaceutical Sciences and [¶]Biochemistry and Biophysics, and [¶¶]Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331

**contributed equally to this work

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2.1 Abstract

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 mechanism(s) by which CTIP2 contributes to either process. CTIP2 complexes that were isolated from SK-N-MC neuroblastoma cells were found to harbor substantial histone deacetylase 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.

2.2 Introduction

CTIP2 (Bcl11b, Rit-1β) is a novel C_2H_2 zinc finger protein that was first isolated and identified as a COUP-TF-interacting protein (Avram et al., 2000). Within the immune

system, CTIP2/Bcl11b is predominantly expressed in mouse thymocytes, and is required for development and survival of $\alpha\beta$ T lymphocytes (Wakabayashi et al., 2003a). Inactivation of CTIP2/Bcl11b by homozygous deletions and point mutations of CTIP2 gene are associated with γ-ray induced thymic lymphomas in mouse (Wakabayashi et al., 2003b; Sakata et al., 2004), and ectopic expression of CTIP2/Bcl11b in HeLa cells results in suppression of cell growth (Wakabayashi et al., 2003b). 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) (MacLeod et al., 2003; Nagel et al., 2003; Bezrookove et al., 2004; Su et al., 2004; Przybylski et al., 2005).

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 post-natal 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 (Leid et al., 2004; Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005). *Ctip2*-null mice exhibit defective axonal projections of corticospinal motor neurons (CSMNs), indicating that CTIP2 plays a critical role in development of the CNS (Arlotta et al., 2005). 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 (Avram et al., 2000,

and unpublished data) or by direct, sequence-specific DNA binding activity (Avram et al., 2002). 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 class II histone deacetylases (HDACs). SIRT1 (sirtuin 1), a class III HDAC, may underlie TSA-insensitive transcriptional repression mediated by CTIPs (Senawong et al., 2003; Senawong et al., 2005), 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 (Xue et al., 1998; Zhang et al., 1999; Yao and Yang 2003; Fujita et al., 2004). The NuRD complex is considered to play a key role in transcriptional repression mediated by sequence specific transcription factors including p53 (Luo et al., 2000), Ikaros (Kim et al., 1999), Hunchback (Kehle et al., 1998), Tramtrack69 (Murawsky et al., 2001), KAP-1 (Schultz et al., 2001), BCL-6 (Fujita et al., 2004), and FOG-1 (Hong et al., 2005).

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.

2.3 Materials and methods

2.3.1 Constructs

The Lex-Gal-Luc reporter and LexA-VP16 constructs were kind gifts from Dr. Malcolm G. Parker (Imperial College, London; Ref (Christian et al., 2004). The Gal4-CTIP2, FLAG-CTIP2, and deletion mutants of the latter were previously described (Senawong et al., 2003). 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 kind gifts from Dr. Danny Reinberg (University of Medicine and Dentistry of New Jersey; Ref (Zhang et al., 1999).

2.3.2 Cell culture

HEK293T cells were cultured on 10 cm plates in high glucose Dulbecco's modified Eagle's medium (Gibco) 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.

2.3.3 Transfection and reporter assays

At approximately 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 h 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.

2.3.4 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.

2.3.5 Immunoprecipitation (IP) 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 h after transfection, cells were lysed with NET-N buffer (150 mM NaCl, 0.5% Nonidet P-40 (NP 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 x *g* for 15 min). Cell lysates (800 μg protein per IP 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% NP 40) at 4^{o}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^{o}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 (Dignam et al., 1983), except buffer C was modified to contain 0.72 M 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 nuclear protein, and in buffer D containing 0.05% NP40. 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 M NaCl, 0.2 mM EDTA, 0.5 mM DTT), and sonicated 3 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.

2.3.6 GST Pull-down experiments

GST pull-down experiments were conducted as described previously (Dowell et al., 1997).

2.3.7 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).

2.3.8 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 Ultrafree centrifugal filter apparatus (10 kDa nominal molecular mass limit), and then applied to an 850 mm x 20 mm Superose 6 size exclusion column (Amersham Biosciences) that had been equilibrated with buffer D containing 1 mM DTT, and calibrated with protein standards (blue dextran, 2000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; bovine serum albumin, 67 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.

2.3.9 Chromatin Immunoprecipitation (ChIP) assays

ChIP assays were conducted as previously described (Senawong et al., 2003; Senawong et al., 2005) with some modifications. Briefly, HEK293T cells were washed sequentially after cross-linking with PBS, buffer I (0.25% TritonX-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 ^{o}C overnight and proteinase K treatment at 45 ^{o}C for 2 h. DNA was recovered by using a Qiaquick Spin kit (Qiagen) and amplified using a forward primer (5'-GTCGAGGGGATGATAATG C-3') upstream of the multimerized 17-mer, and a reverse primer (5'-ACAGTACCGGAATGCCAAG-3') downstream of the promoter but upstream of a transcriptional start site of the luciferase gene. Amplification of *GAPDH* promoter region (negative control) was performed with a forward primer (5'-TCCTCCTGTTTCATCCAAGC-3'), and a reverse primer (5'-TAGTAGCCGGGCCCTACTTT-3'). Conditions of the amplification reactions were as follows; a predenaturation step of 2 min at 94 ^{o}C was followed by 23 (for the reporter) and 31 (for GAPDH) cycles of 94 ^{o}C (denaturation) for 40 s, 56 ^{o}C (annealing) for 45 s, 70 ^{o}C (elongation) for 1 min, and a final elongation step of 5 min at 72 ^{o}C. The resulting 210 bp PCR product of the reporter gene promoter region and 218 bp PCR product of the *GAPDH* 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 (Dynal/Invitrogen).

2.3.10 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 DTT. Eluates were then diluted 30-fold with ChIP dilution buffer, and subjected to the 2nd immunoprecipiation 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'-GTGGTGGACTCTTCTGCGTC- 3'). Amplification of *HMOX-1* proximal promoter was performed using a forward primer 5'-GCCAGACTTTGTTTCCCAAGG-3', and a reverse primer 5'-GAGGAGGCAGGCGTTGACTG –3'.

2.3.11 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 ^{o}C was followed by 35 cycles of 94 ^{o}C (denaturation) for 10 s, 56 ^{o}C (annealing) for 20 s, 70 ^{o}C (elongation) for 20 s, and a final elongation step of 5 min at 72 ^{o}C.

2.3.12 siRNA transfection

Transfections of SK-N-MC cells were performed using Lipofectamine 2000 (Invitrogen) and a siRNA pool targeting CTIP2 (custom synthesized by Dharmacon). Lipofectamine (60μl) as well as siRNA (60 μl of 15 μM SMARTPOOL or nonspecific siRNA) were pre-incubated in Opti-MEM media for 5 min. After pre-incubation, the two reagents were mixed together and allowed to incubate for additional 20 min. Immediately before transfection, SK-N-MC cells were transferred from DMEM media 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 DMEM and

allowed to incubate for another 24 h prior to harvesting for either RNA preparation or protein isolation (see below).

2.3.13 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 two-fold ($p < 0.05$ as determined by 1-way ANOVA), such as *p57KIP2*, were identified, and confirmed to be regulated by CTIP2 by immunoblotting and/or RT-PCR.

2.3.14 Whole cell extract preparation

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

2.4 Results

2.4.1 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 due to CTIP2-mediated recruitment of the histone deacetylase SIRT1 to the template (Senawong et al., 2003). In order 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 (Christian et al., 2004)) in transiently transfected HEK293T cells. Expression of the reporter gene was stimulated by co-expression with LexA-VP16 in order to facilitate evaluation of GAL4-CTIP2-mediated repression. TSA stimulated expression of the reporter gene over seven-fold in the presence of GAL4-DBD (Fig. 2.1*A, lane 2*). Gal4-CTIP2 strongly repressed expression of the reporter gene (Fig. 2.1*A, lane 3*), as previously described within the tk promoter (Senawong et al., 2003). However, in the context of this minimal promoter, Gal4-CTIP2-mediated repression was found to be partially reversed by TSA (Fig. 2.1*A*, compare *lanes 3* and *4*), indicating the possible involvement of class I and class II histone deacetylases (HDACs).

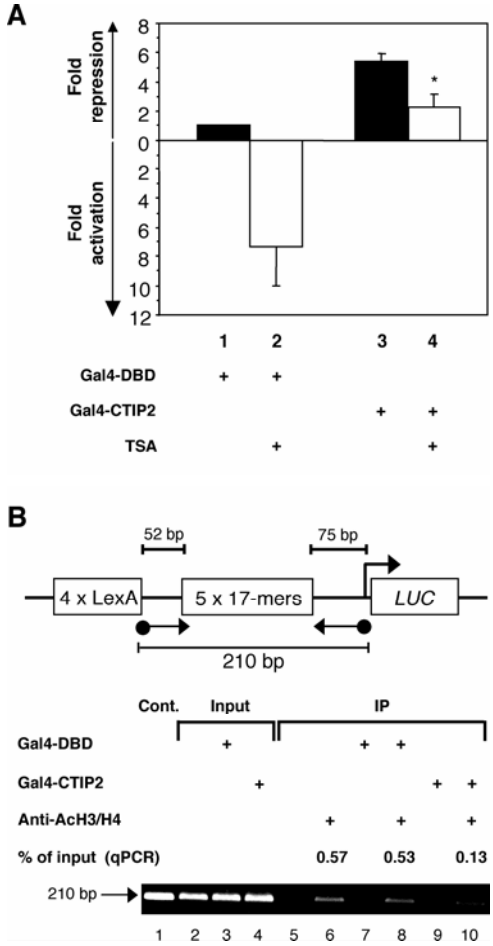

Figure 2.1. **CTIP2-mediated transcriptional repression of a minimal promoter is partially reversed by TSA.** ***A****,* HEK293T cells were co-transfected with 3 μg of Lex-Gal-Luc reporter, 0.1 μg of LexA-VP16 and either 1 μg of Gal4-DBD or Gal4-CTIP2 using the calcium phosphate method. Twenty-four h 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.M. 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 template amplification reactions from samples immunoprecipitated (IP) with or without antibodies specific for acetylated histone H3 and H4 as indicated. The numbers above *lanes 6, 8, and 10* represent quantification of the template present, expressed as percent of input, as determined by qPCR. Results are representative of three independent experiments.

2.4.2 Deacetylation of histone H3/H4 associated with the reporter template in cells expressing GAL4-CTIP2

Chromatin immunoprecipitation (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. 2.1*B, 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 four-fold upon transfection with Gal4-CTIP2 as determined by both conventional and qPCR (Fig. 2.1*B*, 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.

2.4.3 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 ten-fold greater than that observed with control immunoprecipitates (Fig. 2.2*A*, *lanes 1* and *3*). Inclusion of TSA in the *in vitro* assay completely abolished the *in vitro* HDAC activity of FLAG-CTIP2 immunoprecipitates (Fig. 2.2*A*, compare *lanes 1, 2* and *3*).

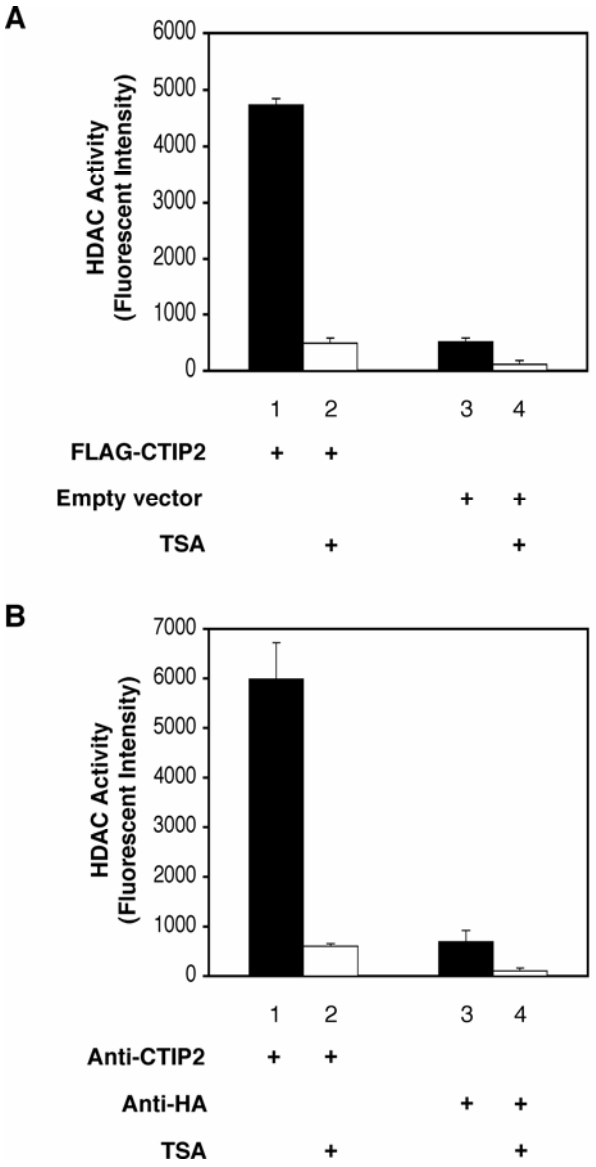

Figure 2.2. **CTIP2 complexes harbor TSA-sensitive HDAC activity *in vitro***. ***A***, HEK293T cells were transiently transfected with a eukaryotic expression vector encoding FLAG-CTIP2 or the corresponding empty vector as indicated. Immunoprecipitations (IPs) were performed on whole cell extracts using an anti-FLAG antibody and the immunoprecipitates were assayed for histone deacetylase activity in the presence (*lane 2*) or absence (*lane 1*) of TSA (0.25 μM). An irrelevant antibody (anti-HA) was used as a control (*lanes 3* and *4*). ***B***, HDAC activity assays performed on CTIP2 immunoprecipitates from SK-N-MC nuclear extracts in the presence (*lane 2*) or absence (*lane 1*) of TSA (0.25 μM). An anti-HA was again used as a control in these experiments (*lanes 3* and *4*). Relative HDAC activities present in *A and B* represent the mean (± S.E.M.) fluorescent intensity values derived from three independent experiments.

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 (Senawong et al., 2003), endogenously express two splice variants of CTIP2 (see Fig. 2.3*B* below). CTIP2 complexes from SK-N-MC cells possessed robust HDAC activity (Fig. 2.2*B*, *lane 1*). In contrast, the immunoprecipitation performed with an irrelevant antibody (anti-HA) exhibited only negligible amounts of HDAC activity (Fig. 2.2*B*, *lane 3*). As observed in transfected HEK293T cells, TSA completely inhibited HDAC activity in CTIP2 immunoprecipitates from SK-N-MC cells (Fig. 2.2*B*, 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 (Smith et al., 2000; Bitterman et al., 2002), a class III HDAC that interacts with CTIP2 in HEK293 and Jurkat cells (Senawong et al., 2003). 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 reversed a fraction of transcriptional repression mediated by GAL4-CTIP2 in transiently transfected HEK293T cells (Fig. 2.1*A*, *lanes 3* and *4*).

2.4.4 CTIP2 associates with the NuRD complex in HEK293T cells

CTIP2 has been recently demonstrated to interact with the NuRD complex in Jurkat cells (Cismasiu et al., 2005). To investigate the physical association between CTIP2 and components of the NuRD complex in HEK293T cells, immunoprecipitation (IP) 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. 2.3*A*, *lane 4*). MTA1 was also detected in MTA2 immunoprecipitates (Fig. 2.3*A*, *lanes 4* and *6*) and this was due to 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. 2.3*A*, *lane 3*). Overexpressed FLAG-CTIP2 was co-immunoprecipitated with MTA2 and the other NuRD components by the anti-MTA2 antibody (Fig. 2.3*A*, *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. 2.3*A*, compare *lanes 1* and *2*). In addition, we observed significantly less Mi-2α/β in the NuRD complexes when CTIP2 was overexpressed (Fig. 2.3*A*, 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. 2.3*A*, compare *lanes 1* and *2* of *last panel*). These data clearly

demonstrate that NuRD complex proteins interact with CTIP2 either directly or indirectly in transfected HEK293T cells.

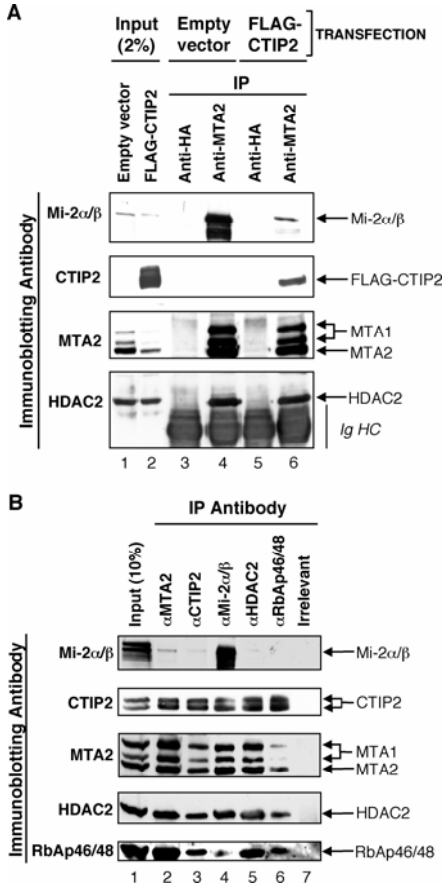

Figure 2.3. **Coimmunoprecipitation of CTIP2 with components of the NuRD complex.** ***A***, FLAG-CTIP2 associates with endogenous NuRD complex in transfected HEK293T cells. Whole cell extracts from HEK293T cells transiently transfected with 10 μg of an expression vector encoding FLAG-CTIP2 or the corresponding empty vector were immunoprecipitated with an irrelevant (anti-HA) or anti-MTA2 antibody as indicated. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting using the indicated antibodies. FLAG-CTIP2 was immunoprecipitated by anti-MTA2 antibody and detected by an anti-CTIP2 monoclonal antibody (25B6). Both MTA1 and MTA2 were detected by the anti-MTA2 antibody as indicated. The positions of Mi-2-α/β, FLAG-CTIP2, MTA1, MTA2 and HDAC2 are indicated. Ig HC = Immunoglobulin heavy chain. ***B***, Association of endogenous CTIP2 with the NuRD complex in SK-N-MC cells. Nuclear extracts from SK-N-MC cells were immunoprecipitated with the indicated antibodies. Immunocomplexes were resolved by SDS-PAGE and analyzed by western blotting using the indicated antibodies. The antibody used for IP and immunoblotting of CTIP2 was anti-CTIP2 antisera. The positions of Mi-2α/β, two splice variants of CTIP2, MTA1, MTA2, HDAC2 and RbAp46/48 are indicated.

2.4.5 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. 2.3*B*, *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. 2.3*B*, *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. 2.4*A*, and *first panel* of Fig. 2.4*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. 2.4*B*), 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. 2.3*B*, *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 was maintained through a high salt extraction and size-exclusion chromatography.

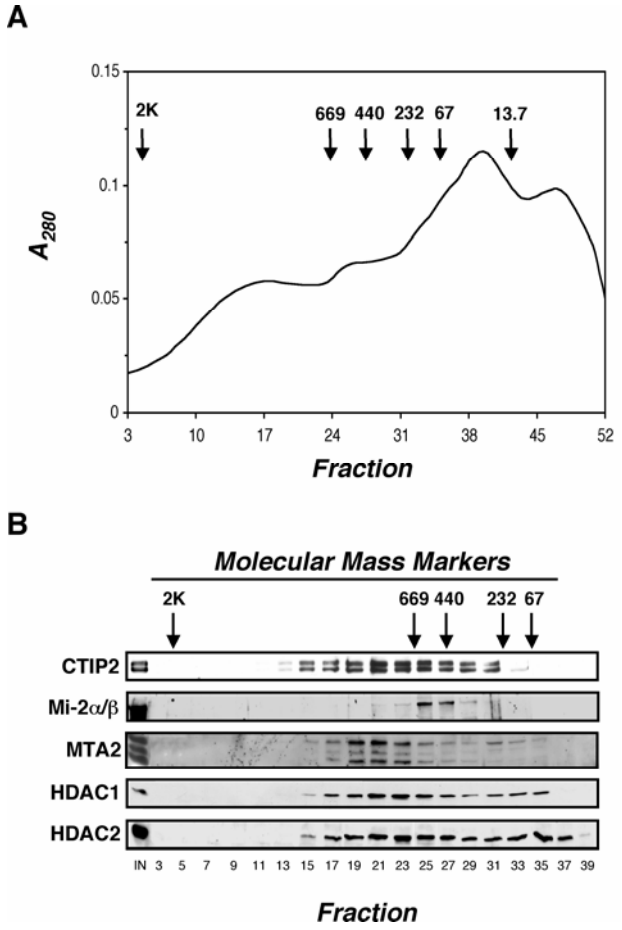

Figure 2.4. **Co-fractionation of CTIP2 and components of NuRD complex on a Superose 6 size-exclusion column**. ***A-B,*** Fractionation of nuclear extracts from SK-N-MC cells on a Supersoe 6 size-exclusion column. ***A,*** The chromatographic elution profile. ***B,*** Analysis of chromatographic fractions by immunoblotting (equal volumes from each fractions were analyzed; IN = 30 μg of nuclear extract protein as input, and fraction numbers are also indicated). The elution positions of calibrating proteins of known molecular weights (kDa) are indicated by downward arrows in both *A and B*.

2.4.6 CTIP2 interacts directly with RbAp46 and RbAp48 *in vitro*

The co-IP results demonstrated that CTIP2 associated with the NuRD complex both in transfected and untransfected cells. To determine the component(s) of the NuRD complex with which CTIP2 may interact directly, IP assays were conducted using lysates of SF9 cells infected with recombinant baculoviruses directing the expression of individual components of the NuRD complex and [^{35}S]-labeled FLAG-CTIP2. FLAG-CTIP2 was found to interact strongly with RbAp46 and RbAp48 (Fig. 2.5*A*, *lanes 7* and *8*), but not with other components of the NuRD complex tested (Fig. 2.5*A*, *lanes 3-6*). This interaction is most likely specific as FLAG-CTIP2 was not immunoprecipitated by non-specific IgG (a negative control; Fig. 2.5*A*, *lane 2*). The efficiency of the IP reactions was confirmed by immunoblotting with cognate antibodies (Fig. 2.5*B*). 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. 2.5*C*, *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. 2.5*C*, *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. 2.5*C*, *lanes 11* and *18*, respectively), whereas CTIP2-(171-350) interacted more weakly (Fig. 2.5*C*, *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 C_2H_2 zinc finger motif and also a proline-rich domain (Avram et al., 2000), appears to be primarily responsible for interaction of CTIP2 with RbAp46 and RbAp48 *in vitro*.

2.4.7 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 (Xue et al., 1998; Zhang et al., 1999). However, the NuRD complex must be recruited to a CTIP2-responsive promoter in a CTIP2-dependent manner in order 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. 2.1), 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. 2.6*A*, compare *lane 7* to *lane 5* of *all panels*). In contrast, very little template was mock-immunoprecipitated (Fig. 2.6*A*, *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. 2.6*B*).

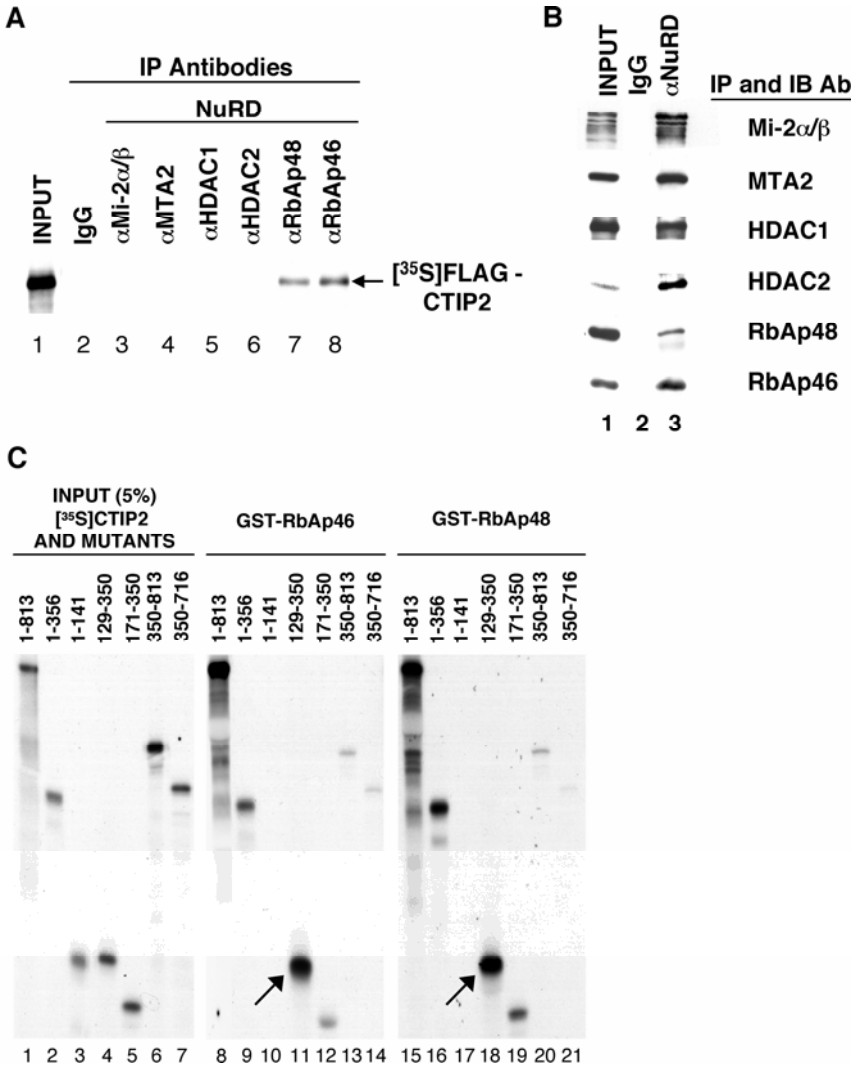

Figure 2.5. **CTIP2 interacts directly with RbAp46 and RbAp48 *in vitro***. ***A-B***, *In vitro* translated and [^{35}S]Met-labeled, full-length FLAG-CTIP2 was mixed with SF9 cell extracts containing individual recombinant NuRD complex proteins and these mixtures were immunoprecipitated with antibodies against the NuRD complex proteins, or non-specific antibody (IgG), as indicated. ***A***, Immunoprecipitates were resolved by SDS-PAGE and the presence of [^{35}S]Met-labeled FLAG-CTIP2 was determined by autoradiography (*lanes 2-8*). Input [^{35}S]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 [^{35}S]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 for simplicity*). After extensive washing, [^{35}S]Met-labeled CTIP2 associated with the affinity resin was resolved by SDS-PAGE and visualized by autoradiography. *Arrows* indicate strong interaction between [^{35}S]Met-labeled CTIP2-(129-350) and GST-RbAp46, -RbAp48 fusion proteins.

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.

2.4.8 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. 2.3*B*), 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 ($CTIP2^{KD}$) or mock ($CTIP2^{Mock}$) siRNAs. The microarray analyses confirmed that CTIP2 knockdown was achieved in $CTIP2^{KD}$ cells (~60% knockdown at the mRNA level; compare *lanes 1* and *2* of Fig. 2.7*A*; see also immunoblot in *top panel* of Fig. 2.7*B*). The expression of a number of genes was increased in the $CTIP2^{KD}$ relative to $CTIP2^{Mock}$ cells, consistent with the previously described role of CTIP2 as a repressor of transcription (Avram et al., 2000; Avram et al., 2002; Senawong et al., 2003). 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 2.1. 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 (Arlotta et al., 2005), it is of interest that two of these genes encode proteins involved in the function of the extracellular matrix. For the purposes of this manuscript, we chose to focus on *p57KIP2*, which encodes a cyclin-dependent kinase inhibitor belonging to the CIP/KIP family (Cunningham and Roussel, 2001).

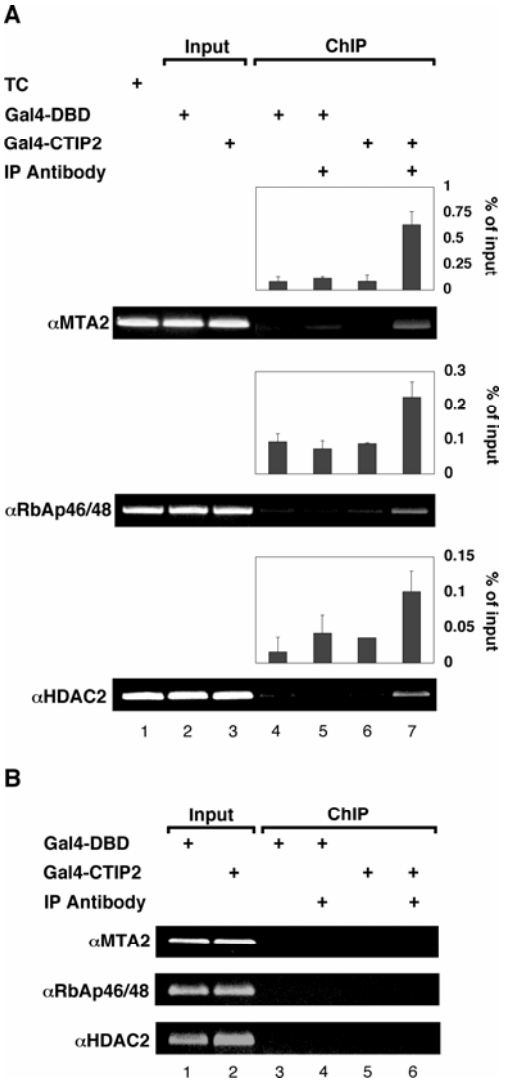

Figure 2.6. **CTIP2-dependent recruitment of endogenous NuRD complex proteins to the promoter template.** ***A***, ChIP assays were performed on HEK293T cells following transient transfection of 3 μg of Lex-Gal-Luc reporter and 5 μg of an expression vector encoding either Gal4-DBD or Gal4-CTIP2. Conventional PCR amplification of a promoter template from samples immunoprecipitated with or without specified antibodies (*lanes 4-7*) is shown *(lower)* together with quantification by qPCR amplification *(upper)* in each panel. *Lane 1* corresponds to a template control (*TC*) in which a reporter plasmid was used directly in the amplification reaction. *Lanes 2* and *3* represent amplification reactions conducted utilizing 5% of the input lysate that was used for IP reactions. The result of conventional PCR (ethidium bromide-stained gels) is a representative of three independent experiments. CTIP2-dependent enrichment of MTA2, RbAp46/48, and HDAC2 on the promoter template was quantified by qPCR and presented as the mean percentage of input ± S.E.M. derived from three independent experiments ***B***, PCR amplification of a genomic *GAPDH* promoter region from samples immunoprecipitated with or without specified antibodies (*lanes 3-6*). Inputs (*lanes 1-2*) were as described in *A*.

Affymetrix microarray analyses revealed that the expression of *p57KIP2* was increased 4-fold in CTIP2^{KD} cells (compare *lanes 3* and *4* of Fig. 2.7*A*), and this, as well as knockdown of CTIP2 protein, was confirmed at the protein level (Fig. 2.7*B*, 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. 2.7*C*, 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.

Table 2.1. **Up-regulated genes in CTIP2^{KD} SK-N-MC cells**

GenBank	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

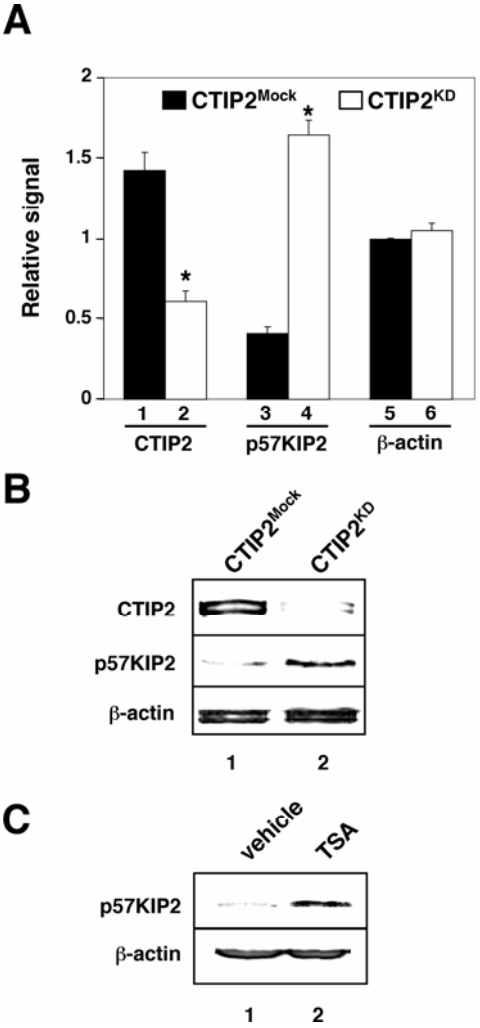

Figure 2.7. **CTIP2 knockdown in SK-N-MC cells results in increased expression of p57KIP2**. SK-N-MC cells were transfected with 100 nM CTIP2 siRNA ($CTIP2^{KD}$) or nonspecific siRNA ($CTIP2^{Mock}$). ***A***, Microarray analyses of mRNA levels of CTIP2, p57KIP2, and β-actin in $CTIP2^{KD}$ and $CTIP2^{Mock}$ cells. The results represent mean mRNA levels (shown as *relative signal*) ± S.E.M. derived from three independent experiments. The difference of mRNA levels between $CTIP2^{KD}$ and $CTIP2^{Mock}$ cells is statistically significance 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 $CTIP2^{KD}$ and $CTIP2^{Mock}$ 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 seven hours, prior to harvesting, whole cell extract preparation and immunoblotting. Again, β-actin serves a loading control.

2.4.9 p57KIP2 is a direct target of CTIP2 in SK-N-MC cells

As the *p57KIP2* proximal promoter region contains several putative CTIP2 binding sites (Avram et al., 2002; Fig. 2.8*A*), ChIP experiments were carried out to determine if CTIP2 is bound to this promoter in SK-N-MC cells. CTIP2 was found to associate strongly with *p57KIP2* promoter, but not with that of *heme oxygenase-1* (*HMOX-1;* Fig. 2.8*B*), even though the latter contains multiple consensus CTIP2 binding sites, and was similarly identified as an induced gene in our transcriptome analyses of $CTIP2^{KD}$ cells (Table 2.1 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 $CTIP2^{KD}$ 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. 2.6). 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 (Zhang et al., 1999) and

our re-ChIP analyses demonstrated the association of MTA2 and CTIP2 with the same fragment of *p57KIP2* promoter (*upper panel of* Fig. 2.8*C*), but not with the *HMOX-1* promoter (*lower panel* of Fig. 2.8*C*). 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. 2.8*D* and *E*) but not that of *HMOX-1* (*lower panels* of Fig. 2.8*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. 2.5), we conclude that CTIP2 recruits the NuRD complex to the *p57KIP2* template resulting in transcriptional repression of this gene under basal conditions.

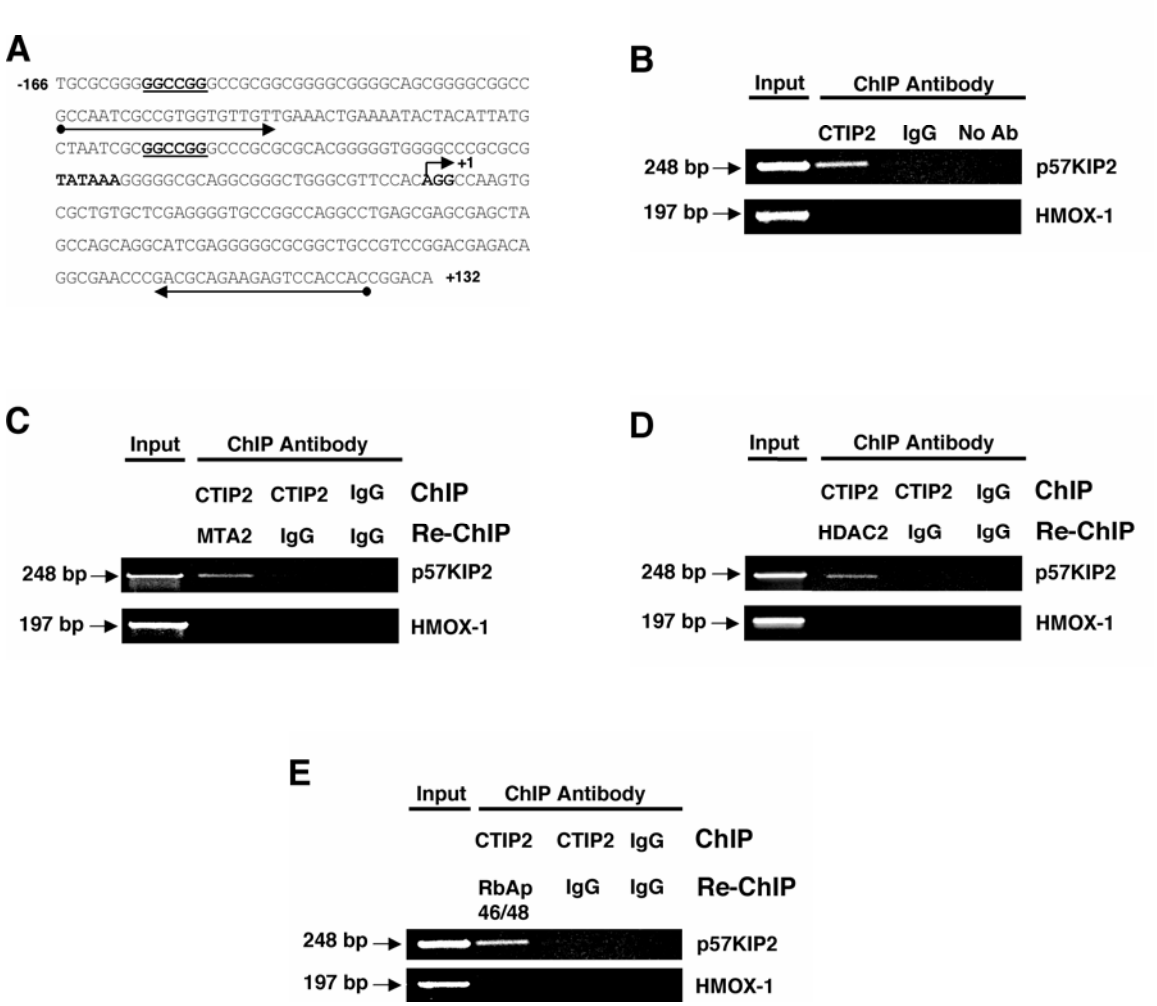

Figure 2.8. **CTIP2 and components of NuRD complex co-associate with *p57KIP2* promoter in SK-N-MC cells**. ***A***, The *p57KIP2* proximal promoter region. Putative CTIP2 binding sites (Ref. 14) are underlined; arrows represent positions of forward and reverse primers for ChIP, and the transcriptional start site is denoted as +1. ***B***, Chromatin immunoprecipitation was performed in SK-N-MC cells using indicated antibodies, and results were analyzed by conventional PCR using primers described in ***A***. The *heme oxygenase-1* (*HMOX-1*) promoter was used as a negative control to demonstrate the specificity of CTIP2 binding to *p57KIP2* promoter. The input lane represents 10% of the total chromatin used for immunoprecipitation. ***C***, ***D, and E***, CTIP2, MTA2, HDAC2 and RbAp46/48 co-occupy the promoter of *p57KIP2* gene. ChIP and re-ChIP studies were performed using the specified antibodies in the orders indicated. The proximal *HMOX-1* promoter was again used as a negative control. ChIP/re-ChIP studies shown are representative of three independent experiments. The sizes of PCR products (*p57KIP2*, 248 bp; *HMOX-1*, 197 bp) are indicated by arrows.

2.5 Discussion

Previously, CTIP1 and CTIP2 were demonstrated to repress transcription in a predominantly TSA-insensitive manner, possibly due to recruitment of SIRT1 to CTIP1/2-responsive promoters, at least in transiently transfected cells, and in the context of the *tk* promoter (Avram et al., 2000; Avram et al., 2002; Senawong et al., 2003; Senawong et al., 2005). 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. 2.1), suggesting 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 II*α*, 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 (Siddiqui et al., 2003). 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 (Hohl and Thiel, 2005). 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 (Hohl and Thiel, 2005). 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^+-dependent HDACs, histone and DNA

methylation may also be involved in transcriptional repression (Blander and Guarente, 2004; Wang et al., 2004). 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 to 1000 kDa (see Fig. 2.4). In contrast, the size of CTIP2 complex in Jurkat cells was found be up to 2000 kDa (Senawong et al., 2003). 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. 2.6), and were found to co-occupy the promoter of an endogenous CTIP2-target gene in SK-N-MC neuroblastoma cells (see Fig. 2.8). This recruitment is likely mediated by the direct interaction of CTIP2 with the histone binding proteins, RbAp46 and/or RbAp48 (see Fig. 2.5). Additionally, many other transcription factors involved in transcriptional repression have been reported to interact with different subunits of the NuRD complex (Kehle et al., 1998; Kim et al., 1999; Luo et al., 2000; Murawsky et al., 2001; Schultz et al., 2001; Fujita et al., 2004; Hong et al., 2005), suggesting the possibility that the NuRD complex is involved in many pathways leading to transcriptional repression. However, it is presently unknown if 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* (Kehle et al., 1998), embryonic patterning, vulva development and signaling in *Caenorrhabditis elegans* (Solari et al., 1999; von Zelewsky et al., 2000), and mouse embryogenesis (Hendrich et al., 2001). 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 (Kantor et al., 2003), 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 (Fujita et al., 2004; Williams et al., 2004).

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 cyclin-dependent kinase (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 (Cunningham and Roussel, 2001). 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-Wiedemann syndrome (Matsuoka et al., 1995).

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 (Dyer and Cepko, 2000). 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 (Dyer and Cepko, 2000). In addition, p57KIP2 is expressed in postmitotic differentiating midbrain dopaminergic neurons and is required for the maturation of these cells (Joseph et al., 2003). 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 (Joseph et al., 2003).

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*.

2.6 Abbreviations

CTIP1 and 2, COUP-TF-interacting proteins 1 and 2; COUP-TF, chicken ovalbumin upstream promoter transcription factor; NuRD, nucleosome remodeling and deacetylation; Sir2, silent information regulator 2; SIRT1, sirtuin 1 or Sir2-like protein 1; TSA, trichostatin A; HEK293T, human embryonic kidney 293T cells; DBD, DNA binding domain; LUC, luciferase; bp, base pair; NAD^+, nicotinamide adenine dinucleotide; GST, glutathione S-transferase; HA, hemagglutinin; IgG, immunoglobulin; kDa, kilodalton; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CSMN, corticospinal motor neuron.

2.7 Acknowledgements

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2.8 References

Arlotta, P., Molyneaux, B. J., et al. (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron **45**(2): 207-21.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Bezrookove, V., van Zelderen-Bhola, S. L., et al. (2004). "A novel t(6;14)(q25-q27;q32) in acute myelocytic leukemia involves the BCL11B gene." Cancer Genet Cytogenet **149**(1): 72-6.

Bitterman, K. J., Anderson, R. M., et al. (2002). "Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1." J Biol Chem **277**(47): 45099-107.

Blander, G. and Guarente, L. (2004). "The Sir2 family of protein deacetylases." Annu Rev Biochem **73**: 417-35.

Chen, B., Schaevitz, L. R., et al. (2005). "Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex." Proc Natl Acad Sci U S A **102**(47): 17184-9.

Christian, M., Tullet, J. M., et al. (2004). "Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140." J Biol Chem **279**(15): 15645-51.

Cismasiu, V. B., Adamo, K., et al. (2005). "BCL11B functionally associates with the NuRD complex in T lymphocytes to repress targeted promoter." Oncogene **24**(45): 6753-64.

Cunningham, J. J. and Roussel, M. F. (2001). "Cyclin-dependent kinase inhibitors in the development of the central nervous system." Cell Growth Differ **12**(8): 387-96.

Dignam, J. D., Lebovitz, R. M., et al. (1983). "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Res **11**(5): 1475-89.

Dowell, P., Peterson, V. J., et al. (1997). "Ligand-induced peroxisome proliferator-activated receptor alpha conformational change." J Biol Chem **272**(3): 2013-20.

Dyer, M. A. and Cepko, C. L. (2000). "p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina." Development **127**(16): 3593-605.

Fujita, N., Jaye, D. L., et al. (2004). "MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation." Cell **119**(1): 75-86.

Hendrich, B., Guy, J., et al. (2001). "Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development." Genes Dev **15**(6): 710-23.

Hohl, M. and Thiel, G. (2005). "Cell type-specific regulation of RE-1 silencing transcription factor (REST) target genes." Eur J Neurosci **22**(9): 2216-30.

Hong, W., Nakazawa, M., et al. (2005). "FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1." Embo J **24**(13): 2367-78.

Joseph, B., Wallen-Mackenzie, A., et al. (2003). "p57(Kip2) cooperates with Nurr1 in developing dopamine cells." Proc Natl Acad Sci U S A **100**(26): 15619-24.

Kantor, B., Makedonski, K., et al. (2003). "Expression and localization of components of the histone deacetylases multiprotein repressory complexes in the mouse preimplantation embryo." Gene Expr Patterns **3**(6): 697-702.

Kehle, J., Beuchle, D., et al. (1998). "dMi-2, a hunchback-interacting protein that functions in polycomb repression." Science **282**(5395): 1897-900.

Kim, J., Sif, S., et al. (1999). "Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes." Immunity **10**(3): 345-55.

Leid, M., Ishmael, J. E., et al. (2004). "CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis." Gene Expr Patterns **4**(6): 733-9.

Luo, J., Su, F., et al. (2000). "Deacetylation of p53 modulates its effect on cell growth and apoptosis." Nature **408**(6810): 377-81.

MacLeod, R. A., Nagel, S., et al. (2003). "Activation of HOX11L2 by juxtaposition with 3'-BCL11B in an acute lymphoblastic leukemia cell line (HPB-ALL) with t(5;14)(q35;q32.2)." Genes Chromosomes Cancer **37**(1): 84-91.

Matsuoka, S., Edwards, M. C., et al. (1995). "p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene." Genes Dev **9**(6): 650-62.

Molyneaux, B. J., Arlotta, P., et al. (2005). "Fezl is required for the birth and specification of corticospinal motor neurons." Neuron **47**(6): 817-31.

Murawsky, C. M., Brehm, A., et al. (2001). "Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex." EMBO Rep **2**(12): 1089-94.

Nagel, S., Kaufmann, M., et al. (2003). "The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2)." Cancer Res **63**(17): 5329-34.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Sakata, J., Inoue, J., et al. (2004). "Involvement of V(D)J recombinase in the generation of intragenic deletions in the Rit1/Bcl11b tumor suppressor gene in gamma-ray-induced thymic lymphomas and in normal thymus of the mouse." Carcinogenesis **25**(6): 1069-75.

Schultz, D. C., Friedman, J. R., et al. (2001). "Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD." Genes Dev **15**(4): 428-43.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Senawong, T., Peterson, V. J., et al. (2005). "BCL11A-dependent recruitment of SIRT1 to a promoter template in mammalian cells results in histone deacetylation and transcriptional repression." Arch Biochem Biophys **434**(2): 316-25.

Siddiqui, H., Solomon, D. A., et al. (2003). "Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition." Mol Cell Biol **23**(21): 7719-31.

Smith, J. S., Brachmann, C. B., et al. (2000). "A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family." Proc Natl Acad Sci U S A **97**(12): 6658-63.

Solari, F., Bateman, A., et al. (1999). "The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning." Development **126**(11): 2483-94.

Su, X. Y., Busson, M., et al. (2004). "Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia." Genes Chromosomes Cancer **41**(3): 243-9.

von Zelewsky, T., Palladino, F., et al. (2000). "The C. elegans Mi-2 chromatin-remodelling proteins function in vulval cell fate determination." Development **127**(24): 5277-84.

Wakabayashi, Y., Watanabe, H., et al. (2003a). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Wakabayashi, Y., Inoue, J., et al. (2003b). "Homozygous deletions and point mutations of the Rit1/Bcl11b gene in gamma-ray induced mouse thymic lymphomas." Biochem Biophys Res Commun **301**(2): 598-603.

Wang, Y., Wysocka, J., et al. (2004). "Linking covalent histone modifications to epigenetics: the rigidity and plasticity of the marks." Cold Spring Harb Symp Quant Biol **69**: 161-9.

Williams, C. J., Naito, T., et al. (2004). "The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development." Immunity **20**(6): 719-33.

Xue, Y., Wong, J., et al. (1998). "NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities." Mol Cell **2**(6): 851-61.

Yao, Y. L. and Yang, W. M. (2003). "The metastasis-associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity." J Biol Chem **278**(43): 42560-8.

Zhang, Y., Ng, H. H., et al. (1999). "Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation." Genes Dev **13**(15): 1924-35.

Chapter 3

The C-Terminal Zinc fingers are Crucial for Self-Association and Transcriptional Repression Mediated by CTIP2

Acharawan Topark-Ngarm[1], and Mark Leid[1,2]

[1]Department of Pharmaceutical Sciences and [2]Environmental Health Sciences Center,

Oregon State University, Corvallis, Oregon 97331

3.1 Abstract

COUP-TF-interacting protein 2 (CTIP2/Rit-1β/BCL11B) is abundantly expressed in the brain and the thymus, in which it is required for development of corticospinal motor neurons and T cells, respectively. CTIP2, as well as a highly related protein CTIP1, are C_2H_2 zinc finger proteins that function by direct, sequence-specific-DNA-binding activity or by recruitment to the promoter template by interaction with chicken ovalbumin upstream promoter transcription factors (COUP-TFs). In this report, we found that CTIP2 directly bound to DNA and participated in self-association in a manner that was different from that of CTIP1. Both the centrally located zinc fingers (ZnF) 3-4 and the C-terminal region containing ZnFs 5-7 appeared to be required for CTIP2 DNA binding activity, however, in this regard the two zinc-binding modules appeared to function differentially. The C-terminal ZnFs mediate self-association of the protein by interacting directly with the proline-rich region located in the amino terminus of the protein. Thus, we believe that mutation of the ZnF5-7 module compromises high affinity DNA binding activity of the protein by disrupting self-association, and this may be relevant to an action of CTIP2 on target genes.

3.2 Introduction

Zinc fingers (ZnFs) are small protein domains that fold around one or more zinc ions. Zinc finger-containing proteins constitute the most abundant protein superfamily in eukaryotes. In the human genome, over 1000 genes have been predicted to encode proteins with ZnFs (Lander et al., 2001). There are more than 10 classes of zinc finger proteins that have been identified and studied to date, based on structure as well as function (Ravasi et al., 2003). The zinc finger proteins are functionally diverse in cellular

processes, which include replication, repair, mRNA stability and processing, transcriptional, translation, metabolism, signaling, cell proliferation, differentiation, and apoptosis (Ravasi et al., 2003). Zinc fingers typically serve as interaction modules that are involved in nucleic acid (DNA and RNA) binding, protein-protein interactions, and binding of biomolecules such as lipids (Laity et al., 2001; Matthews and Sunde, 2002; Hall, 2005).

The C_2H_2 zinc finger is among one of the most common zinc finger domains that has been characterized. The C_2H_2 zinc finger was discovered as a repeated zinc binding motif in *Xenopus* transcription factor IIIA (TFIIIA), which was shown to associate with 5S rRNA and regulate expression of the 5S rRNA gene (Picard and Wegnez, 1979; Pelham and Brown, 1980; Miller et al., 1985). The C_2H_2 zinc finger motif is defined by $\beta\beta\alpha$ structure with a consensus sequence: (Tyr,Phe)-X-Cys-$X_{2,4}$-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His-X_{2-6}, where X is a variable amino acid (Lee et al., 1989; Suzuki and Yagi, 1994). Many of these C_2H_2 zinc finger proteins are transcription factors that function by recognition and binding of specific DNA sequences in the promoter or regulatory regions of target genes.

The sequence specificity for DNA binding is achieved by the contacts made between basic and hydrophobic residues in the α-helix and the nucleotides within the major groove of DNA (Suzuki and Yagi, 1994). The cooperative binding of the α-helices of two or more C_2H_2 zinc fingers arrayed in tandem results in high affinity for DNA binding (Choo et al., 1997). Although the majority of C_2H_2 zinc finger proteins identified to date are implicated in nucleic acid binding, increasing evidences suggest that many members of this superfamily function by mediating structural or functional protein-protein interactions, including dimerization. Some of C_2H_2 zinc finger proteins for example, the

Ikaros family proteins, which are important in the regulation of lymphoid development (Georgopoulos, 2002), can bind to DNA and also to proteins, often via other zinc fingers (Molnar and Georgopoulos, 1994). The proteins in this family contain two clusters of zinc fingers. The N-terminal cluster, containing four zinc fingers, mediates binding to DNA. The C-terminal cluster, containing two ZnFs, mediates either protein homodimerization or heterodimerization with all other family members, resulting in different DNA-binding and transcriptional activities (Molnar and Georgopoulos, 1994; Sun et al., 1996; Morgan et al., 1997; Kelley et al., 1998; Perdomo et al., 2000). Mutant forms of Ikaros, in which only the dimerization zinc fingers are present, can interfere with normal Ikaros function and have been implicated in childhood leukemias (Sun et al., 1999; Nakase et al., 2002; Fujii et al., 2003; Ruiz et al., 2004).

CTIP1 and CTIP2, novel and related C_2H_2 zinc finger proteins, were originally isolated and identified as chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins (Avram et al., 2000). CTIPs are recruited to the promoter template by interaction with COUP-TFs, and potentiate COUP-TFs-mediated transcriptional repression (Avram et al., 2000; unpublished data). By a direct, sequence-specific DNA binding activity, both CTIP1 and CTIP2 are bona fide transcriptional repressors that function independently of COUP-TF family members (Avram et al., 2002).

CTIP2 is highly expressed in the thymus and in the brain (Avram et al., 2000; Leid et al., 2004; Arlotta et al., 2005). A deficiency of CTIP2 results in defective development of T cells and projections of corticospinal motor neurons and the death of mice on the first day after birth (Wakabayashi et al., 2003a; Wakabayashi et al., 2003b; Arlotta et al., 2005 2005). Moreover, rearrangements of the *CTIP2* gene are implicated in human leukemogenesis (Przybylski et al., 2005). Recently, CTIP2 has been shown to

control expression of the proinflammatory cytokine *IL-2* gene (Cismasiu et al., 2006). Finally, CTIP2 has been demonstrated to act as a potent inhibitor of HIV-1 Tat transactivation, leading to inhibition of viral replication (Rohr et al., 2003). These reports underscore the potential impact of CTIP2 on human health. Therefore, additional information regarding properties of CTIP2 is important for the understanding of its implication in these processes.

The specific DNA binding site identified for CTIP proteins is a GC-rich sequence, 5'-GGCCGG-3' (upper strand) which is referred to as the CTIP response element (CTIP RE, Ref (Avram et al., 2002)). The module comprising zinc fingers 3 and 4 (ZnFs 3-4) of CTIP1 is required for recognition of the CTIP RE and also for a self-interaction, which facilitates the binding of protein to the CTIP RE *in vitro* (Avram et al., 2002).

CTIP1 shares high amino acid sequence identity with CTIP2, especially within ZnFs 3-4 of both proteins (93% identity) (Avram et al., 2000). This sequence homology may suggest a common functionality between these two proteins. However, structural distinction at the C-terminus of CTIP2, which contains three additional C_2H_2 zinc fingers, suggests that CTIP2 may exhibit DNA binding and/or self-association activities that are different from those of CTIP1.

In this report, we demonstrate that CTIP2, similar to CTIP1, was found to recognize the CTIP-binding motif in a sequence-specific manner and self-associate *in vitro*. However, we found that CTIP2 exhibited a distinct mode of DNA binding and self-association. Two regions of CTIP2, the ZnF 3-4 module and the ZnFs 5-7 at the C-terminus, both were found to contribute to the DNA binding activity of the protein. However, the module of ZnFs 5-7, not of ZnFs 3-4, appeared to be required for self-association of CTIP2 through its direct interaction with the proline-rich region. Mutations that disrupted the integrity of ZnFs 3 or 4 partially reduced the CTIP2-mediated

transcriptional repression of a reporter gene harboring CTIP2 binding motif. However, disruption of ZnFs 5, 6, or 7 or deletion of all three ZnFs resulted in a marked reduction of repressive activity of CTIP2, likely due to a failure in self-association. Together, these findings suggest that the ZnF5-7 module of CTIP2 is a critical determinant for transcriptional regulatory activity of CTIP2.

3.3 Materials and methods

3.3.1 Synthesis of oligonucleotides

Synthetic oligonucleotides for each strand containing the CTIP RE (5'-GGCCGG-3'; the upper strand) or CTIP RE mutant oligonucleotides (M1-M4) were previously described (Avram et al., 2002). The WT CTIP RE oligonucleotides with 3'-biotin labeling and mutant oligonucleotides: M5-M10 were obtained from Invitrogen.

3.3.2 Constructs

The Gal4-CTIP2 and FLAG-CTIP2 constructs were previously described (Senawong et al., 2003). The constructs used for generating [^{35}S]methionine-labeled proteins were prepared by PCR amplification with primers containing appropriate restriction sites for insertion into pcDNA3(+) or pcDNA3.1/His (Invitrogen), except the construct of FLAG-CTIP2 1-674, which was prepared by digesting FLAG-CTIP2 with XmaI followed by re-ligation. Gal4-CTIP2 1-674 was prepared using an identical approach to FLAG-CTIP2 1-674. The Lex-Gal-Luc reporter and LexA-VP16 constructs were previously described (Topark-Ngarm and Golonzhka et al., 2006). The $(CTIP\ RE)_3$-*tk*-CAT reporter construct was prepared by insertion of annealed oligonucleotides

containing the CTIP RE into the *tk*-CAT reporter (immediately upstream of a *tk* promoter) using standard cloning techniques. All vectors encoding GST-CTIP2 fusion proteins were prepared by PCR amplification using GST-CTIP2 (Avram et al., 2002) as a template followed by insertion into pGEX-2T (Amersham Pharmacia Biotech). All constructs were verified by complete DNA sequencing.

3.3.3 Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was used with the specified oligonucleotides to generate the desired point mutants. All constructs were sequenced for verification.

3.3.4 GST Pull-down experiments

GST pull-down experiments were conducted as described previously (Dowell et al., 1997a). Briefly, equivalent amounts of bacterial expressed GST or GST-CTIP2 fusion proteins previously bound to glutathione-sepharose (Amersham Pharmacia Biotech) were incubated with [^{35}S]methionine-labeled proteins (CTIP2 full-length or truncation mutants) prepared using the TNT transcription-translation system (Promega). After extensive washing, bound proteins were eluted and resolved on denaturing SDS-PAGE gels for analysis by autoradiography.

3.3.4 ABCD assays

In vitro translated and [^{35}S]methionine-labeled proteins were incubated with or without annealed, 3'-biotin-labeled oligonucleotides containing the CTIP RE in binding buffer (10 mM HEPES, pH7.5, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 0.05% Nonidet

P-40) at 4°C for 1 h in a final volume of 100 μl. For competition assays, various non-biotinylated oligonucleotide-containing WT or mutant CTIPRE were additionally included in the reaction. The reaction mixtures were subsequently transferred to a fresh tube containing five microliters of a streptavidin agarose resin pretreated with 1 mg/mL bovine serum albumin in binding buffer for 2 h. The DNA-protein complexes were incubated with the resin for 1 h at 4°C with rotation. At the end of the incubation, the resin was washed 5 times with binding buffer and the bound protein associated with the resin was measured for radioactivity using a liquid scintillation counter. Difference in counts between the reaction with and without the biotinylated CTIP RE oligonucleotides was considered as the DNA binding activity. The relative DNA binding of the protein was equal to the DNA binding activity divided by numbers of methionine present in the protein.

3.3.5 Transfection and reporter assays

HEK293T cells were transfected using the calcium phosphate method and harvested 48 h after transfection. A β-galactosidase expression vector (pCMV-Sport-βGal, Life Technologies) was co-transfected as an internal control, and β-galactosidase activity and total protein concentration were used for normalization across all samples. The relative chloramphenicol acetyltransferase and luciferase activities were determined as described previously (Dowell et al., 1997b).

3.4 Results

3.4.1 CTIP2 is a sequence-specific DNA binding protein

The DNA sequence 5'-GGCCGG-3' (upper strand) was previously identified *in vitro* as a core binding site for CTIP1, which was referred to as the CTIP response element (CTIP RE; Avram et al., 2002). CTIP1 forms multimeric complexes on the CTIP RE, with which the third zinc finger (ZnF3) motif of CTIP1 makes a direct contact (Avram et al., 2002). Likewise, this consensus CTIP RE has been shown to be bound specifically by CTIP2, a highly related protein to CTIP1 (Avram et al., 2002). Although this report suggests that CTIP2 may participate in multimeric formation on the CTIP RE, little is known concerning occurrence of multimerization and the base specificity of CTIP2 for DNA binding.

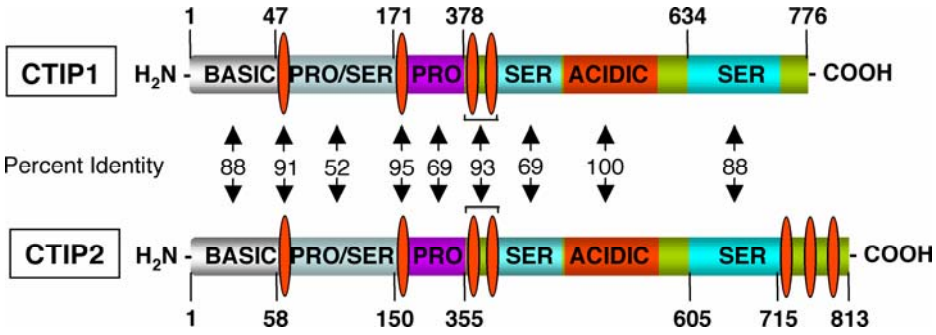


Figure 3.1 **Schematic diagrams of CTIP1 and CTIP2.** CTIP1 and CTIP2 proteins are schematically presented with zinc finger motifs being represented as oval vertical bars. Numbers of amino acid above or below the diagrams and the percentage of identity between each region of CTIP1 and CTIP2 are indicated. The boxes indicate characteristics of each domain.

The CTIP RE is bound by CTIP1 in a sequence-specific manner, and the first four bases of the core CTIP RE appears to be critical determinants for CTIP1 binding (Avram et al., 2002). It is of interest to know if CTIP2 possesses different sequence specificity for DNA binding. To test this hypothesis, we first verified if CTIP2 bound this consensus sequence in Avidin (Streptavidin)-Biotin Complex with DNA (ABCD) binding assay, an approach different from our previous report. The ABCD assay allowed quantification of bound protein to DNA. In this study, a radiolabeled protein bound to the duplex, biotinylated CTIP RE-containing oligonucleotides (Fig. 3.2) was captured on the streptavidin agarose resin through a strong interaction between biotin and streptavidin. The amount of protein remaining bound to the resin after extensive washing would correspond to the extent of the CTIP RE binding by the protein. From the ABCD assay, we indeed confirmed that the biotinylated CTIP RE was bound by CTIP1 and CTIP2 (table 3.1). On the contrary, COUP TF-1, an interacting protein of CTIPs and a member of orphan nuclear receptors (Wang et al., 1989; Ritchie et al., 1990; Qiu et al., 1995; Avram et al., 2000), barely bound to this DNA sequence. We conclude from these findings that the CTIP RE is a sequence specifically bound by CTIP proteins.

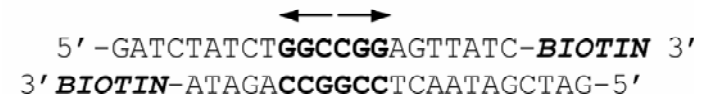


Figure 3.2 **Biotinylated oligonucleotides used in ABCD studies.** Upper and lower strands of oligonucleotides are shown. A consensus CTIP binding site (CTIP RE) is denoted in bold in each strand. Each arrow indicates a half site of the CTIP RE. The biotin labeling at 3'-end is indicated in italicized bold.

PROTEIN	RELATIVE CTIP RE BINDING
CTIP1	366.7 ± 29.4
CTIP2	2138.4 ± 158.4
COUP-TFI	3.0 ± 2.8

Table 3.1. **The binding of [^{35}S]methionine-labeled CTIP1 and CTIP2 to the biotinylated CTIP RE-containing oligonucleotides.** *In vitro* translated and [^{35}S]methionine-labeled, full-length CTIP1, CTIP2, and COUP-TFI were incubated with the annealed biotinylated oligonucleotides containing the CTIP RE before addition of streptavidin agarose resin. After extensive washing, [^{35}S]methionine-labeled CTIP1, CTIP2, and COUP-TFI associated with the resin was measured for radioactivity. The value difference from reactions with and without the biotinylated oligonucleotides divided by numbers of methionine present in the protein was determined as the mean relative binding (± S.E.M.), which was derived from at least three independent experiments.

To determine bases within the 5'-GGCCGG-3' motif required for DNA binding by CTIP2, ABCD assays were performed as described above but in the presence of various non-biotinylated, competitor oligonucleotides, each harboring two mutations in the core CTIP RE motif. If one of these mutants were to harbor mutations at the positions required for CTIP2 binding, we would expect to observe such a mutant oligonucleotide would fail to compete with the biotinylated CTIP RE for the binding to [^{35}S]methionine-labeled CTIP2. The competitor oligonucleotides; wild type CTIP RE, and mutants M1-M4 used in these experiments are shown in Fig. 3.3.

As expected, the non-biotinylated wild-type (WT) CTIP RE inhibited the binding of CTIP2 to the biotinylated CTIP RE (Fig. 3.4*A*, compare *lanes 2 and 3* to *lane1*). The oligonucleotide M3, which was mutated at position 5 and 6 of the CTIP core binding site, also inhibited the CTIP2 binding to the biotinylated CTIP RE somewhat more strongly than or to a similar extent as the non-biotinylated WT CTIP RE (Fig. 3.4*A*, *lanes 1, 2-3, and 8-9*). This inhibition did not exclusively occur due to the fact that the position 5 and 6 was mutated to adenine (A). As when the mutations occurred to cytosine (C) or thymine (T), either of these oligonucleotides was also capable of competing for CTIP2 binding (data not shown). These results indicate that the positions 5 and 6 are not essential for CTIP2 binding. However, the competitors M1 and M2 oligonucleotides, which were mutated at position 1 and 2, and at position 3 and 4, respectively, had a weakly inhibitory effect on the binding of CTIP2 to the biotinylated CTIP RE (Fig. 3.4*A*, *lanes 4-5*, and *lanes 6-7*, respectively), suggesting that these four positions are crucial for CTIP2 binding. Similarly, the M4 oligonucleotide (mutation of all six core positions) minimally inhibited the binding of CTIP2 to the biotinylated CTIP RE (Fig. 3.4*A*, *lanes 10-11*).

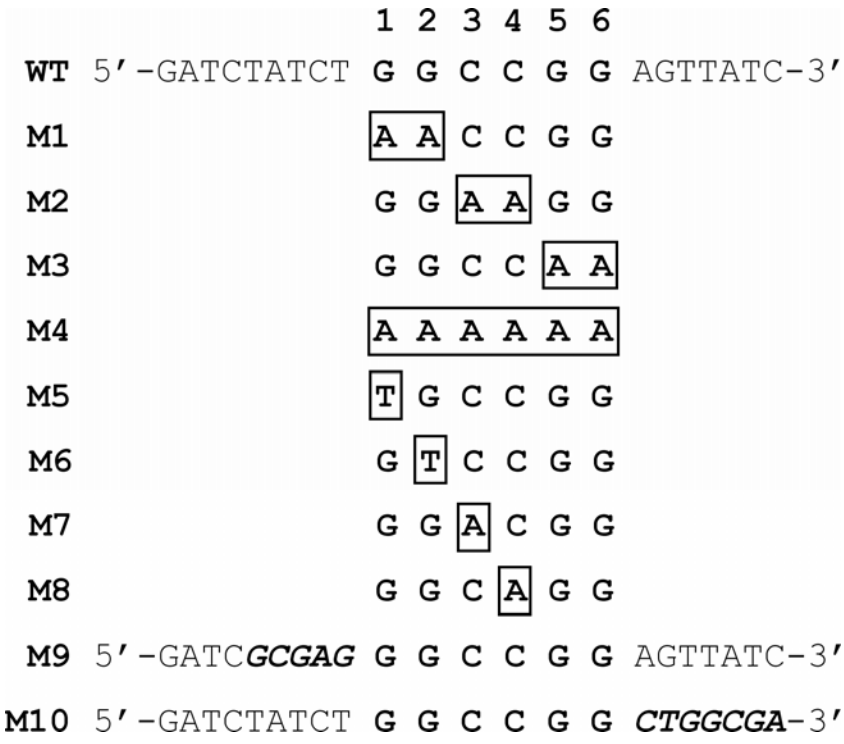


Figure 3.3 **Sequences of competitor oligonucleotides; wild-type (WT) CTIP RE and mutants M1-M10.** The nucleotides that are mutated in each oligonucleotide are boxed. A core CTIP RE is denoted in bold with position numbers. For simplicity, only upper strand is shown and sequences outside the core CTIP binding site of mutants M1-M8, which are identical to those of WT oligonucleotides are not shown. Mutations outside the core CTIP binding site of mutants M9 and M10 are indicated in italicized bold.

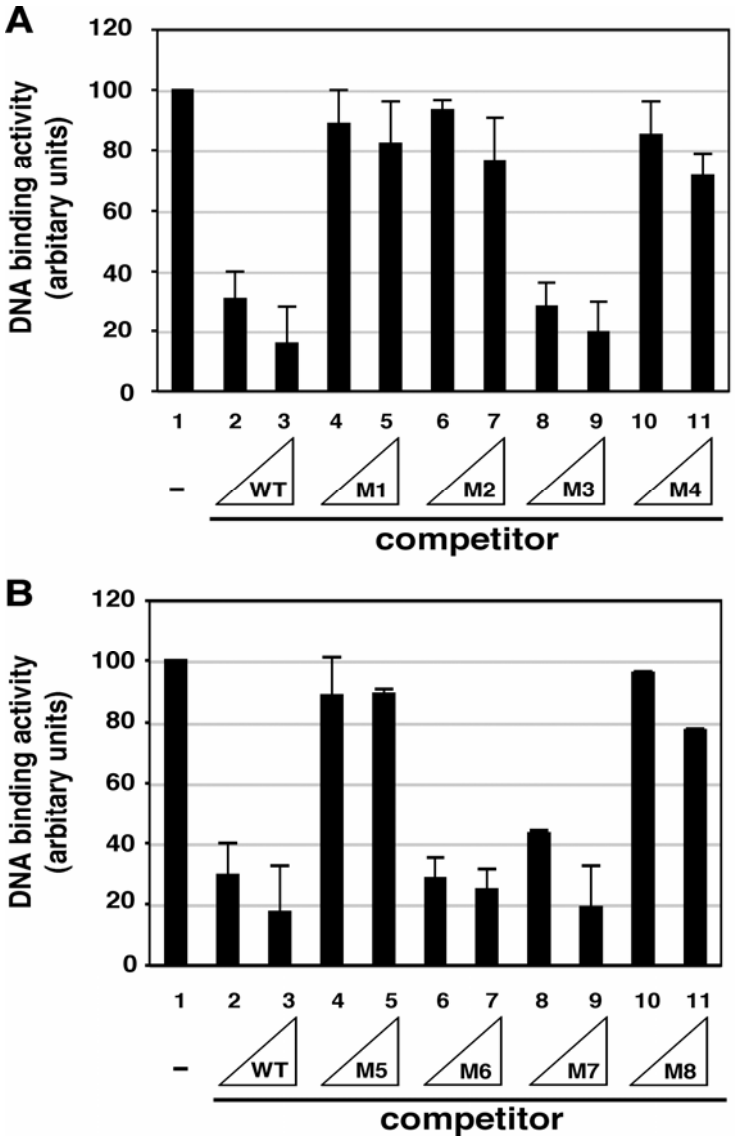


Figure 3.4 **Specificity of CTIP2 binding to the CTIP RE.** ***A,*** ABCD competition assays were carried out to measure the binding of [^{35}S]methionine-labeled CTIP2 to the biotinylated WT CTIP RE in the absence or presence of indicated nonbiotin-labeled competitor CTIP REs. *Lane 1* corresponds to reaction in the absence of non-biotin labeled competitor. *Lanes 2-11* contain nonbiotin-labeled competitor with increasing amounts (10- or 50-fold excess over the concentration of the biotin-labeled CTIP RE) as indicated by a triangle. CTIP RE binding activities were determined relative to *lane 1* and presented as the means ± S.E.M derived from at least three independent experiments. ***B,*** The competition assays were performed and quantified as described in *A,* except that M5, M6, M7, or M8 was used as a competitor.

As the positions outside the core CTIP RE were intact in all of these mutants tested, it is possible that these core-flanking nucleotides may contribute to CTIP2 binding. Thus, we tested two mutant oligonucleotides, each of which had transversion mutations (G $\rightarrow$ T and C $\rightarrow$ A) at sequences immediately upstream or downstream of the core binding site (see M9, and M10 of Fig. 3.3), for the ability to inhibit the interaction between CTIP2 and the biotinylated CTIP RE as described above. We found that these two oligonucleotides, M9 and M10, were able to compete with the biotinylated CTIP RE for CTIP2 binding (data not shown), suggesting that these five nucleotides upstream and seven nucleotides downstream of the core binding site did not contribute to CTIP2 binding, at least under the conditions used herein. Together, these results suggest that CTIP2, similar to CTIP1, binds DNA in a sequence specific manner, in which positions 1, 2, 3, and 4 of the CTIP RE are crucial determinants for CTIP2 binding *in vitro.*

Next, the specific bases within positions 1-4 required for CTIP2 binding was further determined. Mutants M5, M6, M7 and M8, which individually had a transversion mutation at position 1, 2, 3 and 4, respectively (see Fig. 3.3), were examined for the ability to compete with the biotinlyated CTIP RE for CTIP2 binding. The M6 and M7 oligonucleotide mutants (mutation at position 2 and 3, respectively) significantly inhibited the interaction between the biotinylated CTIP RE and CTIP2 to a similar extent as the competitor WT CTIP RE (Fig. 3.4*B*, compare *lane 1* to *lanes 2-3* and to *lanes 6-9*), suggesting that the position 2 and 3 in the core binding site are not important for CTIP2 binding. In contrast, the mutants M5 and M8 (mutation at the position 1 and 4, respectively) minimally inhibited the binding of CTIP2 to the biotinylated CTIP RE, even at a 50-fold excess over the biotinylated CTIP RE (Fig. 3.4*B*, *lanes 4-5*, and *10-11*).

These results demonstrate that the position 1 and 4 are extremely crucial for CTIP2 binding, and CTIP2 is likely to make a direct base contact to these two positions.

3.4.2 Two domains containing the ZnFs 3-4 and the ZNFs 5-7 are required for DNA binding activity of CTIP2

We have established that CTIP2 is a sequence-specific DNA binding protein. We then determined the region (s) of CTIP2 required for DNA binding. A series of CTIP2 truncation mutants were generated as *in vitro* [^{35}S]methionine-labeled proteins and tested for the ability to associate with the CTIP RE. A mutant of CTIP2 containing the ZnFs 3-4 (CTIP2 349-411; *panel F* of Fig. 3.5) interacted with the CTIP RE as strongly as a mutant containing ZnFs 5-7 (CTIP2 661-813; *panel C*), and as a full-length CTIP2 (CTIP2 1-813, *panel A*). Consistently, CTIP2 350-813 that comprised ZnFs 3-7 bound appreciably to the CTIP RE (Fig. 3.5, *panel G*). In contrast, a mutant of CTIP2 containing the first two ZnF motifs at the N-terminus (CTIP2 1-356) did not bind to the CTIP RE (Fig. 3.5, *panel D*). These results suggest that ZnFs 1 and 2 of CTIP 2 are dispensable for DNA binding, whereas the ZnFs 3-4 and the ZnFs 5-7 are the two modules that harbor an intrinsic DNA binding activity. However, CTIP2 350-716 and CTIP2 1-674 mutants, despite of containing ZnFs 3-4, barely bound to the DNA (Fig 3.5, *panel E* and *B*, respectively). Based on these results, we speculated that in the context of full-length CTIP2, the ZnFs 3-4 appeared to be required for DNA binding but might not be sufficient for high-affinity binding.

Accordingly, we generated mutants of CTIP2 in the context of the full-length protein. Each carried a point mutation (cysteine to alanine) at the second Zn (II)-coordinated cysteine that caused the individual disruption of ZnF 3, 4, 5, 6, or 7. These

point mutants were tested for DNA binding activity in the ABCD assays. We found that the mutation in the ZnF 3 or the ZnF 4 (CTIP2 1-813 C359A, *panel H*; CTIP2 1-813 C387A, *panel I* of Fig. 3.5, respectively) resulted in a dramatic reduction of DNA binding activity, suggesting that the ZnFs 3 and 4, in the context of full-length protein, are involved in DNA binding. The mutation in the ZnF 5 (CTIP2 1-813 C719A) did not appear to affect DNA binding of CTIP2 (Fig. 3.5, *panel J*). However, the mutation in the ZnFs 6 or 7 dramatically reduced the CTIP2 DNA binding activity (CTIP2 C747A, *panel K*; CTIP2 C777A, *panel L* of Fig. 3.5, respectively). These results suggest that the ZnFs 6 and 7 are necessary for the CTIP2 DNA binding activity and may explain why deletion of ZnFs 5, 6, 7 all together severely affects DNA binding to a greater extent (CTIP2 1-674; Fig. 3.5, *panel B*). Interestingly, the results from mutations of full-length CTIP2 also indicate that only the ZnF 3-4 module or the ZnF 5-7 module may not be sufficient for high-affinity DNA binding of CTIP2. Together, these results suggest that 1) the region of CTIP2 including ZnFs 1 and 2 do not contribute to DNA interaction 2) the ZnFs 3 and 4 and the motif of ZnFs 5-7 are required for the DNA binding activity of CTIP2. Low-affinity DNA binding observed with a disruption of the ZnFs 3, 4, 6, or 7, or with deletion of ZnFs 5-7 could be due to impaired DNA binding and/or impaired self-association, which is required for high-affinity DNA binding.

3.4.3 CTIP2 participates in self-association in solution

In addition to, or instead of, making direct contacts with DNA, the ZnF 5-7 or the ZnF 3-4 module may be crucial for CTIP2 self-association, a characteristic widely observed in many C_2H_2 zinc finger proteins, such as the Ikaros family members, that

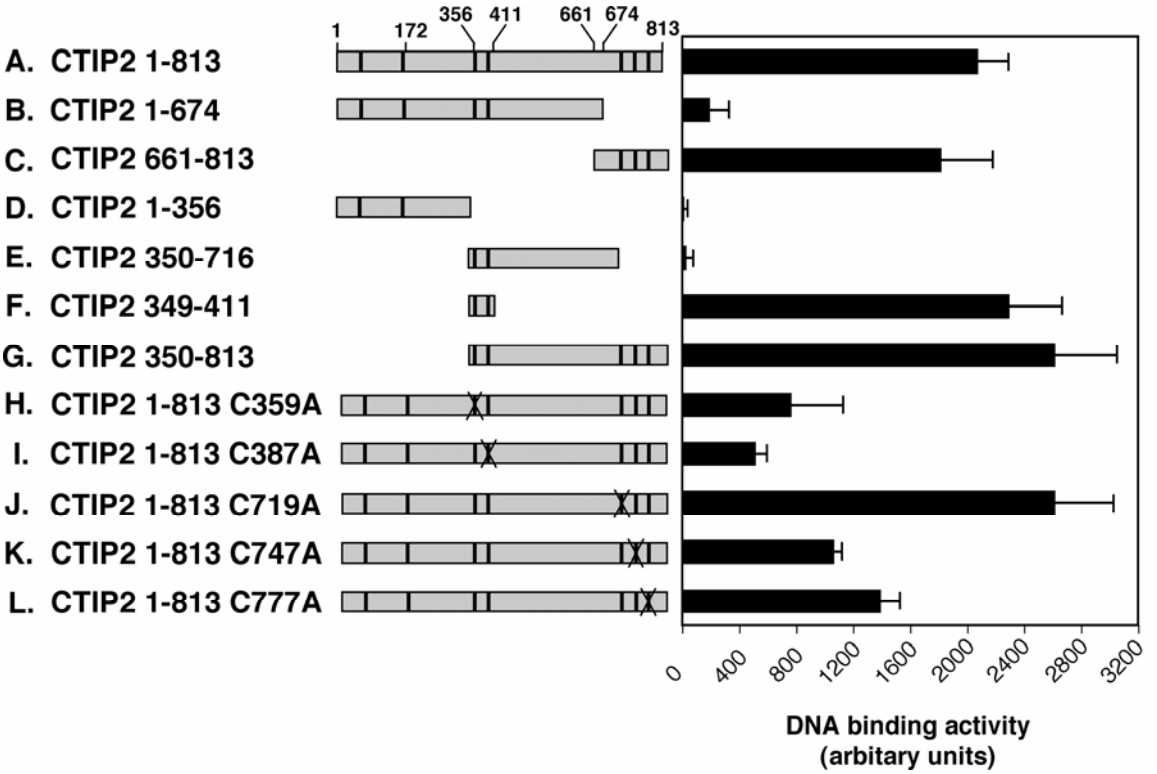


Figure 3.5. **The centrally located ZnFs 3-4 and the ZnF 5-7 module at the C-terminus are required for high-affinity DNA binding of CTIP2.** Full-length, truncation, and point mutants of CTIP2 (*A*-*L*) used for ABCD studies on the right are schematically represented with zinc finger motifs denoted by vertical bars. Disruptions of the individual ZnF by the point mutations are indicated by a cross mark. The binding of [^{35}S]methionine-labeled CTIP2 full-length and mutants to the biotinylated CTIP RE-containing oligonucleotides (see Fig. 3.2) are presented as the mean DNA binding activity ± S.E.M. derived from at least three independent experiments.

harbor a tandem array of ZnFs in a group of two or three (Molnar and Georgopoulos, 1994). Dimerization, although not absolutely necessary for DNA recognition, has been found to enhance the DNA binding affinity of Ikaros family members (Molnar and Georgopoulos, 1994) and nuclear receptors, members of a different class of ZnF proteins (Kumarasiri et al., 1988; Fawell et al., 1990; Drouin et al., 1992; Kuntz and Shapiro, 1997; Takeda et al., 2000). In the previous report, the formation of two CTIP2 · (CTIP RE) complexes with different electrophoretic mobilities has been observed, suggesting the possibility of multimeric-complex formation of CTIP2 upon binding to its binding site (Avram et al., 2002). Together with our above findings, self-association of CTIP2 was thereby studied in details. To test if CTIP2 interacted with itself *in vitro*, GST pull-down assays were carried out by using increasing amounts of affinity purified GST-CTIP2, which was incubated with *in vitro* translated [^{35}S]methionine-labeled full-length CTIP2. Consistent with data in the previous report, we found that *in vitro* translated CTIP2 interacted with GST-CTIP2 in a concentration-dependent manner (Fig. 3.6, *lanes 2-4*), but not with GST alone (*lane 5*). This finding suggests that CTIP2 participates in a self-associative interaction.

3.4.4 The ZnF 5-7 module and the proline-rich region of CTIP2 are involved in self-association

To map the self-interaction interface(s) of CTIP2, a series of GST-CTIP2 fusion proteins and *in vitro* translated, truncated CTIP2 were used in GST pull-down assays. Full-length CTIP2 interacted with GST-CTIP2 173-405 (*panel A* of Fig. 3.7*A*, *lane 5*) and also with GST-CTIP2 715-810 (*lane 7*) more strongly than with GST-CTIP2 405-716

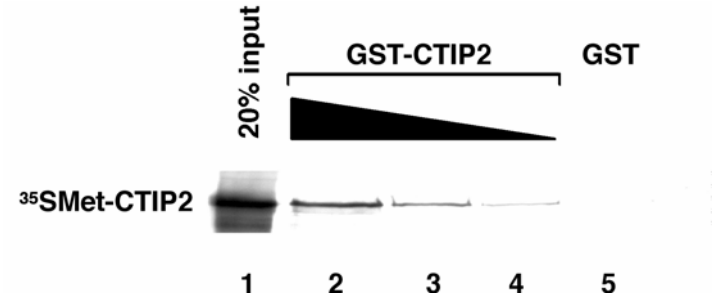
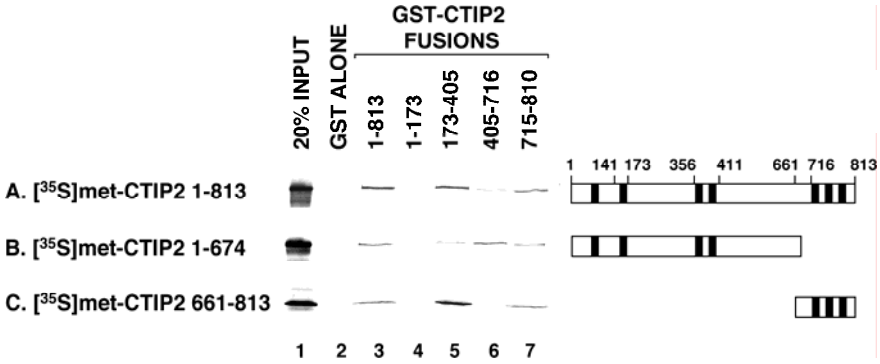


Figure 3.6. **Self-interaction of CTIP2 *in vitro***. GST pull-down experiments were performed using *in vitro* translated and [^{35}S]methionine-labeled CTIP2 and increasing amounts (0.42, 2.12 and 21.2 pmol, denoted by a black triangle) of bacterially expressed and affinity-purified GST-CTIP2 (*lanes 2-4*) or 21.2 pmol of GST (*lane 5*). [^{35}S]methionine-labeled CTIP2 associated with the affinity resin was subjected to SDS-PAGE and determined by autoradiography. Input of [^{35}S]methionine-labeled CTIP2 is shown in *lane 1*. Results shown are representative of at least two experiments.

(*lane 6*), but neither interacted with CTIP2 1-173 (*lane 4*) nor with a control GST (*lane 2*). Similar results were observed with a mutant CTIP2 661-813 (Fig. 3.7*A*, *panel C*). In contrast, we found that CTIP2 lacking the ZnFs 5-7 (CTIP2 1-674) interacted very weakly with either GST-CTIP2 173-405 (panel B of Fig. 3.7*A*, *lane 5*) or -CTIP2 715-810 (*lane 7*), but interacted more strongly with GST-CTIP2 405-716 (*lane 6*). As we did not observe the interaction between GST-CTIP2 1-173 with any [^{35}S]methionine-labeled proteins tested herein, we suggest that amino acids 1-173 of CTIP2 including ZnFs 1-2 are not involved in CTIP2 self-association. Rather, CTIP2 self-association appeared to occur mainly through the interaction between two interfaces: the core region (amino acids 173-405) and the C-terminus of the protein (amino acids 715-810). In fact, we found that GST-CTIP2 full-length apparently interacted with the full-length CTIP2 but minimally interacted with CTIP2 1-674 or with CTIP2 661-813, each of which lacked either the core region or the C-terminus (Fig. 3.7*A*, *lane 3 of all panels*). Thus, these two domains seem to be required for a strong self-interaction of CTIP2.

For a thorough understanding of the CTIP2 self-association, more extensive GST pull-down experiments were performed using GST-CTIP2 full-length and a series of *in vitro* translated, [^{35}S]methionine-labeled CTIP2 truncation mutants. We found that truncation mutants containing the ZnF 1 (CTIP2 1-141) did not interact at all with GST-CTIP2 (*lane 21* of Fig. 3.7*B*). A CTIP2 1-356 mutant consisting of the ZnFs 1-2 and the proline-rich region was found to interact weakly with GST-CTIP2 (Fig. 3.7*B*, *lane 22*). As the mutant containing amino acids 171-350 of CTIP2 interacted with GST-CTIP2 (Fig. 3.7*B*, *lane 29*), this region was likely to mediate the interaction between CTIP2 1-356 and GST-CTIP2. Again, these results confirm that the ZnF 1 and the ZnF 2 of CTIP2 are not involved in self-association, but the region that is relatively rich in proline residues (amino acids 171-350; Avram et al., 2000) is likely to contribute to this process.

A



B

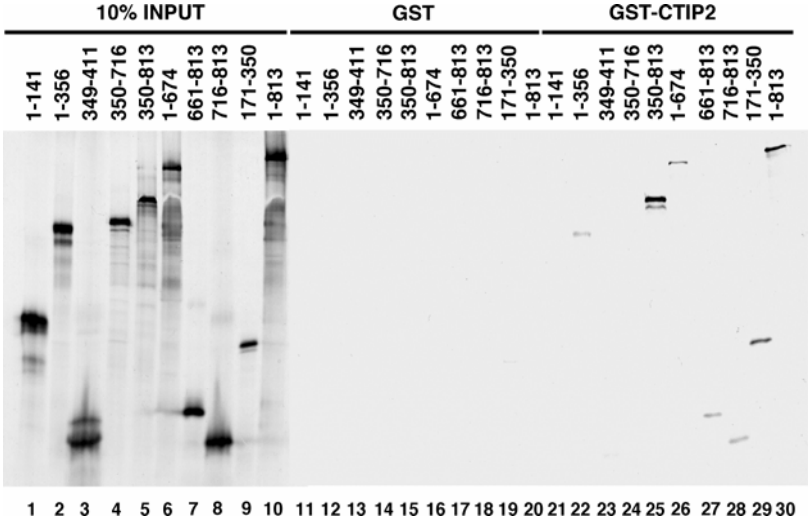


Figure 3.7. **Mapping the self-interaction domain of CTIP2.** ***A,*** Equivalent amounts of GST (*lane 2*) or GST-CTIP2 fusion proteins (*lanes 3-7*) were incubated with indicated [^{35}S]met-labeled CTIP2 proteins. After extensive washing, [^{35}S]met-labeled CTIP2 proteins associated with the affinity resin were determined by SDS-PAGE and autoradiography. [^{35}S]met-labeled CTIP2 proteins used in these studies are schematically represented on the right with zinc finger motifs denoted by vertical bars. ***B*****,** [^{35}S]met-labeled full-length and truncated CTIP2 were incubated with equivalent amounts of GST (*lanes 11-20*) or full-length CTIP2 fused to GST (GST-CTIP2; *lanes 21-30*). [^{35}S]met-labeled proteins associated with the affinity resin were resolved by SDS-PAGE and visualized by autoradiography. Results shown in *A* and *B* are representative of at least two experiments.

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In addition, CTIP2 deletion mutants containing the ZnF 5-7 module (amino acids 716-813) were found to interact with GST-CTIP2 (Fig. 3.7*B*, *lanes 25, 27 and 28*), but not with control GST alone (*lanes 15, 17 and 18*). Consistently, a CTIP2 1-674 mutant lacking this region interacted with GST-CTIP2 more weakly than the full-length CTIP2 (CTIP2 1-813; compare *lanes 26* to *30* of Fig. 3.7*B*). These results confirm the significance of ZnFs 5-7 in CTIP2 self-association as previously shown in Fig. 3.7*A*. A CTIP2 349-411 mutant (consisting of only ZnFs 3-4) did not interact at all with GST-CTIP2 (*lane 23* of Fig. 3.7*B*). A similar result was obtained from a CTIP2 350-716 mutant (*lane 24* of Fig. 3.7*B*) containing the ZnF 3-4 motif (amino acids 349-411) and the acidic-/serine-rich region (amino acids 412-716; Avram et al., 2000), indicating that the ZnF 3-4 module and the acidic-/serine-rich region of CTIP2 are not obligatory for self-association of the protein.

Altogether, the proline-rich region (amino acids 171-350), and the ZnF 5-7 module (amino acids 716-813) are two primary self-association interfaces of CTIP2. In the context of full-length protein, our findings suggest that the ZnF 5-7 module may mediate inter-molecular self-association through the interaction with itself and with the proline-rich region of the partner protein. It is also possible that the interaction between the ZnF 5-7 domain and the proline-rich region occurs intra-molecularly. Such a complex mode of CTIP2 self-association mediated by the ZnF 5-7 module and the proline-rich domain may facilitate the formation of a stable self-interaction complex(es) on the target binding sites.

Given the above findings that the two modules of the ZnF 3-4 and the ZnF 5-7 are both crucial for CTIP2 DNA binding activity, and that the ZnF 3-4 module is not primarily required for self-association, we therefore conclude that the ZnF 3-4 module may play a major role in a direct DNA interaction. The ZnF 5-7A module is, however,

crucial for self-association which is required for high-affinity DNA binding. We proposed a model for self-association and DNA binding of CTIP2 as shown in Fig. 3.8. The functions of the individual domains of CTIP2 are summarized in Table 3.2.

3.4.5 The ZnF 5-7 containing domain is required for transcriptional regulatory activity of CTIP2 in cells

CTIP2 has previously been shown to be a transcriptional repressor that functions by tethering to targeted promoters either through COUP-TF (chicken ovalbumin upstream promoter transcription factor) nuclear receptors, or a heterologous DNA binding domain (Avram et al., 2000; Senawong et al., 2003). In addition, CTIP2 appears to bind the CTIP RE directly and mediates transcriptional repression (Avram et al., 2002). If the hypothesis that the ZnF 5-7 region of CTIP2 is important for self-association and DNA binding is true, lack of this region must result in failure of CTIP2 to repress transcription through the CTIP RE due to an inability to self-associate and bind DNA. To determine if the ZnF 5-7 module of CTIP2 plays a role in CTIP2-mediated transcriptional repression, full-length CTIP2 and the truncation mutant lacking the ZnFs 5-7 (CTIP2 1-674), which did not bind to the CTIP RE with high affinity (see *panel B* of Fig. 3.5), were assessed for the ability to mediate repression of a downstream CAT reporter gene driven by a thymidine kinase (*tk*) promoter containing the CTIP REs (Avram et al., 2000) in transiently transfected HEK293T cells (Fig. 3.9*A*). Within a range of concentrations tested, full-length CTIP2 did not repress transcription from an empty reporter (*tk*-CAT; compare *lanes 2-4* to *lane 1* of Fig. 3.9*B*), but was found to repress transcription from the reporter harboring the CTIP REs ((CTIP RE)$_3$-*tk*-CAT) in a concentration-dependent manner (compare *lanes 9-11* to *lane 8*). These results suggest that transcriptional activity of CTIP2 occurs upon its specific binding to this CTIP RE element.

Table 3.2. **The summary of DNA binding and self-associative activities of domains of CTIP2**

DOMAIN	AA RESIDUES	DNA BINDING	SELF-ASSOCIATION
ZnFs 1-2	1-172	no	no
Proline-rich region	173-350	no	associates with the ZnF 5-7 motif
ZnFs 3-4	351-404	yes	no
Acidic-/serine-rich region	405-715	no	no
ZnFs 5-7	716-813	yes	self-associates and associates with the proline-rich region

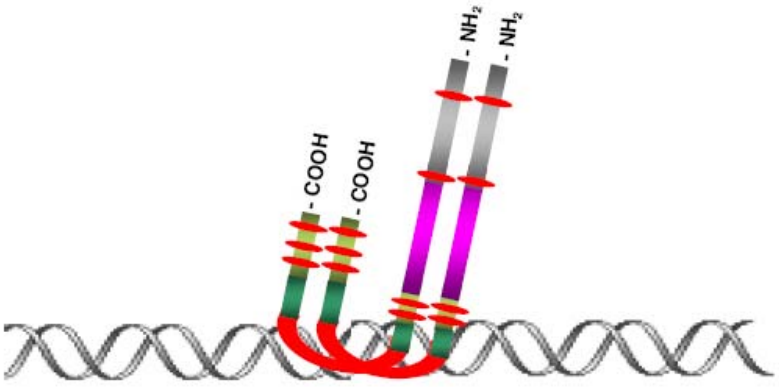

Figure 3.8. **A proposed model for DNA binding and self-association of CTIP2.** Two monomers of CTIP2 are schematically shown with different domains being represented by color boxes and zinc fingers being denoted by red vertical bars. We propose that, at least, two monomers of CTIP2 participate in self-association, which is mediated by the two interaction interfaces: the C-terminus of the protein including the ZnFs 5-7 and the proline-rich region. Self-association may enhance the DNA binding, which occurs primarily by a direct interaction between the ZnF 3-4 module and the CTIP response element.

In contrast, a CTIP2 1-674 mutant which lacked the ZnFs 5-7 neither repressed transcription from the (CTIP RE)$_3$-*tk*-CAT reporter (Fig. 3.9*B*, *lanes 12-14*), nor repressed transcription from the empty *tk*-CAT reporter (*lanes 5-7*), suggesting that the region comprising of the ZnFs 5-7 is crucial for the regulatory activity of CTIP2 in this context, possibly due to its requirement for self-association and DNA binding.

3.4.6 The ZnF 5-7 domain does not harbor an autonomous repressive activity

Although the above results suggest that the ZnF 5-7 module may harbor self-associative and DNA binding activities which are required for regulatory function of CTIP2, there is another possibility that this region may confer an autonomous repressive activity. To test this possibility, the CTIP2 1-674 mutant and the mutant that mainly contained the ZnFs 5-7 (CTIP2 661-813) were examined for ability to repress transcription of a luciferase reporter gene driven by a minimal promoter (Lex-Gal-Luc; (Christian et al., 2004; Topark-Ngarm and Golonzhka et al., 2006) when transiently transfected into HEK293T cells. Given the above finding that CTIP2 1-674 protein did not bind DNA well, we therefore needed to fuse such a protein to a Gal4-DBD (a heterologous DNA binding domain), which would allow the protein tethering to DNA through Gal4-binding sites located upstream of the promoter (Fig. 3.10*A*). This way the repressive activity could be assessed without the interference from its inability for DNA binding. In order to facilitate evaluation of transcriptional repression mediated by Gal4-CTIP2 fusion proteins, expression of the reporter gene was stimulated by co-expression with LexA-VP16 (Christian et al., 2004).

We found that Gal4-DBD alone did not repress transcription of the LUC reporter gene (*lane 2*; Fig. 3.10*B*), but Gal4-CTIP2 1-674 was able to repress

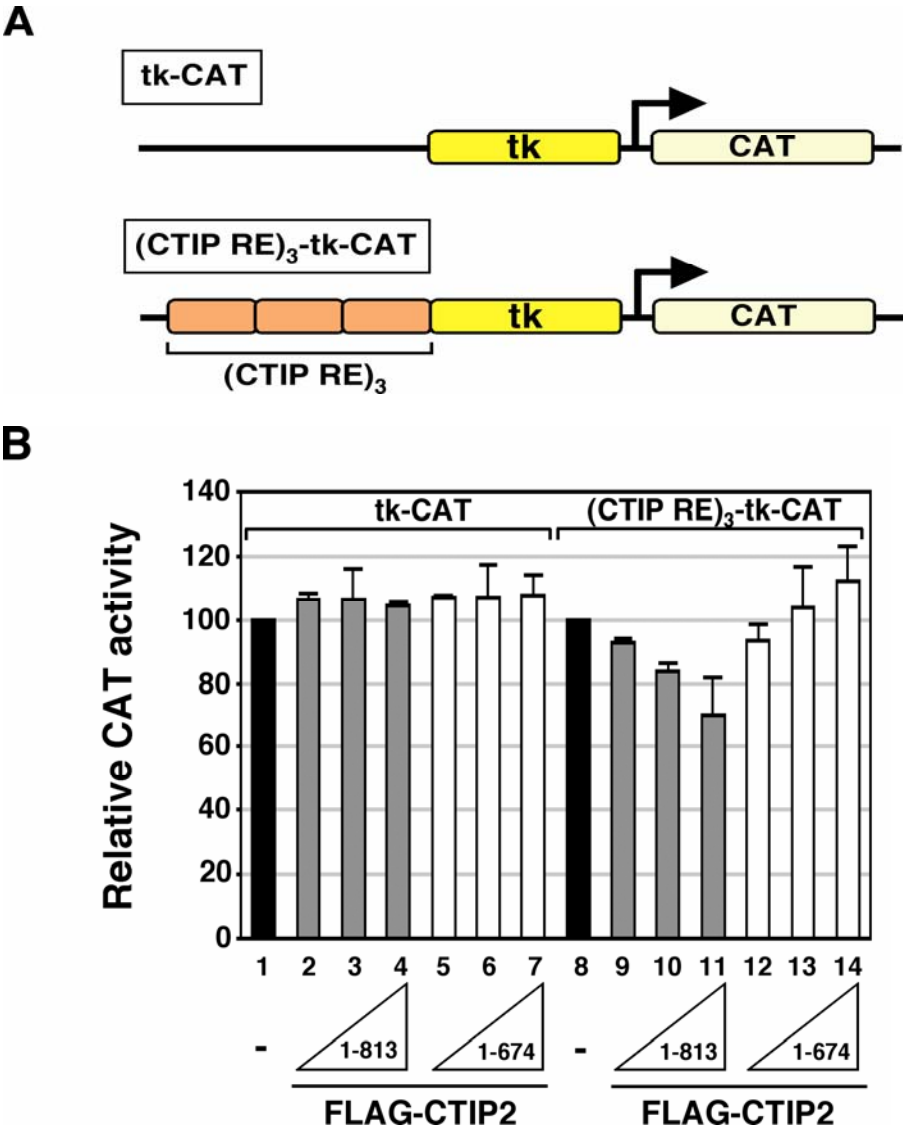

Figure 3.9. **Requirement of the ZnFs 5-7 for the transcriptional regulatory activity of CTIP2 in transiently transfected HEK293T cells. *A*,** Schematic diagrams of *tk*-CAT reporters. *(Top)* without (*tk*-CAT) or *(Bottom)* with three copies of CTIP RE located immediately upstream of a *tk* promoter of a *CAT* reporter gene ((CTIP RE)$_3$-*tk*-CAT). ***B,*** HEK293T cells were co-transfected in 10 cm plate with 4 μg of either control *tk*-CAT or (CTIP RE)$_3$-*tk*-CAT reporter and with increasing amounts (0.5, 5, 15 ng; indicated by a triangle) of expression vector encoding either full-length (grey bars) or amino acids 1-674 (white bars) of FLAG-CTIP2. Transfection efficiency was normalized by use of a co-transfected β-galactosidase expression vector and protein concentration. The CAT activity determined in the presence of empty vector transfection was taken to be 100 (*lanes 1* and *8*; black bars) and that against which all other determined CAT activities (*lanes 2-7*, and *lanes 9-14*, respectively) were compared. The results shown here represent means ± S.E.M. of three independent experimental determinations.

transcription to similar extent as Gal4-CTIP2 full-length (compare *lanes 5-6* and *lanes 3-4*, respectively, to *lane 1*). However, we did not observed statistically significant repression by Gal4-CTIP2 661-813 (Fig. 3.10*B*, *lanes 7-8*). Considering that Gal4-CTIP2 661-813 fails to repress transcription, we strongly suggest that the ZnF 5-7 module does not harbor an autonomous repressive activity.

3.4.7 The integrity of ZnFs 5-7 is a crucial determinant for transcriptional regulatory activity of CTIP2

We have shown above that the DNA binding and/or self-associative activities of the ZnF 5-7 module are required for the CTIP2-mediated transcriptional repression. We then determined if the ZnF 3 and 4, which were also found to be necessary for CTIP2 DNA binding activity *in vitro*, were crucial for CTIP2 functionality as a transcriptional repressor in cells. If these individual ZnFs contribute to this process, any mutation that disrupts the integrity of the ZnF would result in a failure or compromise in transcriptional repression activity. It was also of interest to test if disruptions of ZnF 5, 6, or 7 individually would differently affect CTIP2-mediated transcriptional repression. Thus, the mutants that caused the individual ZnF disruption as described above were examined for repression activity on the $(CTIP\ RE)_3$-*tk*-CAT reporter in transiently transfected HEK293T cells. Given the findings that ZnF1 and 2 did not play a role in DNA binding and self-associative interaction, therefore such a mutation in either the ZnF1 or the ZnF2 was not generated and tested herein. In the reporter assay, we found that the ZnF3 (C359A) and the ZnF4 (C387A) mutants, both of which minimally bound to the DNA; however, moderately repressed transcription when compared to the wild-type counterpart (Fig. 3.11, compare *lanes 3* and *4* to *lane 2*). In contrast, we found that mutations that disrupted the integrity of the ZnF5 (C719A), ZnF6 (C747A), or ZnF7 (C777A), markedly

affected the transcriptional activity of CTIP2 with the greatest effect found in the ZnF 6 mutation (Fig. 3.11, compare *lanes 5, 6* and *7* to *lane 2*). It appeared that these three ZnFs were likely to work coordinately, not exclusively, in CTIP2-mediated transcriptional repression because deletion of all three ZnFs completely abolished the transcriptional repressive activity of CTIP2 as described above (see CTIP2 1-674 in Fig. 3.9).

Taken together, the ZnFs 5-7 at the C-terminus of CTIP2 are crucial for transcriptional repression, likely due to their necessity for self-association and DNA binding.

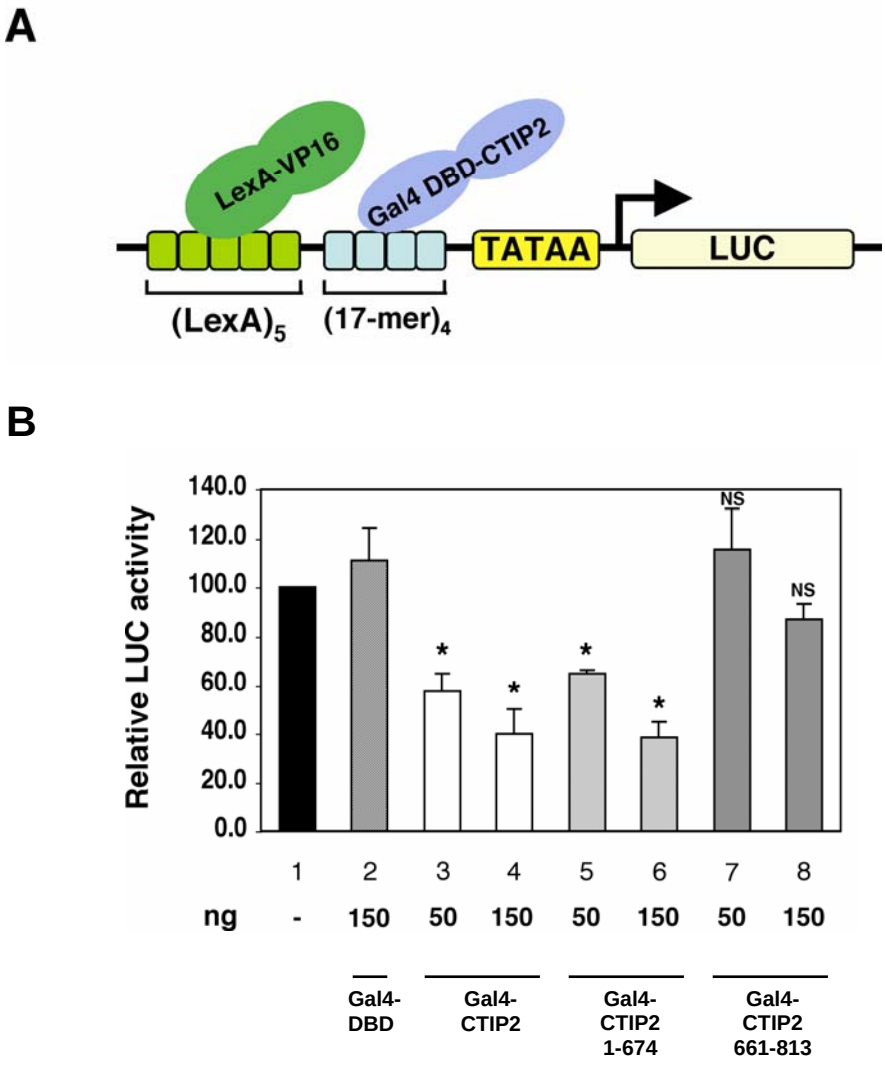

Figure 3.10. **The ZnF 5-7 domain of CTIP2 does not confer the intrinsic repressive activity.** ***A,*** A Schematic diagram of a Lex-Gal-Luc reporter illustrating LexA and Gal4 (17-mer) binding sites, to where LexA-VP16 and Gal4-fusion proteins i.e., Gal4-CTIP2 binds. Both of these binding sites are upstream of the *LUC* reporter gene driven by a minimal promoter. ***B,*** HEK293T cells were transfected with 3 μg of Lex-Gal-Luc reporter, 0.1 μg of LexA-VP16 and indicated amounts of expression vector encoding Gal4-DBD or Gal4-CTIP2 fusion proteins. Light units were normalized across all samples by expression of β-galactosidase and presented as means of LUC activity ± S.E.M. relative to *lane 1*. The presented data were derived from three independent experiments. Statistical significance is indicated by asterisks ($p < 0.05$, Student's *t* test) when comparing against either *lane 1* or *lane 2*. NS = nonstatistical significance.

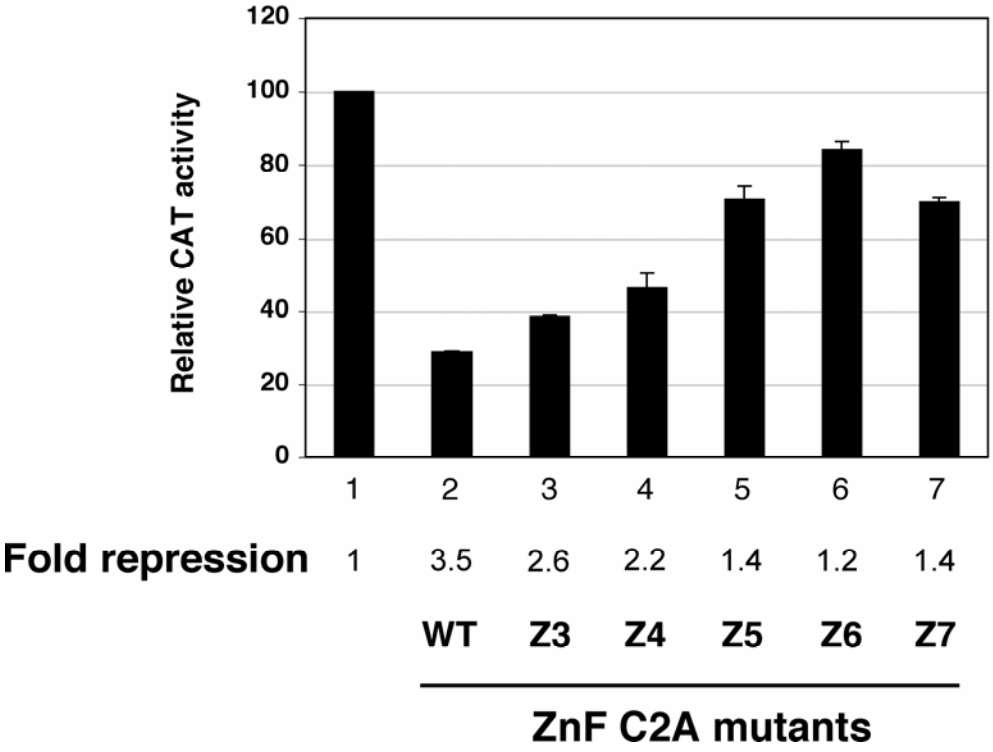

Figure 3.11. **Dramatic reduction of repressive activity of CTIP2 by the mutation in ZnF 5, 6 and 7.** HEK293T cells were co-transfected with 4 μg of (CTIP RE)$_3$-tk-CAT reporter and 1μg of empty vector or an expression vector encoding either wild-type (WT) FLAG-CTIP2 or cysteine to alanine (C2A) mutants; mutation in an individual ZnF is denoted by Z followed by the number of ZnF in which mutation occurs. Transfection efficiency was normalized by use of a co-transfected β-galactosidase expression vector and protein concentration. The CAT activity determined in the presence of empty vector transfection was taken to be 100 (*lane 1*) and that against which all other determined CAT activities (*lanes 2-7*) were compared. Fold repression in all lanes was determined relative to *lane 1*. The results shown here represent means ± S.E.M. of three independent experimental determinations.

3.5 Discussion

Zinc finger proteins play crucial roles in a variety of biological activities such as development, differentiation, tumor suppression, and immunological responses (Ravasi et al., 2003). Many studies have demonstrated that ZnFs may function in homotypic and heterotypic, protein-protein interactions, which enhance the affinity (homo- and heterotypic) and/or DNA binding specificity (heterotypic) of numerous transcription factors that harbor these structural motifs (Fawell et al., 1990; Molnar and Georgopoulos, 1994; Tzameli and Zannis, 1996; Kuntz and Shapiro, 1997; Tsai and Reed, 1998; Haelens et al., 2003; McCarty et al., 2003).

CTIP2 is a transcriptional repressor that contains multiple C_2H_2 zinc fingers (Avram et al., 2000). Here we demonstrated that CTIP2, similar to the highly related CTIP1 (Avram et al., 2000), bound directly to DNA in a sequence-specific manner and was found to self-associate *in vitro (*see Table 3.1 and Fig. 3.4-3.7). However, CTIP2 exhibited complex modes of DNA binding and self-interaction, which were distinct from those of CTIP1. The central region of CTIP2 comprising the ZnFs 3-4 was found to be necessary for DNA binding activity. Unlike those in CTIP1, the ZnF 3-4 module in CTIP2 was not primarily required for self-association. Rather, the C-terminal domain of CTIP2 containing the ZnFs 5-7 confers an intrinsic self-associative activity which appears to be obligatory for high-affinity DNA binding (see Fig. 3.5 and 3.7). The ZnF 5-7 region was found to interact with itself and also with the proline-rich region, at least in the absence of DNA (see Fig. 3.7), suggesting that oligomerization of CTIP2 may occur prior to DNA binding.

Mutations that disrupted the integrity of the ZnF3 (C359A) or the ZnF4 (C387A) negatively affected, but did not eliminate, the ability of CTIP2 to bind DNA *in vitro* (Fig. 3.5). However, these same mutations only partially reduced CTIP2-mediated transcriptional repression of an artificial target gene in cells (Fig. 3.11). These findings suggest at least two non-mutually exclusive possibilities. First, the residual DNA binding activity of CTIP2 C359A and C387A may be sufficient to allow the protein to bind DNA and repress transcription in cells. Second, other cellular factors may interact with the DNA-bound form of the CTIP2 and stabilize a complex that binds DNA with affinity lower than that of wild-type protein. In contrast, disruption of the ZnF 5, 6, or 7 or deletion of all these three ZnFs considerably affected the repressive activity of CTIP2 (see Fig. 3.9 and 3.11), and this could be explained by a lack of DNA binding, most likely due to a lack of self-association.

Moreover, self-association of CTIP2 may contribute to CTIP2-mediated transcriptional repression by other mechanism(s). Indeed, several studies have shown that dimerization of ZnF proteins is subject to regulation by cellular signaling pathways. For example, formation of homodimerization of Mel-18 –a polycomb protein that has tumor suppressor gene-like activity and negatively regulates transcription–has been shown to be inhibited by PKC phosphorylation. Although the consequence of this inhibition on its function is presently unknown, the authors suggest that equilibrium between monomers and dimers might control the function of the class II polycomb protein complex containing Mel-18 by regulating the subunit composition of the complex (Fujisaki et al., 2003). In another report, Fernandes and colleagues have demonstrated that the C-terminus of p50E4F –a cellular factor that is regulated by adenovirus E1A oncoproteins–mediates both sequence-specific DNA binding and dimerization and is

regulated by phosphorylation (Rooney et al., 1998). Thus, further studies will be necessary to investigate if self-association of CTIP2 and/or repression of its target genes are influenced by cellular signaling pathways. In addition, self-association may be a mode used by the protein to recruit other transcriptional regulators to support its function. It has been shown that the recruitment of nuclear receptor corepressor (NCoR) prevents dissociation of homodimer complex of the thyroid hormone receptor from a target hormone response element (Makowski et al., 2003). Corepressor recruitment by the C-terminal dimerization domain of CTIP2, however, is unlikely to be operant in our case as we clearly demonstrate that this domain does not harbor intrinsic transcriptional repressive activity (see Fig. 3.10). However, the proline-rich region of CTIP2, which interacts with the ZnF5-7 module, has been shown by our group to associate directly with two repressors: the class III histone deacetylase SIRT1 (Senawong et al., 2003), and with components of the NuRD corepressor complex (Topark-Ngarm and Golonzhka et al., 2006), both of which likely underlie CTIP2-mediated transcriptional repression in a cellular context. Thus, it is conceivable that the association between proline-rich and the C-terminal ZnF domain of CTIP2 may stabilize the corepressor binding to protein in cells, and this may be an area of future investigation.

Together, these findings define pivotal roles of ZnFs in transcriptional repression mediated by CTIP2. Understanding sequence-specific DNA binding and protein-protein interaction properties of CTIP2 is an important step toward the goal of elucidating transcriptional targets and functions of CTIP2 in various cellular and promoter contexts.

3.6 Abbreviations

CTIP1 and CTIP2, COUP-TF-interacting proteins 1 and 2; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CTIP RE, CTIP response element; ZnF, zinc finger; NuRD, nucleosome remodeling and deacetylation; Sir2, silent information regulator 2; SIRT1, sirtuin 1 or Sir2-like protein 1; HEK293T, human embryonic kidney 293T cells; DBD, DNA binding domain; CAT, chloramphenicol acetyltransferase, LUC, luciferase; bp, base pair; AA, amino acid; GST, glutathione S-transferase; WT, wild-type; ABCD, Avidin (Streptavidin)-Biotin Complex with DNA

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3.8 References

Arlotta, P., Molyneaux, B. J., et al. (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron **45**(2): 207-21.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Choo, Y., Castellanos, A., et al. (1997). "Promoter-specific activation of gene expression directed by bacteriophage-selected zinc fingers." J Mol Biol **273**(3): 525-32.

Christian, M., Tullet, J. M., et al. (2004). "Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140." J Biol Chem **279**(15): 15645-51.

Cismasiu, V. B., Ghanta, S., et al. (2006). "BCL11B participates in the activation of IL2 gene expression in CD4+ T lymphocytes." Blood **108**(8): 2695-702.

Dowell, P., Ishmael, J. E., et al. (1997a). "p300 functions as a coactivator for the peroxisome proliferator-activated receptor alpha." J Biol Chem **272**(52): 33435-43.

Dowell, P., Peterson, V. J., et al. (1997b). "Ligand-induced peroxisome proliferator-activated receptor alpha conformational change." J Biol Chem **272**(3): 2013-20.

Drouin, J., Sun, Y. L., et al. (1992). "Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor." Mol Endocrinol **6**(8): 1299-309.

Fawell, S. E., Lees, J. A., et al. (1990). "Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor." Cell **60**(6): 953-62.

Fujii, K., Ishimaru, F., et al. (2003). "Over-expression of short isoforms of Helios in patients with adult T-cell leukaemia/lymphoma." Br J Haematol **120**(6): 986-9.

Fujisaki, S., Ninomiya, Y., et al. (2003). "Dimerization of the Polycomb-group protein Mel-18 is regulated by PKC phosphorylation." Biochem Biophys Res Commun **300**(1): 135-40.

Georgopoulos, K. (2002). "Haematopoietic cell-fate decisions, chromatin regulation and ikaros." Nat Rev Immunol **2**(3): 162-74.

Haelens, A., Verrijdt, G., et al. (2003). "DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation." Biochem J **369**(Pt 1): 141-51.

Hall, T. M. (2005). "Multiple modes of RNA recognition by zinc finger proteins." Curr Opin Struct Biol **15**(3): 367-73.

Kelley, C. M., Ikeda, T., et al. (1998). "Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors." Curr Biol **8**(9): 508-15.

Kumarasiri, M. H., Shapiro, L. E., et al. (1988). "Cell cycle dependence of thyroid hormone nuclear receptors in cultured GC cells: relationship to nuclear matrix." Endocrinology **122**(5): 1897-904.

Kuntz, M. A. and Shapiro, D. J. (1997). "Dimerizing the estrogen receptor DNA binding domain enhances binding to estrogen response elements." J Biol Chem **272**(44): 27949-56.

Laity, J. H., Lee, B. M., et al. (2001). "Zinc finger proteins: new insights into structural and functional diversity." Curr Opin Struct Biol **11**(1): 39-46.

Lander, E. S., Linton, L. M., et al. (2001). "Initial sequencing and analysis of the human genome." Nature **409**(6822): 860-921.

Lee, M. S., Gippert, G. P., et al. (1989). "Three-dimensional solution structure of a single zinc finger DNA-binding domain." Science **245**(4918): 635-7.

Leid, M., Ishmael, J. E., et al. (2004). "CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis." Gene Expr Patterns **4**(6): 733-9.

Makowski, A., Brzostek, S., et al. (2003). "Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor." Mol Endocrinol **17**(2): 273-86.

Matthews, J. M. and Sunde, M. (2002). "Zinc fingers--folds for many occasions." IUBMB Life **54**(6): 351-5.

McCarty, A. S., Kleiger, G., et al. (2003). "Selective dimerization of a C2H2 zinc finger subfamily." Mol Cell **11**(2): 459-70.

Miller, K. G., Tower, J., et al. (1985). "A complex control region of the mouse rRNA gene directs accurate initiation by RNA polymerase I." Mol Cell Biol **5**(3): 554-62.

Molnar, A. and Georgopoulos, K. (1994). "The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins." Mol Cell Biol **14**(12): 8292-303.

Morgan, B., Sun, L., et al. (1997). "Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation." Embo J **16**(8): 2004-13.

Nakase, K., Ishimaru, F., et al. (2002). "Overexpression of novel short isoforms of Helios in a patient with T-cell acute lymphoblastic leukemia." Exp Hematol **30**(4): 313-7.

Pelham, H. R. and Brown, D. D. (1980). "A specific transcription factor that can bind either the 5S RNA gene or 5S RNA." Proc Natl Acad Sci U S A **77**(7): 4170-4.

Perdomo, J., Holmes, M., et al. (2000). "Eos and pegasus, two members of the Ikaros family of proteins with distinct DNA binding activities." J Biol Chem **275**(49): 38347-54.

Picard, B. and Wegnez, M. (1979). "Isolation of a 7S particle from Xenopus laevis oocytes: a 5S RNA-protein complex." Proc Natl Acad Sci U S A **76**(1): 241-5.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Qiu, Y., Krishnan, V., et al. (1995). "Isolation, characterization, and chromosomal localization of mouse and human COUP-TF I and II genes." Genomics **29**(1): 240-6.

Ravasi, T., Huber, T., et al. (2003). "Systematic characterization of the zinc-finger-containing proteins in the mouse transcriptome." Genome Res **13**(6B): 1430-42.

Ritchie, H. H., Wang, L. H., et al. (1990). "COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily." Nucleic Acids Res **18**(23): 6857-62.

Rohr, O., Marban, C., et al. (2003). "Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells." J Leukoc Biol **74**(5): 736-49.

Rooney, R. J., Rothammer, K., et al. (1998). "Mutational analysis of p50E4F suggests that DNA binding activity is mediated through an alternative structure in a zinc finger domain that is regulated by phosphorylation." Nucleic Acids Res **26**(7): 1681-8.

Ruiz, A., Jiang, J., et al. (2004). "Overexpression of the Ikaros 6 isoform is restricted to t(4;11) acute lymphoblastic leukaemia in children and infants and has a role in B-cell survival." Br J Haematol **125**(1): 31-7.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Sun, L., Goodman, P. A., et al. (1999). "Expression of aberrantly spliced oncogenic ikaros isoforms in childhood acute lymphoblastic leukemia." J Clin Oncol **17**(12): 3753-66.

Sun, L., Liu, A., et al. (1996). "Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development." Embo J **15**(19): 5358-69.

Suzuki, M. and Yagi, N. (1994). "DNA recognition code of transcription factors in the helix-turn-helix, probe helix, hormone receptor, and zinc finger families." Proc Natl Acad Sci U S A **91**(26): 12357-61.

Takeda, K., Ichiki, T., et al. (2000). "Peroxisome proliferator-activated receptor gamma activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells." Circulation **102**(15): 1834-9.

Topark-Ngarm, A., Golonzhka, O., et al. (2006). "CTIP2 associates with the NuRD complex on the promoter of p57KIP2, a newly identified CTIP2 target gene." J Biol Chem **281**(43): 32272-83.

Tsai, R. Y. and Reed, R. R. (1998). "Identification of DNA recognition sequences and protein interaction domains of the multiple-Zn-finger protein Roaz." Mol Cell Biol **18**(11): 6447-56.

Tzameli, I. and Zannis, V. I. (1996). "Binding specificity and modulation of the ApoA-I promoter activity by homo- and heterodimers of nuclear receptors." J Biol Chem **271**(14): 8402-15.

Wakabayashi, Y., Watanabe, H., et al. (2003a). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Wakabayashi, Y., Inoue, J., et al. (2003b). "Homozygous deletions and point mutations of the Rit1/Bcl11b gene in gamma-ray induced mouse thymic lymphomas." Biochem Biophys Res Commun **301**(2): 598-603.

Wang, L. H., Tsai, S. Y., et al. (1989). "COUP transcription factor is a member of the steroid receptor superfamily." Nature **340**(6229): 163-6.

Chapter 4

Transcriptional Repression of Id2 by CTIP2

Acharawan Topark-Ngarm[1], Philippe Kastner[2], Olga Golonzhka[3], Valerie Peterson[1], and Mark Leid[1,4]

[1]Departments of Pharmaceutical Sciences and [3]Biochemistry and Biophysics, and [4]Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331, and [2]the Institut de Génétique et de Biologie Moléculaire et Cellulaire in Illkirch, France

4.1 Abstract

Chicken ovalbumin upstream promoter **t**ranscription factor (COUP-TF)-**i**nteracting **p**rotein **2** (CTIP2) is a C_2H_2 zinc-finger transcriptional repressor that functions by direct, sequence-specific DNA binding activity or by recruitment to the promoter template by interaction with COUP proteins. Inactivation of CTIP2 has been implicated in the etiology of lymphoid malignancies, suggesting that CTIP2 may function as a tumor suppressor. Analyses of *CTIP2*-deficient mice have also demonstrated the role for CTIP2 in T cell development. However, the molecular mechanism(s) and transcriptional targets of CTIP2 in T cells have not yet been identified. Here we demonstrate that *Id2,* a gene that encodes **I**nhibitor of **D**NA binding and/or differentiation **2**, is a newly identified target gene of CTIP2 in T cell developmental pathways. CTIP2 was found to bind directly to the putative CTIP RE in the promoter region of *Id2 in vitro* and recruited to the promoter template of *Id2* in mouse thymocytes and human T cell leukemia Jurkat cells. CTIP2 repressed transcription activity of a *tk* promoter containing a multimerized CTIP RE and also of a natural mouse *Id2* promoter harboring a putative CTIP RE in mammalian cells. These results suggest that a direct association of CTIP2 with the promoter of *Id2* leads to a transcriptional repression of *Id2.*

4.2 Introduction

The Id family proteins in mammals is comprised of 4 related members, designated Id1, Id2, Id3 and Id4, and belong to the helix-loop-helix (HLH) family of transcription factors (Norton, 2000). The HLH proteins share a highly conserved HLH motif and an adjacent basic region. The conserved HLH domain primarily mediates homo- and hetero-dimerization. The adjacent region of highly basic residues is essential for DNA binding of HLH dimers at canonical E boxes (CANNTG), N boxes (CACNAG), or Ets sites (GGAA/T) present in the promoter of regulated genes (Massari and Murre, 2000; Zebedee and Hara, 2001). However, the Id proteins lack such a DNA-binding region, and instead function solely by dimerization with other members of the family, principally the basic HLH (bHLH) proteins, and prevent them from binding to DNA (Massari and Murre, 2000). As most bHLH proteins positively regulate numbers of genes during cell differentiation, the term “Id” refers to inhibition of both DNA binding and differentiation. Besides acting as dominant negative regulators of cell differentiation, the Id proteins are important parts of various biological processes including development, cell cycle and tumorigenesis (Lasorella et al., 2001; Yokota, 2001; Zebedee and Hara, 2001; Ruzinova and Benezra, 2003; Sikder et al., 2003; Lasorella et al., 2005; Perk et al., 2005).

During the process of lymphocyte development, hematopoeitc stem cells (HSC) give rise to common lymphoid cell progenitors (CLP), which develop into three distinct cell types: B and T lymphocytes and natural killer (NK) cells (Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005). T cell development occurs in thymus where CLPs enter and then commit to become NK cell- or T cell-lineage. The committed T cell

progenitors begin an ordered pattern of differentiation defined by the rearrangement of TCR γ, δ, and β loci and the acquisition or loss of cell surface markers (Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005). The earliest T cell progenitors are present as the CD4 and CD8 double negative population, and then progress through four DN stages, DN1-DN4, characterized by the expression profile of cell surface markers, CD44 and CD25, and the TCR rearrangement status (Godfrey and Zlotnik, 1993). Between the DN2 and the DN3 stages, the pro-T cells become committed to expression of either the αβ or γδ TCR (Passoni et al., 1997; Haks et al., 2005; Hayes et al., 2005). However, it remains unclear how the αβ/γδ commitment occurs. The γδ T cells are the predominant T-cell population during early fetal development in mice, but start declining relative to αβ T cells just before birth and by adulthood make up only approximately 10% of T cells. Only αβ T cells progress to DN4 stage, before becoming immature single positive (ISP), double positive (DP), and single positive (SP), based on the CD4 and CD8 expression (Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005). Changes in gene expression, notably transcription factors, have been shown to correlate with these developmental steps (Rothenberg and Dionne, 2002; Rothenberg and Taghon, 2005).

In the thymus, Id2 is abundantly expressed (Ikawa et al., 2001; Rivera and Murre, 2001; Quong et al., 2002; Anderson, 2006). Transgenic mice with overexpressed Id2 protein in T cells show an early developmental block at the $CD4^-$ $CD8^-$ stage (Morrow et al., 1999). In contrast, *Id2*-deficient mice lack lymph nodes and display a block in NK cell development, but exhibit normal αβ T cell development (Yokota et al., 1999; Ikawa et al., 2001; Yokota, 2001). These phenotypes of *Id2*-deficient mice are complimentary to those of *mice* lacking E2A, an E-box binding protein (Bain et al., 1997;

Bain et al., 1999). Since the Id proteins act as dominant-negative HLH protein, the ratio of E proteins to Id proteins may ultimately determine cell fate decision in T cell development.

CTIP2/*Bcl11b*/*Rit1* is a tumor suppressor gene that encodes a C_2H_2 zinc finger transcription factor (Avram et al., 2000; Wakabayashi et al., 2003b). The high level of CTIP2 expression in thymocytes is correlated with its significant roles in T cell survival and development. *CTIP2*-deficient mice exhibit the interruption of the $\alpha\beta$ thymocyte development at the DN3 or the ISP stages, a similar phenotype to that of Id2 overexpressed-mice (Morrow et al., 1999; Wakabayashi et al., 2003a). Loss of CTIP2 also results in profound apoptosis in thymus of neonatal mice, but does not affect developmental impairment of the $\gamma\delta$ lineages (Wakabayashi et al., 2003a). Transgenic expression of TCR$\alpha\beta$ in CTIP2-deficient mice rescues apoptosis detected in the DN4 cells but fails to promote transition from the DN3 stage to the DP stage of development, suggesting that CTIP2 may act on many pathways (Inoue et al., 2006). Although the growing evidence illustrates the significance of CTIP2 in these processes, little is known concerning the molecular mechanism(s), and transcriptional targets of CTIP2 in T cell development.

Here we found that CTIP2 was associated with the *Id2* promoter, likely through a direct binding to a putative CTIP2 binding site. We also found that CTIP2 repressed the activity of natural *Id2* promoter. By performing a transcriptome analyses in $\gamma\delta$ and pooled DN thymocytes of *CTIP2*-null mice, the expression of *Id2* was found to be up-regulated. Therefore, we suggest that the association of CTIP2 with the *Id2* promoter subsequently leads to negative regulation of *Id2* in the early process of thymocyte development.

4.3 Material and methods

4.3.1 Synthesis of oligonucleotides

Synthetic oligonucleotides for each strand containing the control CTIP RE (5'-GGCCGG-3'; the upper strand) and mutated CTIP RE (M1-M4) were previously described (Avram et al., 2002). The 3'-biotinylated control CTIP RE and Id2-CTIP RE-containing oligonucleotides were obtained from Invitrogen. The Id2-CTIP RE oligonucleotides were designed to be identical to sequence located at 2.7 kb upstream of transcriptional start site (TSS) of mouse *Id2* gene, covering a conserved putative CTIP binding site (-2.7 kb Id2-CTIP RE), and were designed to have a GATC overhang for subsequent cloning.

4.3.2 Constructs

FLAG-CTIP2 and FLAG-CTIP1 constructs were previously described (Senawong et al., 2003; Senawong et al., 2005). The $(CTIP\ RE)_3$-*tk*-CAT and $(Id2\text{-}CTIP\ RE)_3$-*tk*-CAT reporter constructs were prepared by insertion of annealed-mutimerized control CTIP RE and Id2-CTIP RE oligonucleotides with GATC overhang into a pBLCAT2 vector (*tk*-CAT reporter) at BamH I sites located immediately upstream of a thymidine kinase (*tk*) promoter using standard cloning techniques. All constructs were verified by complete DNA sequencing. The *tk* promoter was removed from pBLCAT2 vector using BamH I and Bgl II or BamH I and Xho I restriction enzymes. The BamH I/ Bgl II fragment of pBLCAT2 was re-ligated to reconstitute a promoterless-CAT reporter construct. The BamH I/ Xho I promoterless-vector was used for subsequent cloning as described below. The -2.9 kb Id2-CAT and the -2.7 kb Id2-CAT reporter constructs were

prepared by amplification of a 5' region of mouse *Id2* gene ranging from -2.9 kb or -2.7 kb to +152 bp, respectively, relative to TSS using appropriate primers and a DNA template from mouse ES cells (Expand long template PCR system, Roche). These amplified fragments were cloned into pCR 2.1 vector using TA cloning kit (Invitrogen). An insert in the pCR 2.1 vector was verified by sequencing before being removed and cloned into BamH I/ Xho I sites of the promoterless-CAT reporter construct.

4.3.3 ABCD assays

In vitro translated and [^{35}S]methionine-labeled proteins were incubated with or without annealed, 3'-biotin-labeled oligonucleotides containing the control CTIP RE or the Id2-CTIP RE in binding buffer (10 mM HEPES, pH7.5, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40) at 4^{o}C for 1 h in a final volume of 100 μl. For competition assays, various non-biotinylated oligonucleotides were additionally included in the reaction. The reaction mixtures were subsequently transferred to a fresh tube containing five microliters of a streptavidin agarose resin pretreated with 1 mg/mL bovine serum albumin in binding buffer for 2 h. The DNA-protein complexes were incubated with the resin for 1 h at 4^{o}C with rotation. At the end of the incubation, the resin was washed 5 times with binding buffer and the bound protein associated with the resin was measured for radioactivity using a liquid scintillation counter.

4.3.4 Transfection and reporter assays

HEK293T cells were transfected using the calcium phosphate method and harvested 48 h after transfection. A β-galactosidase expression vector (pCMV-Sport-βGal, Life Technologies) was co-transfected as an internal control, and β-galactosidase

activity and total protein concentration were used for normalization across all samples. The relative chloramphenicol acetyltransferase was determined as described previously (Dowell et al., 1997).

4.3.5 Chromatin Immunoprecipitation (ChIP)

ChIP assays were conducted essentially as previously described (Topark-Ngarm and Golonzhka et al., 2006) using extracts from Jurkat cells and mouse thymocytes that were immunoprecipitated with anti-CTIP2 or non-specific IgG. The enrichment of a DNA template was analyzed by conventional PCR using primers specific to region surrounding the CTIP2 binding site of mouse *Id2* promoter, forward: 5'-TGAGTGACGGCGCGGTT-3'; reverse: 5'-TGTGTCCATTCCGCCCGT-3'. Amplification of *HO-1* was performed using a forward primer 5'-GCCAGACTTTGTTTCCCAAG-3', and a reverse primer 5'-GAGGAGGCAGGCGTTGAC-3'.

4.3.6 Alignment of mouse and human *Id2* promoter regions

The alignment was performed using Consite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/).

4.3.7 Affymetrix microarray analysis

Cells were sorted for the microarray analysis and thymocytes from one WT and two mutant, newborn mice (pooled) were labeled with anti-CD8-FITC, anti-CD4-PE, and anti-γδTCR-Cy5. $CD4^-CD8^-\gamma\delta+$ (>10^5 cells) and $CD4^-CD8^-\gamma\delta-$ (~ 25,000) cells were sorted with a DiVa high speed cell sorter (BD). Sort purity was greater than 98%. RNA was extracted from WT or CTIP2 sorted cells using the micro-RNeasy kit (Qiagen).

Yields were approximately 10 ng for the γδ+ populations and less then 2 ng for the γδ- cells. cRNA probe synthesis was performed using two consecutive T7 polymerase-mediated amplification steps, according to standard Affymetrix protocols. Size distribution of the amplified products was similar for all samples and within the normal range, but the yield was suboptimal for the γδ- samples. Probes were hybridized on Affymetrix U74Av2 chips. Data were analyzed with the MAS5 software. Note that the arrays hybridized with probes derived from the γδ- samples generated weak signals which prevented detection of a large fraction of genes (~15% of "present" calls compared with ~ 40% for the γδ+ samples).

4.4 Results

4.4.1 *Id2* is one of CTIP2 target genes in the thymocytes of *CTIP2*-null mice

The data from our previous transcriptome analyses in SK-N-MC neuroblastoma cells, which highly express CTIP2, have demonstrated a number of CTIP2 target genes (Topark-Ngarm and Golonzhka et al., 2006). In addition to expression in SK-N-MC cells, CTIP2 has been shown to be abundantly expressed in mouse thymocytes (Wakabayashi et al., 2003a). Studies in *CTIP2*-null mice revealed importance roles of CTIP2 in T cell development (Wakabayashi et al., 2003a; Inoue et al., 2006). However, the precise function(s) and target genes of CTIP2 in thymocytes have not yet been clarified. Accordingly, we performed transcriptome analyses on the thymocytes of wild-type (WT) and *Bcl11b*/*CTIP2*-null mice ($Bcl11b^{L-/L-}$). The affymetrix microarray analyses revealed

that a number of genes were up-regulated and approximately 15 genes were down-regulated in *CTIP2*-null mice relative to WT mice (Fig. 4.1*A*).

One of these up-regulated genes was an **i**nhibitor of **D**NA binding and/or differentiation **2** (*Id2*; Fig. 4.1*B*). *Id2* encodes a helix-loop-helix (HLH) protein that dimerizes with and inhibits DNA binding activity of bHLH transcription factors that positively regulate differentiation of various cell types (Norton, 2000; Quong et al., 2002; O'Toole et al., 2003; Murre, 2005; Rothschild et al., 2006). The data from microarray analyses showed that the expression of *Id2* was increased almost 3-fold in the $\gamma\delta^-$ cells and 4-fold in the $\gamma\delta^+$ cells of the *CTIP2*-null mice when compared to WT counterparts (Fig. 4.1*C*). These data suggest that *CTIP2* negatively regulates *Id2* in mouse thymocytes.

The derepressed expression of *Id2* in *CTIP2*-null mice described herein may explain a similar phenotype between overexpression of Id2 in thymus and *CTIP2*-deficient mice (Morrow et al., 1999; Wakabayashi et al., 2003a; Inoue et al., 2006). Therefore, we chose to study Id2 in details for the purposes of this manuscript.

4.4.2 *Id2* is a direct target of CTIP2 in human Jurkat cells and in mouse thymocytes

The above data have demonstrated that *Id2* is a target gene of CTIP2. However, the microarray analyses cannot distinguish whether *Id2* is directly or indirectly regulated by CTIP2. If *Id2* is a direct target of CTIP2, CTIP2 should be bound to the promoter region of *Id2.* Within a range of 10 kilobases (kb) of mouse and human promoter, three and eight putative CTIP2 binding sites (5'-GGCCGG-3'; on lower strand) are present, respectively (Avram et al., 2002). However, there is only one CTIP2 binding site that is 100% conserved between the two species (Fig. 4.2*A*).

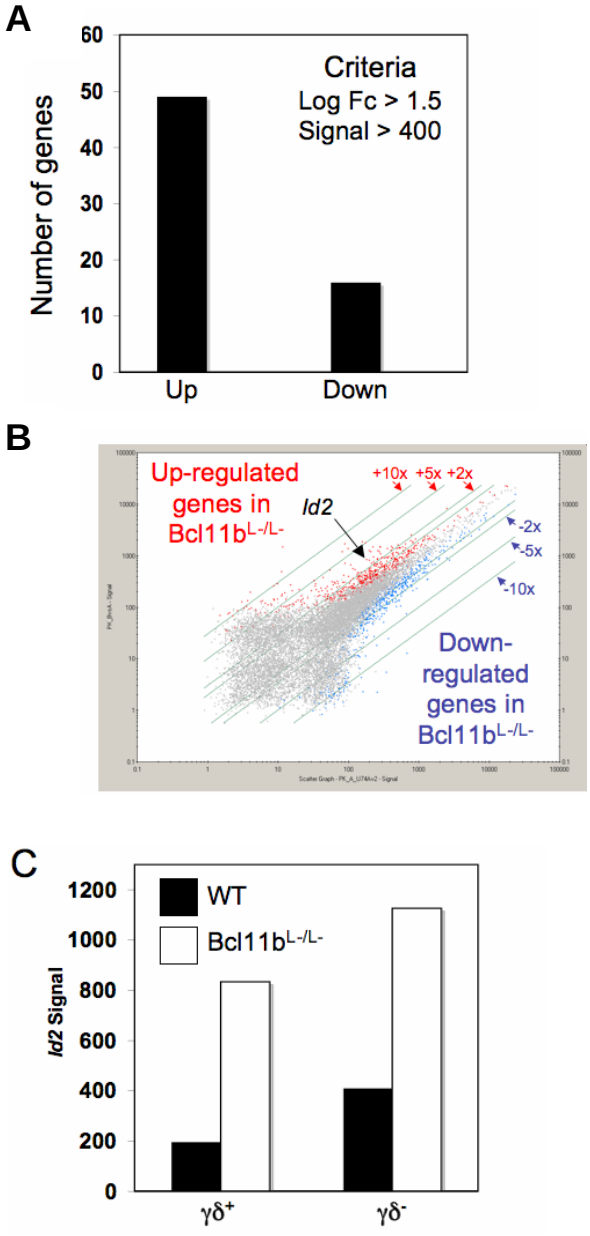


Figure 4.1. **Transcriptome analysis of CTIP2 (Bcl11b) mutant thymocytes. *A*,** Numbers of genes that are up- and down-regulated in Bcl11b$^{L-/L-}$ mice. The cutoff criteria used are indicated. ***B*,** Scatter plot representing RNA levels in wt (abscissa) and Bcl11b$^{L-/L-}$ mice (ordinate). Red and blue points indicate genes that are up- and down-regulated in the absence of Bcl11b and that corresponding to Id2 is indicated. ***C,*** Up-regulation of Id2 mRNA in CD4^{-}CD8^{-} γδ^{+} and CD4^{-}CD8^{-} γδ^{-} T cells from Bcl11b mutants.

This CTIP2 binding site is located at approximately 2.7 kb upstream of transcriptional start site (TSS) of the mouse promoter and hereafter is referred to as a -2.7 kb Id2-CTIP2 RE (Fig. 4.2*B*).

To determine if CTIP2 binds to this promoter region, chromatin Immunoprecipitation (ChIP) experiments were performed in a human T-cell leukemia Jurkat cell line and mouse T cells, both of which were shown to express high levels of two splice variants of CTIP2 (Senawong et al., 2003). We found that CTIP2 was associated with the promoter region of *Id2* containing the -2.7 kb Id2-CTIP2 RE in mouse thymocytes, but not with that of the control *heme oxygenase-1* (*HO-1)* gene (Fig. 4.2*C*, *middle* and *bottom panels*). The similar results were obtained from human Jurkat cells (Fig. 4.2*C*, *top panel*). These findings suggest that *Id2* is a direct target of CTIP2 in mouse T cells and human Jurkat cells, in which CTIP2 is associated with this promoter region of *Id2*.

4.4.3 CTIP2 directly binds to a putative CTIP RE-containing sequence derived from the mouse *Id2* promoter

Next, ABCD assays were performed to determine if CTIP2 directly binds to the -2.7 kb Id2-CTIP2 RE using *in vitro* translated CTIP2 and the synthesized oligonucleotides having the sequence identical to that of the mouse *Id2* promoter covering the -2.7 kb Id2-CTIP RE (Id2-CTIP RE; Fig. 4.3*A*). The oligonucleotides, containing the originally identified CTIP RE and previously shown to be bound by CTIP2 (Avram et al., 2002), were used as the positive control (Biotin-CTIP RE; Fig. 4.3*A*).

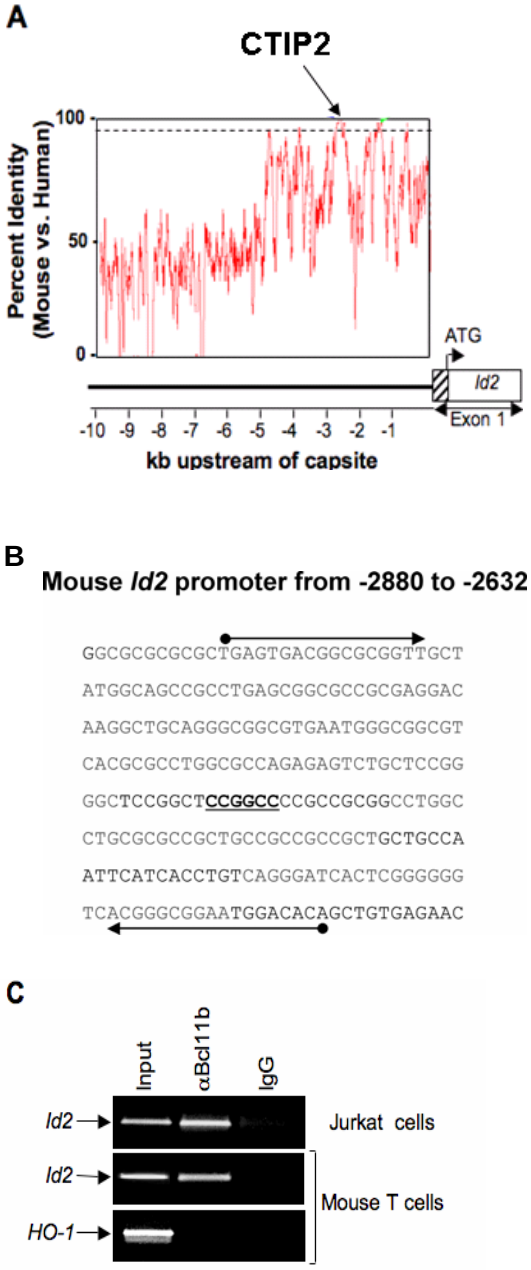


Figure 4.2. **Interaction of CTIP2 with the *Id2* promoter region.** ***A,*** Alignment of human and mouse *Id2* promoter regions from the TSS sites to -10 kb. The area of high sequence identity is noted that harbors a CTIP2 binding site. ***B*,** Region surrounding CTIP2 binding site in mouse *Id2* promoter (coordinates indicated; only the upper strand is shown for clarity). The CTIP2 binding site (5' -GGCCGG- 3' on lower strand) is indicated in bold and underlined. PCR primers to be used in ChIP assays are indicated by arrows. ***C***, ChIP analyses using extracts from Jurkat cells and mouse thymocytes that were immunoprecipitated with anti-Bcl11b (CTIP2) or non-specific IgG as indicated.

We found that CTIP2 binding to the Biotin-Id2 CTIP RE was relatively weaker than the binding to the control Biotin-CTIP RE (*lanes 6* and *1* of Fig. 4.3*B*, respectively). These results were confirmed by competition assays performed using non-biotinylated oligonucleotides as competitors (Fig. 4.3*B*, *lanes 2-5* and *7-10*). As transcription factors trend to respond instantaneously to various changes in cells, the low-affinity binding of CTIP2 to its binding site within the promoter of *Id2* is not surprising.

In chapter 3, we have established that CTIP2 binds to the control Biotin-CTIP RE, in which the first four positions of the core CTIP RE are important for CTIP2 recognition (also see below in Fig. 4.4*C*). To examine if CTIP2 bound to the Biotin-Id2 CTIP RE in the similar manner, the ABCD competition assays were performed using non-biotinylated oligonucleotides M1-M4 as competitors (Fig. 4.4*A*), as previously described in Chapter 3. We found that competitor WT and M3 were capable of competing with the Biotin-Id2 CTIP RE for CTIP2 binding (Fig. 4.4*B*, compare *lanes 2-3* and *8-9* to *lane 1*), but competitors M1, M2, and M4 were not. Similar results were obtained from separate ABCD assays using the control Biotin-CTIP RE (Fig. 4.4*C*).

Together, we conclude that CTIP2 directly binds to the putative CTIP RE, which is located at 2.7 kb upstream of TSS, and that the four positions within the core motif of Id2-CTIP RE are likely to be crucial determinants for CTIP2 recognition *in vitro.* We note that the competitor oligonucleotides used herein are defined in the context of the previously identified CTIP RE. Therefore, we cannot exclude the possibility that CTIP2 may also recognize other positions of the putative binding site within the *Id2* promoter. Given the data from transcriptome analyses of the thymocytes of *CTIP2*-null mice and ChIP assays, it is likely that transcriptional repression of *Id2* mediated by CTIP2 may be a consequence from a direct association of CTIP2 to this promoter region of *Id2.*

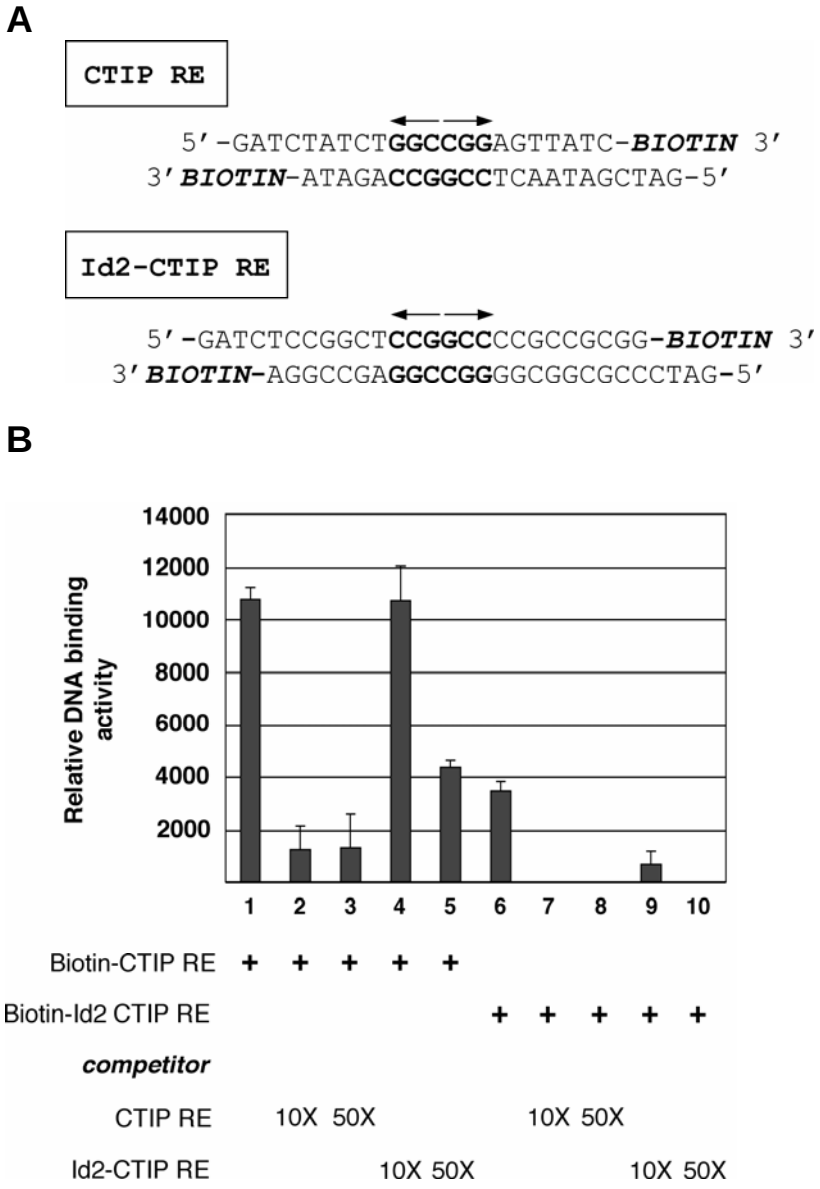
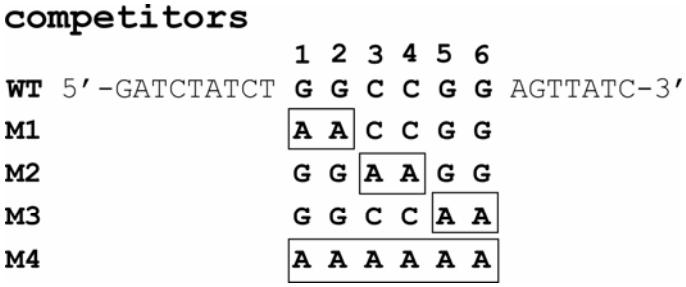
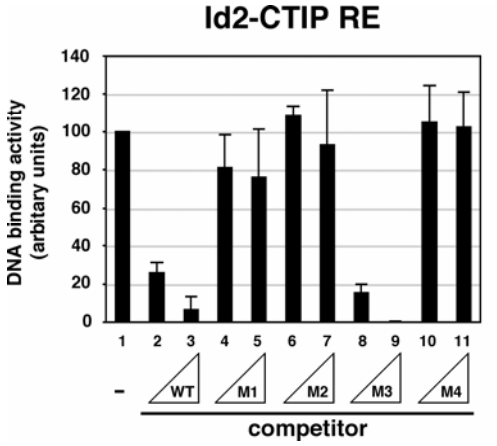


Figure 4.3. ***In vitro*, direct binding of CTIP2 to the oligonucleotides containing the putative CTIP RE derived from the mouse *Id2* promoter. *A,*** Biotinylated CTIP RE and Id2-CTIP RE oligonucleotides used in ABCD assays in *B* are shown. A core consensus CTIP binding site (CTIP RE) is denoted in bold in each strand. Each arrow indicates a half site of the CTIP RE. The biotin labeling at 3'-end is indicated in italicized bold. ***B,*** *In vitro* translated and [^{35}S]met-labeled, full-length CTIP2 were incubated with indicated biotinylated oligonucleotides, in the absence or presence of competitor oligonucleotides, before addition of streptavidin agarose resin. After extensive washing, [^{35}S]met-labeled CTIP2 associated with the resin was measured for radioactivity and determined as relative DNA binding activity. The data shown here were derived from at least three independent experiments and are presented as the means ± S.E.M.

A



B



C

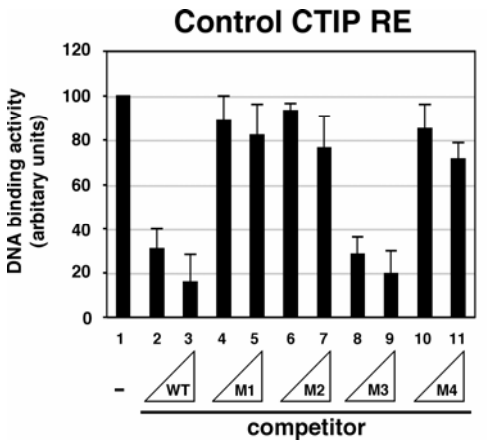


Figure 4.4. **Specificity of CTIP2 binding to the Id2-CTIP RE.** ***A,*** Sequences of competitor oligonucleotides; wild-type (WT) CTIP RE and mutants M1-M4. The nucleotides that are mutated in each oligonucleotide are boxed. A core CTIP RE is denoted in bold with position numbers. For simplicity, only upper strand is shown, and sequences outside the core CTIP binding site which are identical in all oligonucleotides are not shown. ***B****,* ABCD competition assays were carried out to measure the binding of [^{35}S]met-labeled CTIP2 to the biotinylated Id2-CTIP RE, in the absence or presence of nonbiotinylated competitor CTIP REs, as indicated. *Lane 1* corresponds to reaction in the absence of a competitor. *Lanes 2-11* contain nonbiotinylated competitors with increasing amounts (10- or 50-fold excess over the concentration of the biotinylated Id2-CTIP RE), as denoted by a triangle. CTIP RE binding activities were determined relative to lane 1 and are presented as the means ± S.E.M derived from at least three independent experiments. ***C,*** The competition assays were performed and quantified as described in *B,* except that the control biotinylated CTIP RE were used.

4.4.4 CTIP2 represses transcription from the *tk* promoter harboring the CTIP RE derived from the *Id2* promoter in HEK293T cells

Next, the -2.7 kb CTIP2 binding site in the mouse *Id2* promoter was examined to determine if it confers CTIP2 responsiveness in cells. We carried out reporter assays on HEK293T cells that had been transiently transfected with an expression vector encoding Flag-CTIP2 and a *tk*-CAT reporter containing either a multimerized putative Id2-CTIP RE or a multimerized control-CTIP RE ((Id2-CTIP RE)$_3$-*tk*-CAT or (CTIP RE)$_3$-*tk*-CAT, respectively; Fig. 4.5*A*). We found that CTIP2 repressed transcription from (Id2-CTIP RE)$_3$-*tk*-CAT reporter in a concentration-dependent manner relative to an empty tk-CAT reporter (Fig. 4.5*B*, *lanes 6-8*). This repressive effect of CTIP2 on (Id2-CTIP RE)$_3$-*tk*-CAT reporter was not as strong as that on (CTIP RE)$_3$-*tk*-CAT reporter (Fig. 4.5*B*, *lanes 2-4*). Considering that CTIP2 bound to the Id2-CTIP RE with relatively lower affinity (see Fig. 4.3*B* above), this may explain the weaker repressive effect of CTIP2 on the promoter containing the Id2-CTIP RE. However, these results indicate that CTIP2 represses transcription from the *tk* promoter containing the putative CTIP RE derived from the *Id2* promoter.

4.4.5 CTIP2 represses the activity of mouse *Id2* promoter containing the CTIP RE

To investigate if the CTIP RE was responsible for the repressive effect of CTIP2 on natural mouse *Id2* promoter, two different portions of the mouse *Id2* promoter were cloned into the promoterless-CAT reporter. One fragment was ranging from -2.9 kb to +152 bp and the other was from -2.7 kb to +152 bp relative to TSS (named -2.9 kb Id2 and -2.7 kb Id2, respectively; Fig. 4.6*A* and *B*). The -2.9 kb Id2 fragment contained the

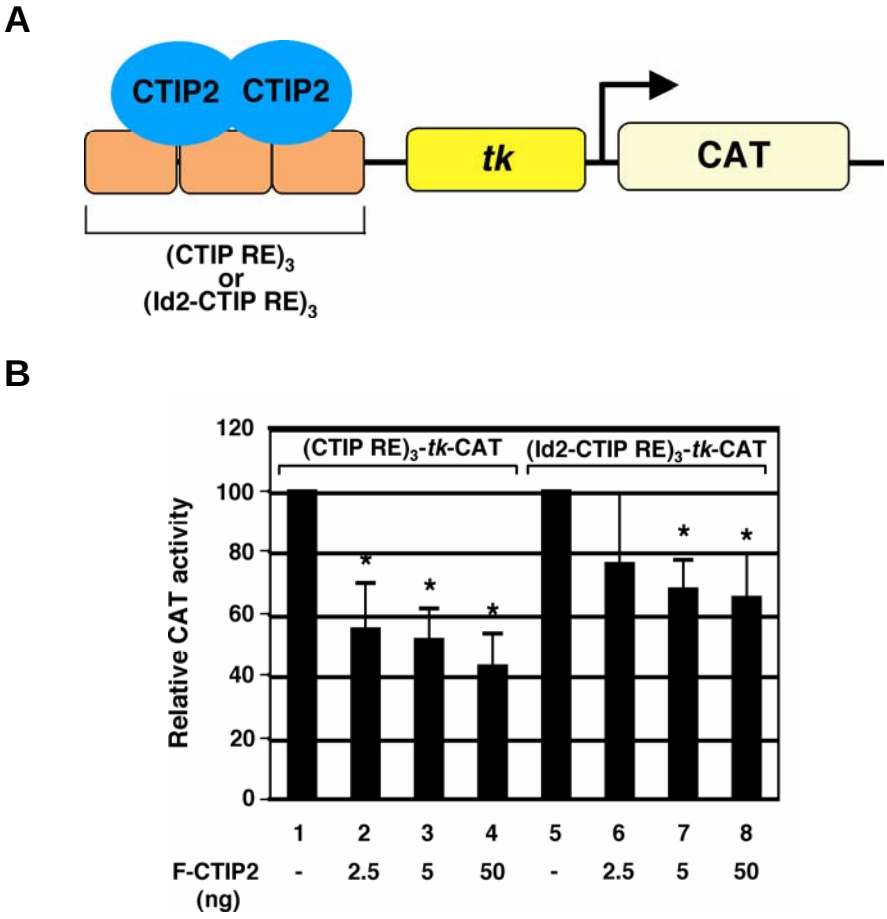


Figure 4.5. **CTIP2 represses transcription from the CTIP RE derived from the *Id2* promoter in HEK293T cells. *A,*** Schematic diagram of (CTIP RE)$_3$-*tk*-CAT or (Id2-CTIP RE) $_3$-*tk*-CAT reporter, both of which harbor three copies of the control CTIP RE or the Id2-CTIP RE immediately upstream of a *tk* promoter. ***B,*** HEK293T cells were co-transfected in 6-well plate with 1 μg of either (CTIP RE)$_3$-*tk*-CAT, or (Id2-CTIP RE) $_3$-*tk*-CAT reporter with indicated amounts of expression vector encoding Flag-CTIP2 (F-CTIP2). Transfection efficiency was normalized by use of a co-transfected β-galactosidase expression vector and protein concentration. The absolute CAT activities in all samples were calculated relative to the sample contained the control empty *tk*-CAT reporter with the correspond amount of F-CTIP2. The determined CAT activity in the absence of F-CTIP2 plasmid (*lanes 1 or 5*) was taken to be 100 and that against which all other determined CAT activities (*lanes 2-4* or *lanes 6-8*, respectively) were compared; asterisks indicate statistical significance. The results shown here represent the means ± S.E.M. of three independent experimental determinations.

conserved, putative CTIP2 binding site (Id2 CTIP RE), whereas the -2.7 kb Id2 fragment did not. These two reporters are hereafter referred to as the -2.9 kb Id2-CAT and the -2.7 kb Id2-CAT, respectively (Fig. 4.6*C*). The effect of CTIP2 on transcription from these two reporters was assessed in transiently transfected HEK293T cells (Fig. 4.7*A*). The CAT activity from the promoterless-CAT reporter was barely detectable, confirming that the basal transcriptional activity associated with the *tk* promoter was eliminated (data not shown). An insertion of the -2.9 kb Id2 fragment into a promoterless-CAT reporter stimulated the basal transcriptional activity of the reporter gene 24-fold (Fig. 4.7*B*, compare *lane 6* to *lane 1*). However, an insertion of the -2.7 kb Id2 fragment only increased the basal transcriptional activity approximately 3-fold (Fig. 4.7*B*, compare *lane 2* to *lane 1*). The enormous stimulation of the basal transcription observed from the -2.9 kb Id2-CAT reporter suggests that the additional 200 bp-region at the 5' end may contain positive regulatory binding sites for transcription factors that can enhance the promoter activity. From an analysis of this 200 bp-region, we indeed found three E-box sites (Fig. 4.6*A*). The E-box elements are largely represented across the whole genome in eukaryotes, in which they have been identified in promoter and enhancer elements (Massari and Murre, 2000). A number of transcription factors bind to the E-box sites and are capable of enhancing tissue-specific expression of genes involved in proliferation, differentiation, tissue-specific responses, cell death, and the circadian clock (Littlewood and Evan, 1990; Mutoh et al., 2000; Munoz and Baler, 2003; Appelbaum and Gothilf, 2006; Parker et al., 2006). Therefore, it is conceivable that these 3 E-box motifs present in the -2.9 kb-Id2-CAT may be bound by transcriptional factors that are endogenously expressed in HEK293T cells and responsible for an activation of the transcription.

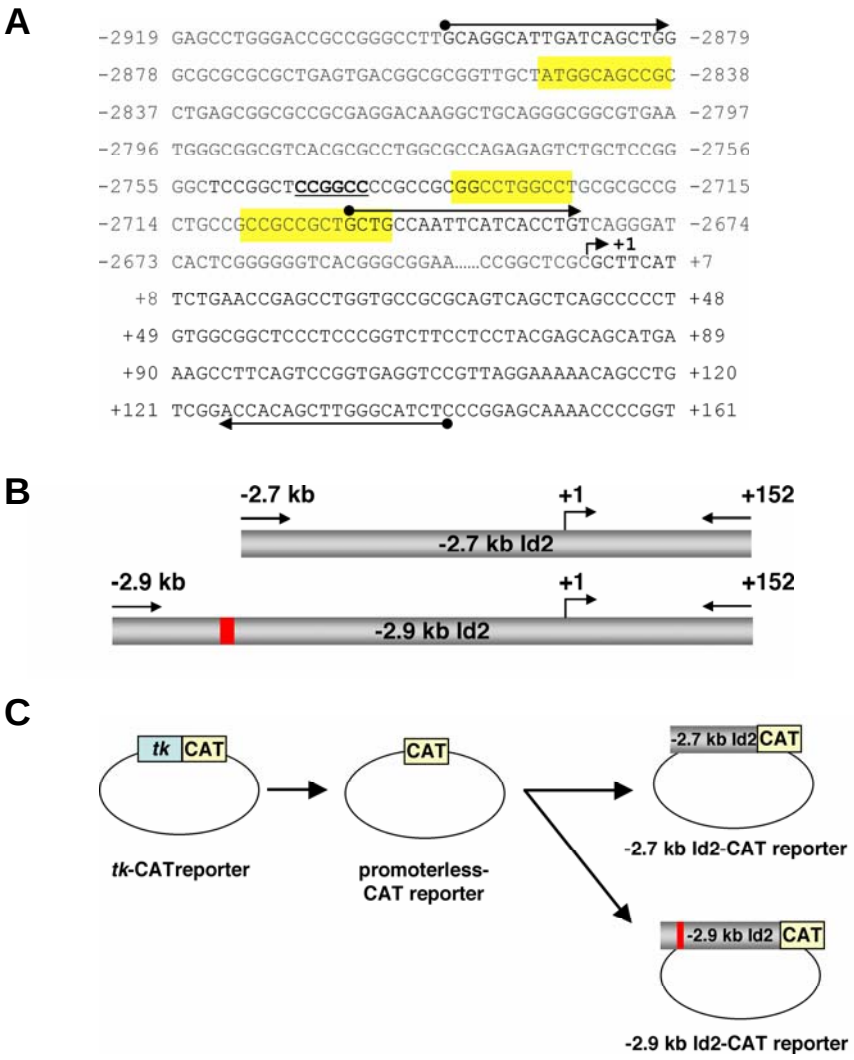


Figure 4.6. **Cloning of the mouse *Id2* promoter into the promoterless-CAT reporter.** ***A,*** Region of the mouse *Id2* promoter surrounding a putative CTIP2 binding site, located approximately at 2.7 kb upstream of transcriptional start site (TSS) of mouse *Id2* (only the upper strand is shown for clarity). The CTIP2 binding site (5'-GGCCGG-3' on lower strand) is indicated in bold and underlined. Arrows represent positions of forward and reverse primers used in PCR amplification of the -2.7 kb Id2 or the -2.9 kb Id2 fragments. The TSS site is denoted as +1 and E boxes are indicated in yellow. Note that the sequence is discontinuous for simplicity. ***B,*** Schematic representations of PCR products of the -2.7 kb Id2 and the -2.9 kb Id2. The putative CTIP RE within the -2.9 kb Id2 fragment is represented by a red bar. ***C,*** A diagram illustrating a stepwise cloning of the -2.7 kb Id2-CAT and the -2.9 kb Id2-CAT reporters. The *tk* promoter was removed from *tk*-CAT reporter to generate a promoterless-CAT reporter. Subsequently, either the -2.7 kb Id2 or the -2.9 kb Id2 fragment was cloned into the promoterless-CAT reporter, generating the -2.7 kb Id2-CAT or the -2.9 kb Id2-CAT, respectively.

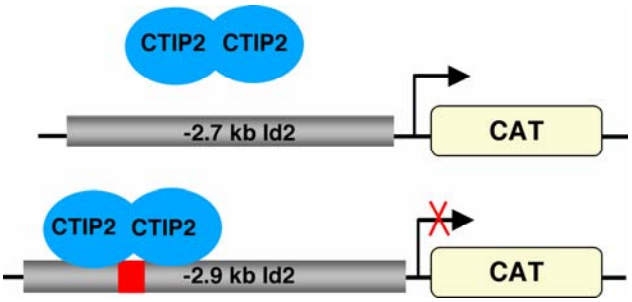
We found that CTIP2 repressed transcription from the -2.9 kb Id2-CAT reporter in concentration-dependent manner (*lanes 7-9* of Fig. 4.7*B*). This concentration-dependent repression was also obtained from the -2.7 kb Id2-CAT (*lanes 3-5* of Fig. 4.7*B*). The repressive effect of CTIP2 on the -2.7 kb Id2-CAT was not totally unexpected as several potential CTIP2 binding sites, although not 100% conserved between mouse and human, are found in the promoter region of mouse *Id2* between -2 kb and TSS (see Fig. 4.2*A*). Therefore, CTIP2 may also bind directly to these binding sites. Moreover, it is possible that the -2.7 kb Id2 region also contains other functional regulatory binding sites which are bound by CTIP2 indirectly. However, the magnitude of repression mediated by CTIP2 observed from the -2.9 kb Id2-CAT was markedly more than that observed from the -2.7 kb Id2-CAT, suggesting that the conserved CTIP RE was responsible, at least in part, for repressive effect of CTIP2 on the mouse *Id2* promoter in cells.

As mentioned above that CTIP2 as well as CTIP1 was capable of binding to the CTIP RE, we therefore determined if CTIP1 produced the similar repressive effect on the -2.9 kb Id2-CAT reporter. Unlike CTIP2, we found that CTIP1 insignificantly suppressed the transcription from the -2.9 kb Id2-CAT reporter (Fig. 4.7*C,* compare *lanes 5-6* to *lane 1*), indicating that the mouse *Id2* promoter harbors a specific responsiveness to CTIP2.

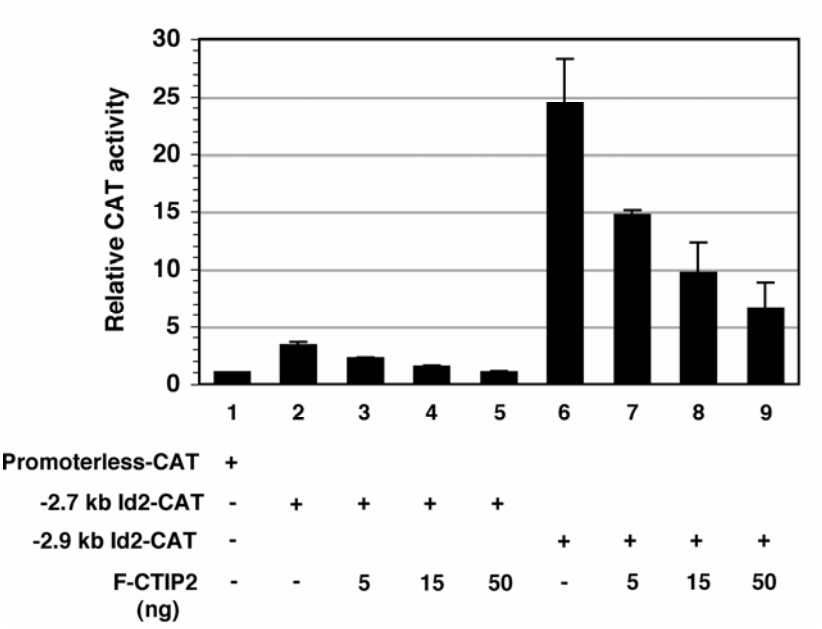
Altogether, we conclude that transcriptional repression of *Id2,* a newly identified CTIP2 target gene in $\gamma\delta$ and DN thymocytes of CTIP2-null mice*,* is the functional outcome of a direct interaction of CTIP2 with the *Id2* promoter template.

Figure 4.7

A


CTIP2 CTIP2
-2.7 kb Id2
CAT
CTIP2 CTIP2
-2.9 kb Id2
CAT

B


Relative CAT activity
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9
Promoterless-CAT + - - - - - - - - -
-2.7 kb Id2-CAT - + + + + - - - -
-2.9 kb Id2-CAT - - - - - + + + +
F-CTIP2 (ng) - - 5 15 50 - 5 15 50

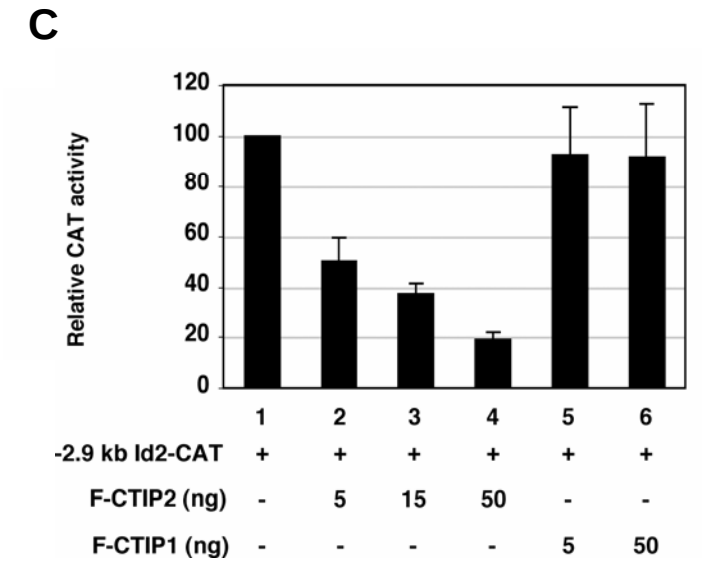


Figure 4.7. **Repressive effect of CTIP2 on the mouse *Id2* promoter. *A,*** Schematic diagram of reporter assays on HEK293T cells co-transfected with either *(top)* the -2.7 kb Id2-CAT or *(bottom)* the -2.9 kb Id2-CAT reporter and an expression vector encoding F-CTIP2. Reporter assays in *B and C*: HEK293T cells were co-transfected in 6-well plate with 1 μg of either the -2.7 kb Id2-CAT or the -2.9 kb Id2-CAT reporter and indicated amounts of expression vector encoding F-CTIP2 or F-CTIP1. The amounts of DNA in all lanes were equalized by an empty vector. Transfection efficiency was normalized by use of a co-transfected β-galactosidase expression vector and protein concentration. The results shown here represent the means ± S.E.M. of three independent experimental determinations. ***B,*** The CAT activities determined in the each lane was relative to *lane 1*, which was set to be 1. ***C,*** The CAT activities determined as described in *B.*

4.5 Discussion

The development of T lymphocytes is a complex and ordered process, which is dependent on the temporal activation of lineage-specific genes, productive antigen receptor rearrangements, as well as the coordination of survival and proliferation with developmental progression (Quong et al., 2002; Rothenberg and Taghon, 2005). CTIP2 has been previously shown to be required for differentiation and survival of αβ thymocytes. *CTIP2*-deficient mice die at P0, the day of birth, and display developmental arrest of αβ cells at an early stage. However, γδ thymocyte production is not perturbed (Wakabayashi et al., 2003a). An arrest at DN3 stage in the CTIP2 mutant mice may be ascribed to lack of expression of pre-T cell receptor (TCR) complex on the cell surface (Wakabayashi et al., 2003a). However, transgenic expression of TCRαβ in *CTIP2*-deficient mice partially rescues apoptosis, but fails to promote transition from the DN3 to the DP stage (Inoue et al., 2006). Compound mice lacking CTIP2 and tumor suppressor p53 exhibit a partial rescue of T cell development; thymocytes from these mice progress from the DN3 to the ISP stage, but not to the DP stage (Okazuka et al., 2005). This suggest that CTIP2 affects not only pre-TCR signaling but also many other signaling pathways required for DN thymocytes to successfully proceed toward DP cells.

In this report, transcriptome analyses of γδ and pooled DN cells thymocytes of new born, *CTIP2*-null mice revealed a number of CTIP2 target genes (see Fig. 4.1*A*). More genes are up-regulated in *CTIP2*-deficient mice, consistent with the previously described role of CTIP2 as a transcriptional repressor (Avram et al., 2002; Senawong et al., 2003; Topark-Ngarm and Golonzhka et al., 2006).The inhibitor of DNA binding and/or differentiation 2, *Id2* is newly identified herein as one of the CTIP2 target genes in both DN and γδ thymocytes (see Fig. 4.1*B* and *C*).

The promoter regions of the mouse and human *Id2* genes contain several putative CTIP2 binding sites. We found that CTIP2 bound directly to a binding site that is conserved between mouse and human promoters (see Fig. 4.2 to 4.4). We speculate that mouse and human share a common pathway of Id2 regulation by CTIP2. Consistent with the data from mouse transcriptome analyses in $\gamma\delta$ and pooled DN thymocytes from *CTIP2*-deficient mice, CTIP2 suppressed the activity of *Id2* promoter fused to a reporter gene in HEK293T cells (see Fig. 4.5 and 4.7). However, the molecular mechanism(s) underlying CTIP2-mediated transcriptional repression of *Id2* remains to be investigated.

Gene expression that occurs during T cell development is controlled, at least in part, by a class of transcription factors known as the basic helix-loop-helix E-proteins, whose activities are opposed by Id proteins, members from another class of HLH family (Quong et al., 2002; Rothenberg and Taghon, 2005). The function of E proteins is discontinuous in thymus. E2A in particular is most essential prior to the DP stage (Engel et al., 2001; Pan et al., 2002). Mice lacking E2A exhibit a partial block at the DN1 to DN2 transition (Bain et al., 1997). Inhibition of E2A, through expression of a dominant negative form of HEB or an Id1 transgene, also decreases Vβ to DJβ rearrangement, a critical process for pre-T cell receptor (TCR) expression at the DN3 stage in $\alpha\beta$ T cell development (Barndt et al., 2000; Kim et al., 2002). In contrast, deficiency of Id2 in mice does not affect development of $\alpha\beta$ T cells (Yokota et al., 1999; Ikawa et al., 2001), while forced expression of Id2 blocks this developmental process (Morrow et al., 1999).

Id2 is of great interest for us as overexpression of Id2 in thymus results in a phenotype quite similar to that of CTIP2-null mice (arrest in $\alpha\beta$ T cell development) (Morrow et al., 1999). Id2 interacts with and inhibits the DNA binding activity of the E2A proteins, E12 and E47 (Benezra et al., 1990; Voronova and Lee, 1994), proteins that

have been demonstrated to play a crucial roles in T cell development (Bain et al., 1997; Bain et al., 1999a; Bain et al., 1999b; Engel et al., 2001). In addition, both ectopic overexpression of Id2 in T cells (Morrow et al., 1999) and E2A knockout (Bain et al., 1997) results in aggressive T cell lymphomas, which is also of interest for us as CTIP2 has been proposed to function as a tumor suppressor gene (MacLeod et al., 2003; Nagel et al., 2003; Wakabayashi et al., 2003b; Bezrookove et al., 2004; MacLeod et al., 2004; Su et al., 2004; Okazuka et al., 2005; Przybylski et al., 2005).

We hypothesize that CTIP2, by suppressing expression of Id2 and allowing the E2A proteins to function properly in T cell developmental progression, is necessary for successful transition through the pre-TCR checkpoint. Therefore, it follows that lack of CTIP2 may result in inappropriate expression of Id2, followed by Id2-mediated inhibition of E2A protein function. Although CTIP2 may facilitate E2A function in the developmental pathway leading to $\alpha\beta$ T cells, CTIP2 is clearly not required for the proper development of $\gamma\delta$ T cells (Wakabayashi et al., 2003a).

This report provides important evidence that one of the key regulators in T cell development, Id2, is negatively regulated by CTIP2. It will be of great interest to study if loss of Id2 expression in *CTIP2*-null mice may rescue the arrest in $\alpha\beta$ T cell development. Also, detailed studies on *CTIP2*-null mice are needed to clarify the role (s) of this protein *in vivo.*

4.6 Abbreviations

The abbreviations used are Id, Inhibitor or DNA binding and/or differentiation 2; HLH, helix-loop-helix; CTIP2, COUP-TF-interacting proteins 2; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HEK293T, human embryonic kidney 293T cells; DBD, DNA binding domain; LUC, luciferase; bp, base pair; kb, kilobase pair; ChIP, chromatin immunoprecipitation; *HO-1*, Heme oxygenase-1; WT, wild type

4.7 Acknowledgments

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4.8 References

Anderson, M. K. (2006). "At the crossroads: diverse roles of early thymocyte transcriptional regulators." Immunol Rev **209**: 191-211.

Appelbaum, L. and Gothilf, Y. (2006). "Mechanism of pineal-specific gene expression: the role of E-box and photoreceptor conserved elements." Mol Cell Endocrinol **252**(1-2): 27-33.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Bain, G., Engel, I., et al. (1997). "E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas." Mol Cell Biol **17**(8): 4782-91.

Bain, G., Quong, M. W., et al. (1999a). "Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47." J Exp Med **190**(11): 1605-16.

Bain, G., Romanow, W. J., et al. (1999b). "Positive and negative regulation of V(D)J recombination by the E2A proteins." J Exp Med **189**(2): 289-300.

Barndt, R. J., Dai, M., et al. (2000). "Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB." Mol Cell Biol **20**(18): 6677-85.

Benezra, R., Davis, R. L., et al. (1990). "The protein Id: a negative regulator of helix-loop-helix DNA binding proteins." Cell **61**(1): 49-59.

Bezrookove, V., van Zelderen-Bhola, S. L., et al. (2004). "A novel t(6;14)(q25-q27;q32) in acute myelocytic leukemia involves the BCL11B gene." Cancer Genet Cytogenet **149**(1): 72-6.

Dowell, P., Peterson, V. J., et al. (1997). "Ligand-induced peroxisome proliferator-activated receptor alpha conformational change." J Biol Chem **272**(3): 2013-20.

Engel, I., Johns, C., et al. (2001). "Early thymocyte development is regulated by modulation of E2A protein activity." J Exp Med **194**(6): 733-45.

Godfrey, D. I. and Zlotnik, A. (1993). "Control points in early T-cell development." Immunol Today **14**(11): 547-53.

Haks, M. C., Lefebvre, J. M., et al. (2005). "Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage." Immunity **22**(5): 595-606.

Hayes, S. M., Li, L., et al. (2005). "TCR signal strength influences alphabeta/gammadelta lineage fate." Immunity **22**(5): 583-93.

Ikawa, T., Fujimoto, S., et al. (2001). "Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2." Proc Natl Acad Sci U S A **98**(9): 5164-9.

Inoue, J., Kanefuji, T., et al. (2006). "Expression of TCR alpha beta partly rescues developmental arrest and apoptosis of alpha beta T cells in Bcl11b-/- mice." J Immunol **176**(10): 5871-9.

Kim, D., Xu, M., et al. (2002). "Helix-loop-helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF-kappaB activities." Immunity **16**(1): 9-21.

Lasorella, A., Rothschild, G., et al. (2005). "Id2 mediates tumor initiation, proliferation, and angiogenesis in Rb mutant mice." Mol Cell Biol **25**(9): 3563-74.

Lasorella, A., Uo, T., et al. (2001). "Id proteins at the cross-road of development and cancer." Oncogene **20**(58): 8326-33.

Littlewood, T. D. and Evan, G. I. (1990). "The role of myc oncogenes in cell growth and differentiation." Adv Dent Res **4**: 69-79.

MacLeod, R. A., Nagel, S., et al. (2004). "BCL11B rearrangements probably target T-cell neoplasia rather than acute myelocytic leukemia." Cancer Genet Cytogenet **153**(1): 88-9.

MacLeod, R. A., Nagel, S., et al. (2003). "Activation of HOX11L2 by juxtaposition with 3'-BCL11B in an acute lymphoblastic leukemia cell line (HPB-ALL) with t(5;14)(q35;q32.2)." Genes Chromosomes Cancer **37**(1): 84-91.

Massari, M. E. and Murre, C. (2000). "Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms." Mol Cell Biol **20**(2): 429-40.

Morrow, M. A., Mayer, E. W., et al. (1999). "Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages." Mol Immunol **36**(8): 491-503.

Munoz, E. and Baler, R. (2003). "The circadian E-box: when perfect is not good enough." Chronobiol Int **20**(3): 371-88.

Murre, C. (2005). "Helix-loop-helix proteins and lymphocyte development." Nat Immunol **6**(11): 1079-86.

Mutoh, H., Ratineau, C., et al. (2000). "Review article: transcriptional events controlling the terminal differentiation of intestinal endocrine cells." Aliment Pharmacol Ther **14 Suppl 1**: 170-5.

Nagel, S., Kaufmann, M., et al. (2003). "The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2)." Cancer Res **63**(17): 5329-34.

Norton, J. D. (2000). "ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis." J Cell Sci **113 (Pt 22)**: 3897-905.

O'Toole, P. J., Inoue, T., et al. (2003). "Id proteins negatively regulate basic helix-loop-helix transcription factor function by disrupting subnuclear compartmentalization." J Biol Chem **278**(46): 45770-6.

Okazuka, K., Wakabayashi, Y., et al. (2005). "p53 prevents maturation of T cell development to the immature CD4-CD8+ stage in Bcl11b-/- mice." Biochem Biophys Res Commun **328**(2): 545-9.

Pan, L., Hanrahan, J., et al. (2002). "An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus." J Immunol **168**(8): 3923-32.

Parker, M. H., Perry, R. L., et al. (2006). "MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation." Mol Cell Biol **26**(15): 5771-83.

Passoni, L., Hoffman, E. S., et al. (1997). "Intrathymic delta selection events in gammadelta cell development." Immunity **7**(1): 83-95.

Perk, J., Iavarone, A., et al. (2005). "Id family of helix-loop-helix proteins in cancer." Nat Rev Cancer **5**(8): 603-14.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Quong, M. W., Romanow, W. J., et al. (2002). "E protein function in lymphocyte development." Annu Rev Immunol **20**: 301-22.

Rivera, R. and Murre, C. (2001). "The regulation and function of the Id proteins in lymphocyte development." Oncogene **20**(58): 8308-16.

Rothenberg, E. V. and Dionne, C. J. (2002). "Lineage plasticity and commitment in T-cell development." Immunol Rev **187**: 96-115.

Rothenberg, E. V. and Taghon, T. (2005). "Molecular genetics of T cell development." Annu Rev Immunol **23**: 601-49.

Rothschild, G., Zhao, X., et al. (2006). "E Proteins and Id2 converge on p57Kip2 to regulate cell cycle in neural cells." Mol Cell Biol **26**(11): 4351-61.

Ruzinova, M. B. and Benezra, R. (2003). "Id proteins in development, cell cycle and cancer." Trends Cell Biol **13**(8): 410-8.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Senawong, T., Peterson, V. J., et al. (2005). "BCL11A-dependent recruitment of SIRT1 to a promoter template in mammalian cells results in histone deacetylation and transcriptional repression." Arch Biochem Biophys **434**(2): 316-25.

Sikder, H., Huso, D. L., et al. (2003). "Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors." Cancer Cell **4**(4): 291-9.

Su, X. Y., Busson, M., et al. (2004). "Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia." Genes Chromosomes Cancer **41**(3): 243-9.

Topark-Ngarm, A., Golonzhka, O., et al. (2006). "CTIP2 associates with the NuRD complex on the promoter of p57KIP2, a newly identified CTIP2 target gene." J Biol Chem **281**(43): 32272-83.

Voronova, A. F. and Lee, F. (1994). "The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation." Proc Natl Acad Sci U S A **91**(13): 5952-6.

Wakabayashi, Y., Watanabe, H., et al. (2003a). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Wakabayashi, Y., Inoue, J., et al. (2003b). "Homozygous deletions and point mutations of the Rit1/Bcl11b gene in gamma-ray induced mouse thymic lymphomas." Biochem Biophys Res Commun **301**(2): 598-603.

Yokota, Y. (2001). "Id and development." Oncogene **20**(58): 8290-8.

Yokota, Y., Mansouri, A., et al. (1999). "Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2." Nature **397**(6721): 702-6.

Zebedee, Z. and Hara, E. (2001). "Id proteins in cell cycle control and cellular senescence." Oncogene **20**(58): 8317-25.

Chapter 5

Summary

Chicken ovalbumin upstream promoter **t**ranscription factor (COUP-TF)-**i**nteracting **p**rotein **2** (CTIP2) is a novel C_2H_2 zinc finger protein that was identified in 2000 (Avram et al., 2000). CTIP2 has been implicated in lymphoid malignancies (Bernard et al., 2001; Sakata et al., 2004; Przybylski et al., 2005) and development of T lymphocytes (Wakabayashi et al., 2003; Inoue et al., 2006) and the central nervous system (Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005). However, the precise cellular function(s) and the molecular mechanism(s) underlying the contribution of CTIP2 in these processes remain to be investigated. CTIP2 is a transcriptional repressor that functions by at least two mechanisms. CTIP2 interacts with and stimulates transcriptional repression of COUP-TF family members (Avram et al., unpublished data). CTIP2 also represses transcription independently of COUP-TF proteins by direct DNA binding activity to a motif that is related to the canonical GC box (Avram et al., 2002).

The study of biochemical properties and the elucidation of the molecular mechanism(s) underlying CTIP2-mediated transcriptional repression would represent a step closer toward the understanding of cellular functions of CTIP2 protein, and provide a foundation for understanding the role of CTIP2 in biology. The studies described herein were designed to contribute additional knowledge concerning the transcriptional regulatory activity of CTIP2 through the functional characterization of DNA binding, self-association, and identification of CTIP2-associated protein(s) and and transcriptional targets of this important transcription factor.

In this study, we have demonstrated that CTIP2 repressed transcription through the use of class I histone deacetylases (HDAC) in human neuroblastoma cells. CTIP2 complexes isolated from SK-N-MC neuroblastoma cells were found to harbor histone deacetylase (HDAC) activity that was conferred by HDAC1 and/or HDAC2, both of which are components of the **Nu**cleosome **R**emodeling and Histone **D**eacetylation (NuRD) complex. In addition, size-exclusion chromatography revealed that CTIP2 and the NuRD proteins were components of a large complex in nuclear extracts of SK-N-MC cells. CTIP2 was shown to interact with the NuRD complex via direct binding to two core components of this complex, RbAp46 and RbAp48. The RbAp46/48 interface of CTIP2 was identified and contained within the proline-rich region of CTIP2. This region has previously been shown to interact with SIRT1, a class III HDAC (Senawong et al., 2003), suggesting that such a region may act as a key regulatory domain to interact with both class I (the NuRD complex) and class III (SIRT1) HDACs. The NuRD complex was recruited to the artificial promoter template and a promoter of a cyclin-dependent kinase inhibitor, *p57KIP2* in a CTIP2-dependent manner. *p57KIP2* was identified herein as a novel CTIP2 target gene in SK-N-MC cells. These results suggest that CTIP2 may repress transcription of *p57KIP2* by recruitment of the NuRD complex to the promoter template and this may be relevant to a subset of genes in a neuron-like context.

In addition to interacting with the components of the NuRD complex, the proline-rich region of CTIP2 was found to interact with the ZnF5-7 module located at the C-terminus of the protein. The C-terminal region as well as the centrally located zinc fingers (ZnF) 3-4 appeared to be required for CTIP2 DNA binding activity, however, in this regard the two zinc-binding modules appeared to function differentially. The CTIP2 ZnF3-4 module appeared to be involved in direct interaction with DNA, whereas the ZnF5-7 module was found to play a role in self-association of the protein. The disruption

of ZnF 5, 6, or 7 or deletion of all these three ZnFs considerably affected the repressive activity of CTIP2, which was likely due to disruption of CTIP2 self-association, which compromised the ability of the protein to bind DNA with high affinity. These results suggest that self-association of CTIP2 is critical for transcriptional regulatory activity of the protein.

Regarding the functional significance of CTIP2 in T cell development, transcriptome analyses of γδ and pooled DN thymocytes from new born, *CTIP2*-null mice revealed an up-regulation of *Id2*. A gene product of *Id2* has been demonstrated to oppose the activity of E proteins (Quong et al., 2002; Rothenberg and Taghon, 2005), which are required for early events in T cell development (Engel et al., 2001; Pan et al., 2002). CTIP2 was found on both the mouse and human *Id2* promoters, and our *in vitro* evidence suggested that this interaction was direct. Consistent with the data from mouse transcriptome analyses, CTIP2 suppressed the activity of *Id2* promoter fused to a reporter gene in human 293T cells. These results suggest that *Id2* is a direct transcriptional target of CTIP2. As overexpression of Id2 in thymus results in a phenotype quite similar to that of *CTIP2*-null mice (arrest in αβ T cell development) (Morrow et al., 1999, Wakabayashi et al., 2003), suppression of Id2 expression by CTIP2 may be necessary for a proper T cell development.

In summary, these results suggest that CTIP2 represses transcription via at least two mechanisms involving two different classes of HDACs (class I and III). In addition to recruiting a class III SIRT1, CTIP2 recruits the NuRD complexes including class I HDACs to the target promoter template. The recruitments of different HDAC complexes is as a function of promoter, and possibly cellular, context. The transcriptional regulatory activity of CTIP2 requires the C-terminal ZnFs for a self-association and for binding to

the target sequences. In the light of results demonstrating that the expression of *Id2* and *p57KIP2* genes are repressed by CTIP2, CTIP2-mediated transcriptional repression may contribute to the function(s) of CTIP2 during development of T cells and the central nervous system.

References

Arlotta, P., Molyneaux, B. J., et al. (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron **45**(2): 207-21.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Bernard, O. A., Busson-LeConiat, M., et al. (2001). "A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia." Leukemia **15**(10): 1495-504.

Chen, B., Schaevitz, L. R., et al. (2005). "Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex." Proc Natl Acad Sci U S A **102**(47): 17184-9.

Engel, I., Johns, C., et al. (2001). "Early thymocyte development is regulated by modulation of E2A protein activity." J Exp Med **194**(6): 733-45.

Inoue, J., Kanefuji, T., et al. (2006). "Expression of TCR alpha beta partly rescues developmental arrest and apoptosis of alpha beta T cells in Bcl11b-/- mice." J Immunol **176**(10): 5871-9.

Molyneaux, B. J., Arlotta, P., et al. (2005). "Fezl is required for the birth and specification of corticospinal motor neurons." Neuron **47**(6): 817-31.

Pan, L., Hanrahan, J., et al. (2002). "An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus." J Immunol **168**(8): 3923-32.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Quong, M. W., Romanow, W. J., et al. (2002). "E protein function in lymphocyte development." Annu Rev Immunol **20**: 301-22.

Rothenberg, E. V. and Taghon, T. (2005). "Molecular genetics of T cell development." Annu Rev Immunol **23**: 601-49.

Sakata, J., Inoue, J., et al. (2004). "Involvement of V(D)J recombinase in the generation of intragenic deletions in the Rit1/Bcl11b tumor suppressor gene in gamma-ray-induced thymic lymphomas and in normal thymus of the mouse." Carcinogenesis **25**(6): 1069-75.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Wakabayashi, Y., Watanabe, H., et al. (2003). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Bibliography

Aasland, R., Gibson, T. J., et al. (1995). "The PHD finger: implications for chromatin-mediated transcriptional regulation." Trends Biochem Sci **20**(2): 56-9.

Anderson, M. K. (2006). "At the crossroads: diverse roles of early thymocyte transcriptional regulators." Immunol Rev **209**: 191-211.

Appelbaum, L. and Gothilf, Y. (2006). "Mechanism of pineal-specific gene expression: the role of E-box and photoreceptor conserved elements." Mol Cell Endocrinol **252**(1-2): 27-33.

Arlotta, P., Molyneaux, B. J., et al. (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron **45**(2): 207-21.

Avram, D., Fields, A., et al. (2000). "Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors." J Biol Chem **275**(14): 10315-22.

Avram, D., Fields, A., et al. (2002). "COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein." Biochem J **368**(Pt 2): 555-63.

Bain, G., Engel, I., et al. (1997). "E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas." Mol Cell Biol **17**(8): 4782-91.

Bain, G., Quong, M. W., et al. (1999a). "Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47." J Exp Med **190**(11): 1605-16.

Bain, G., Romanow, W. J., et al. (1999b). "Positive and negative regulation of V(D)J recombination by the E2A proteins." J Exp Med **189**(2): 289-300.

Bardwell, V. J. and Treisman, R. (1994). "The POZ domain: a conserved protein-protein interaction motif." Genes Dev **8**(14): 1664-77.

Barndt, R. J., Dai, M., et al. (2000). "Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB." Mol Cell Biol **20**(18): 6677-85.

Becker, P. B. and Horz, W. (2002). "ATP-dependent nucleosome remodeling." Annu Rev Biochem **71**: 247-73.

Bellefroid, E. J., Marine, J. C., et al. (1995). "Emergence of the ZNF91 Kruppel-associated box-containing zinc finger gene family in the last common ancestor of anthropoidea." Proc Natl Acad Sci U S A **92**(23): 10757-61.

Benezra, R., Davis, R. L., et al. (1990). "The protein Id: a negative regulator of helix-loop-helix DNA binding proteins." Cell **61**(1): 49-59.

Bernard, O. A., Busson-LeConiat, M., et al. (2001). "A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia." Leukemia **15**(10): 1495-504.

Bertrand, D. (2002). "Neuronal Nicotinic Acetylcholine Receptors and Epilepsy." Epilepsy Curr **2**(6): 191-193.

Bezrookove, V., van Zelderen-Bhola, S. L., et al. (2004). "A novel t(6;14)(q25-q27;q32) in acute myelocytic leukemia involves the BCL11B gene." Cancer Genet Cytogenet **149**(1): 72-6.

Bitterman, K. J., Anderson, R. M., et al. (2002). "Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1." J Biol Chem **277**(47): 45099-107.

Blander, G. and Guarente, L. (2004). "The Sir2 family of protein deacetylases." Annu Rev Biochem **73**: 417-35.

Bouras, T., Fu, M., et al. (2005). "SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1." J Biol Chem **280**(11): 10264-76.

Chen, B., Schaevitz, L. R., et al. (2005). "Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex." Proc Natl Acad Sci U S A **102**(47): 17184-9.

Choo, Y., Castellanos, A., et al. (1997). "Promoter-specific activation of gene expression directed by bacteriophage-selected zinc fingers." J Mol Biol **273**(3): 525-32.

Christian, M., Tullet, J. M., et al. (2004). "Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140." J Biol Chem **279**(15): 15645-51.

Ciofani, M., Schmitt, T. M., et al. (2004). "Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation." J Immunol **172**(9): 5230-9.

Cismasiu, V. B., Adamo, K., et al. (2005). "BCL11B functionally associates with the NuRD complex in T lymphocytes to repress targeted promoter." Oncogene **24**(45): 6753-64.

Cismasiu, V. B., Ghanta, S., et al. (2006). "BCL11B participates in the activation of IL2 gene expression in CD4+ T lymphocytes." Blood **108**(8): 2695-702.

Cosgrove, M. S. and Wolberger, C. (2005). "How does the histone code work?" Biochem Cell Biol **83**(4): 468-76.

Cress, W. D. and Seto, E. (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

Cunningham, J. J. and Roussel, M. F. (2001). "Cyclin-dependent kinase inhibitors in the development of the central nervous system." Cell Growth Differ **12**(8): 387-96.

Dangond, F., Hafler, D. A., et al. (1998). "Differential display cloning of a novel human histone deacetylase (HDAC3) cDNA from PHA-activated immune cells." Biochem Biophys Res Commun **242**(3): 648-52.

David, G., Alland, L., et al. (1998). "Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein." Oncogene **16**(19): 2549-56.

Denu, J. M. (2005). "The Sir 2 family of protein deacetylases." Curr Opin Chem Biol **9**(5): 431-40.

Dignam, J. D., Lebovitz, R. M., et al. (1983). "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Res **11**(5): 1475-89.

Dowell, P., Ishmael, J. E., et al. (1997a). "p300 functions as a coactivator for the peroxisome proliferator-activated receptor alpha." J Biol Chem **272**(52): 33435-43.

Dowell, P., Peterson, V. J., et al. (1997b). "Ligand-induced peroxisome proliferator-activated receptor alpha conformational change." J Biol Chem **272**(3): 2013-20.

Drouin, J., Sun, Y. L., et al. (1992). "Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor." Mol Endocrinol **6**(8): 1299-309.

Dyer, M. A. and Cepko, C. L. (2000). "p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina." Development **127**(16): 3593-605.

Eckner, R., Arany, Z., et al. (1994). "The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family." Cold Spring Harb Symp Quant Biol **59**: 85-95.

Elfring, L. K., Daniel, C., et al. (1998). "Genetic analysis of brahma: the Drosophila homolog of the yeast chromatin remodeling factor SWI2/SNF2." Genetics **148**(1): 251-65.

Emiliani, S., Fischle, W., et al. (1998). "Characterization of a human RPD3 ortholog, HDAC3." Proc Natl Acad Sci U S A **95**(6): 2795-800.

Engel, I., Johns, C., et al. (2001). "Early thymocyte development is regulated by modulation of E2A protein activity." J Exp Med **194**(6): 733-45.

Fawell, S. E., Lees, J. A., et al. (1990). "Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor." Cell **60**(6): 953-62.

Fischer, D. D., Cai, R., et al. (2002). "Isolation and characterization of a novel class II histone deacetylase, HDAC10." J Biol Chem **277**(8): 6656-66.

Fischle, W., Emiliani, S., et al. (1999). "A new family of human histone deacetylases related to Saccharomyces cerevisiae HDA1p." J Biol Chem **274**(17): 11713-20.

Frye, R. A. (1999). "Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity." Biochem Biophys Res Commun **260**(1): 273-9.

Fujii, K., Ishimaru, F., et al. (2003). "Over-expression of short isoforms of Helios in patients with adult T-cell leukaemia/lymphoma." Br J Haematol **120**(6): 986-9.

Fujisaki, S., Ninomiya, Y., et al. (2003). "Dimerization of the Polycomb-group protein Mel-18 is regulated by PKC phosphorylation." Biochem Biophys Res Commun **300**(1): 135-40.

Fujita, N., Jaye, D. L., et al. (2004). "MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation." Cell **119**(1): 75-86.

Gao, L., Cueto, M. A., et al. (2002). "Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family." J Biol Chem **277**(28): 25748-55.

Georgopoulos, K. (2002). "Haematopoietic cell-fate decisions, chromatin regulation and ikaros." Nat Rev Immunol **2**(3): 162-74.

Godfrey, D. I. and Zlotnik, A. (1993). "Control points in early T-cell development." Immunol Today **14**(11): 547-53.

Greenbaum, S. and Zhuang, Y. (2002). "Regulation of early lymphocyte development by E2A family proteins." Semin Immunol **14**(6): 405-14.

Grozinger, C. M., Hassig, C. A., et al. (1999). "Three proteins define a class of human histone deacetylases related to yeast Hda1p." Proc Natl Acad Sci U S A **96**(9): 4868-73.

Haelens, A., Verrijdt, G., et al. (2003). "DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation." Biochem J **369**(Pt 1): 141-51.

Haks, M. C., Lefebvre, J. M., et al. (2005). "Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage." Immunity **22**(5): 595-606.

Hall, T. M. (2005). "Multiple modes of RNA recognition by zinc finger proteins." Curr Opin Struct Biol **15**(3): 367-73.

Hayes, J. J. and Hansen, J. C. (2001). "Nucleosomes and the chromatin fiber." Curr Opin Genet Dev **11**(2): 124-9.

Hayes, S. M., Li, L., et al. (2005). "TCR signal strength influences alphabeta/gammadelta lineage fate." Immunity **22**(5): 583-93.

Heemskerk, M. H., Blom, B., et al. (1997). "Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3." J Exp Med **186**(9): 1597-602.

Hendrich, B., Guy, J., et al. (2001). "Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development." Genes Dev **15**(6): 710-23.

Hernandez-Hoyos, G., Anderson, M. K., et al. (2003). "GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation." Immunity **19**(1): 83-94.

Hohl, M. and Thiel, G. (2005). "Cell type-specific regulation of RE-1 silencing transcription factor (REST) target genes." Eur J Neurosci **22**(9): 2216-30.

Hong, W., Nakazawa, M., et al. (2005). "FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1." Embo J **24**(13): 2367-78.

Hu, E., Chen, Z., et al. (2000). "Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor." J Biol Chem **275**(20): 15254-64.

Huang, T. T., Zong, Y., et al. (2003). "TCR-mediated hyper-responsiveness of autoimmune Galphai2(-/-) mice is an intrinsic naive CD4(+) T cell disorder selective for the Galphai2 subunit." Int Immunol **15**(11): 1359-67.

Hubbert, C., Guardiola, A., et al. (2002). "HDAC6 is a microtubule-associated deacetylase." Nature **417**(6887): 455-8.

Humphrey, G. W., Wang, Y., et al. (2001). "Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1." J Biol Chem **276**(9): 6817-24.

Ikawa, T., Fujimoto, S., et al. (2001). "Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2." Proc Natl Acad Sci U S A **98**(9): 5164-9.

Inoue, J., Kanefuji, T., et al. (2006). "Expression of TCR alpha beta partly rescues developmental arrest and apoptosis of alpha beta T cells in Bcl11b-/- mice." J Immunol **176**(10): 5871-9.

Joseph, B., Wallen-Mackenzie, A., et al. (2003). "p57(Kip2) cooperates with Nurr1 in developing dopamine cells." Proc Natl Acad Sci U S A **100**(26): 15619-24.

Kadam, S. and Emerson, B. M. (2003). "Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes." Mol Cell **11**(2): 377-89.

Kantor, B., Makedonski, K., et al. (2003). "Expression and localization of components of the histone deacetylases multiprotein repressory complexes in the mouse preimplantation embryo." Gene Expr Patterns **3**(6): 697-702.

Kao, H. Y., Ordentlich, P., et al. (1998). "A histone deacetylase corepressor complex regulates the Notch signal transduction pathway." Genes Dev **12**(15): 2269-77.

Kehle, J., Beuchle, D., et al. (1998). "dMi-2, a hunchback-interacting protein that functions in polycomb repression." Science **282**(5395): 1897-900.

Kelley, C. M., Ikeda, T., et al. (1998). "Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors." Curr Biol **8**(9): 508-15.

Kelly, T. J., Simancek, P., et al. (1998). "Identification and characterization of a single-stranded DNA-binding protein from the archaeon Methanococcus jannaschii." Proc Natl Acad Sci U S A **95**(25): 14634-9.

Kim, J., Sif, S., et al. (1999). "Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes." Immunity **10**(3): 345-55.

Kim, D., Xu, M., et al. (2002). "Helix-loop-helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF-kappaB activities." Immunity **16**(1): 9-21.

Kornberg, R. D. and Lorch, Y. (1999). "Chromatin-modifying and -remodeling complexes." Curr Opin Genet Dev **9**(2): 148-51.

Krishna, S. S., Majumdar, I., et al. (2003). "Structural classification of zinc fingers: survey and summary." Nucleic Acids Res **31**(2): 532-50.

Kumarasiri, M. H., Shapiro, L. E., et al. (1988). "Cell cycle dependence of thyroid hormone nuclear receptors in cultured GC cells: relationship to nuclear matrix." Endocrinology **122**(5): 1897-904.

Kuntz, M. A. and Shapiro, D. J. (1997). "Dimerizing the estrogen receptor DNA binding domain enhances binding to estrogen response elements." J Biol Chem **272**(44): 27949-56.

Laity, J. H., Lee, B. M., et al. (2001). "Zinc finger proteins: new insights into structural and functional diversity." Curr Opin Struct Biol **11**(1): 39-46.

Lander, E. S., Linton, L. M., et al. (2001). "Initial sequencing and analysis of the human genome." Nature **409**(6822): 860-921.

Langley, E., Pearson, M., et al. (2002). "Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence." Embo J **21**(10): 2383-96.

Lasorella, A., Rothschild, G., et al. (2005). "Id2 mediates tumor initiation, proliferation, and angiogenesis in Rb mutant mice." Mol Cell Biol **25**(9): 3563-74.

Lasorella, A., Uo, T., et al. (2001). "Id proteins at the cross-road of development and cancer." Oncogene **20**(58): 8326-33.

Lee, M. S., Gippert, G. P., et al. (1989). "Three-dimensional solution structure of a single zinc finger DNA-binding domain." Science **245**(4918): 635-7.

Leid, M., Ishmael, J. E., et al. (2004). "CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis." Gene Expr Patterns **4**(6): 733-9.

Li, J., Wang, J., et al. (2000). "Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3." Embo J **19**(16): 4342-50.

Littlewood, T. D. and Evan, G. I. (1990). "The role of myc oncogenes in cell growth and differentiation." Adv Dent Res **4**: 69-79.

Lorick, K. L., Jensen, J. P., et al. (1999). "RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination." Proc Natl Acad Sci U S A **96**(20): 11364-9.

Losson, R. (1997). "KRAB zinc finger proteins and nuclear receptors: a possible cross-talk." Biol Chem **378**(7): 579-81.

Luger, K., Rechsteiner, T. J., et al. (1997). "Characterization of nucleosome core particles containing histone proteins made in bacteria." J Mol Biol **272**(3): 301-11.

Lundblad, J. R., Kwok, R. P., et al. (1995). "Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP." Nature **374**(6517): 85-8.

Luo, J., Su, F., et al. (2000). "Deacetylation of p53 modulates its effect on cell growth and apoptosis." Nature **408**(6810): 377-81.

Luo, K., Stroschein, S. L., et al. (1999). "The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling." Genes Dev **13**(17): 2196-206.

MacLeod, R. A., Nagel, S., et al. (2004). "BCL11B rearrangements probably target T-cell neoplasia rather than acute myelocytic leukemia." Cancer Genet Cytogenet **153**(1): 88-9.

MacLeod, R. A., Nagel, S., et al. (2003). "Activation of HOX11L2 by juxtaposition with 3'-BCL11B in an acute lymphoblastic leukemia cell line (HPB-ALL) with t(5;14)(q35;q32.2)." Genes Chromosomes Cancer **37**(1): 84-91.

Makowski, A., Brzostek, S., et al. (2003). "Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor." Mol Endocrinol **17**(2): 273-86.

Margueron, R., Trojer, P., et al. (2005). "The key to development: interpreting the histone code?" Curr Opin Genet Dev **15**(2): 163-76.

Martens, J. A. and Winston, F. (2003). "Recent advances in understanding chromatin remodeling by Swi/Snf complexes." Curr Opin Genet Dev **13**(2): 136-42.

Massari, M. E. and Murre, C. (2000). "Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms." Mol Cell Biol **20**(2): 429-40.

Matsuoka, S., Edwards, M. C., et al. (1995). "p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene." Genes Dev **9**(6): 650-62.

Matthews, J. M. and Sunde, M. (2002). "Zinc fingers--folds for many occasions." IUBMB Life **54**(6): 351-5.

McCarty, A. S., Kleiger, G., et al. (2003). "Selective dimerization of a C2H2 zinc finger subfamily." Mol Cell **11**(2): 459-70.

Mersfelder, E. L. and Parthun, M. R. (2006). "The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure." Nucleic Acids Res **34**(9): 2653-62.

Metivier, R., Penot, G., et al. (2003). "Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter." Cell **115**(6): 751-63.

Michishita, E., Park, J. Y., et al. (2005). "Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins." Mol Biol Cell **16**(10): 4623-35.

Miller, K. G., Tower, J., et al. (1985). "A complex control region of the mouse rRNA gene directs accurate initiation by RNA polymerase I." Mol Cell Biol **5**(3): 554-62.

Molnar, A. and Georgopoulos, K. (1994). "The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins." Mol Cell Biol **14**(12): 8292-303.

Molyneaux, B. J., Arlotta, P., et al. (2005). "Fezl is required for the birth and specification of corticospinal motor neurons." Neuron **47**(6): 817-31.

Morgan, B., Sun, L., et al. (1997). "Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation." Embo J **16**(8): 2004-13.

Morrow, M. A., Mayer, E. W., et al. (1999). "Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages." Mol Immunol **36**(8): 491-503.

Munoz, E. and Baler, R. (2003). "The circadian E-box: when perfect is not good enough." Chronobiol Int **20**(3): 371-88.

Murawsky, C. M., Brehm, A., et al. (2001). "Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex." EMBO Rep **2**(12): 1089-94.

Murre, C. (2005). "Helix-loop-helix proteins and lymphocyte development." Nat Immunol **6**(11): 1079-86.

Mutoh, H., Ratineau, C., et al. (2000). "Review article: transcriptional events controlling the terminal differentiation of intestinal endocrine cells." Aliment Pharmacol Ther **14** Suppl 1: 170-5.

Nagel, S., Kaufmann, M., et al. (2003). "The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2)." Cancer Res **63**(17): 5329-34.

Nakamura, T., Yamazaki, Y., et al. (2000). "Evi9 encodes a novel zinc finger protein that physically interacts with BCL6, a known human B-cell proto-oncogene product." Mol Cell Biol **20**(9): 3178-86.

Nakase, K., Ishimaru, F., et al. (2002). "Overexpression of novel short isoforms of Helios in a patient with T-cell acute lymphoblastic leukemia." Exp Hematol **30**(4): 313-7.

Nawijn, M. C., Ferreira, R., et al. (2001). "Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice." J Immunol **167**(2): 715-23.

Norton, J. D. (2000). "ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis." J Cell Sci **113** (Pt 22): 3897-905.

Okazuka, K., Wakabayashi, Y., et al. (2005). "p53 prevents maturation of T cell development to the immature CD4-CD8+ stage in Bcl11b-/- mice." Biochem Biophys Res Commun **328**(2): 545-9.

O'Toole, P. J., Inoue, T., et al. (2003). "Id proteins negatively regulate basic helix-loop-helix transcription factor function by disrupting subnuclear compartmentalization." J Biol Chem **278**(46): 45770-6.

Pai, S. Y., Truitt, M. L., et al. (2003). "Critical roles for transcription factor GATA-3 in thymocyte development." Immunity **19**(6): 863-75.

Pan, L., Hanrahan, J., et al. (2002). "An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus." J Immunol **168**(8): 3923-32.

Parker, M. H., Perry, R. L., et al. (2006). "MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation." Mol Cell Biol **26**(15): 5771-83.

Passoni, L., Hoffman, E. S., et al. (1997). "Intrathymic delta selection events in gammadelta cell development." Immunity **7**(1): 83-95.

Pear, W. S. and Radtke, F. (2003). "Notch signaling in lymphopoiesis." Semin Immunol **15**(2): 69-79.

Pelham, H. R. and Brown, D. D. (1980). "A specific transcription factor that can bind either the 5S RNA gene or 5S RNA." Proc Natl Acad Sci U S A **77**(7): 4170-4.

Perdomo, J., Holmes, M., et al. (2000). "Eos and pegasus, two members of the Ikaros family of proteins with distinct DNA binding activities." J Biol Chem **275**(49): 38347-54.

Perk, J., Iavarone, A., et al. (2005). "Id family of helix-loop-helix proteins in cancer." Nat Rev Cancer **5**(8): 603-14.

Peterson, C. L. and Laniel, M. A. (2004). "Histones and histone modifications." Curr Biol **14**(14): R546-51.

Petersson, K., Ivars, F., et al. (2002). "The pT alpha promoter and enhancer are direct targets for transactivation by E box-binding proteins." Eur J Immunol **32**(3): 911-20.

Picard, B. and Wegnez, M. (1979). "Isolation of a 7S particle from Xenopus laevis oocytes: a 5S RNA-protein complex." Proc Natl Acad Sci U S A **76**(1): 241-5.

Przybylski, G. K., Dik, W. A., et al. (2005). "Disruption of the BCL11B gene through inv(14)(q11.2q32.31) results in the expression of BCL11B-TRDC fusion transcripts and is associated with the absence of wild-type BCL11B transcripts in T-ALL." Leukemia **19**(2): 201-8.

Qiu, Y., Krishnan, V., et al. (1995). "Isolation, characterization, and chromosomal localization of mouse and human COUP-TF I and II genes." Genomics **29**(1): 240-6.

Quong, M. W., Romanow, W. J., et al. (2002). "E protein function in lymphocyte development." Annu Rev Immunol **20**: 301-22.

Radtke, F., Wilson, A., et al. (2004). "Notch signaling in T- and B-cell development." Curr Opin Immunol **16**(2): 174-9.

Ravasi, T., Huber, T., et al. (2003). "Systematic characterization of the zinc-finger-containing proteins in the mouse transcriptome." Genome Res **13**(6B): 1430-42.

Ritchie, H. H., Wang, L. H., et al. (1990). "COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily." Nucleic Acids Res **18**(23): 6857-62.

Rivera, R. and Murre, C. (2001). "The regulation and function of the Id proteins in lymphocyte development." Oncogene **20**(58): 8308-16.

Robey, E. A. and Bluestone, J. A. (2004). "Notch signaling in lymphocyte development and function." Curr Opin Immunol **16**(3): 360-6.

Rohr, O., Marban, C., et al. (2003). "Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells." J Leukoc Biol **74**(5): 736-49.

Rooney, R. J., Rothammer, K., et al. (1998). "Mutational analysis of p50E4F suggests that DNA binding activity is mediated through an alternative structure in a zinc finger domain that is regulated by phosphorylation." Nucleic Acids Res **26**(7): 1681-8.

Rothenberg, E. V. and Dionne, C. J. (2002). "Lineage plasticity and commitment in T-cell development." Immunol Rev **187**: 96-115.

Rothenberg, E. V. and Taghon, T. (2005). "Molecular genetics of T cell development." Annu Rev Immunol **23**: 601-49.

Rothschild, G., Zhao, X., et al. (2006). "E Proteins and Id2 converge on p57Kip2 to regulate cell cycle in neural cells." Mol Cell Biol **26**(11): 4351-61.

Ruiz, A., Jiang, J., et al. (2004). "Overexpression of the Ikaros 6 isoform is restricted to t(4;11) acute lymphoblastic leukaemia in children and infants and has a role in B-cell survival." Br J Haematol **125**(1): 31-7.

Ruzinova, M. B. and Benezra, R. (2003). "Id proteins in development, cell cycle and cancer." Trends Cell Biol **13**(8): 410-8.

Saha, A., Wittmeyer, J., et al. (2006). "Chromatin remodelling: the industrial revolution of DNA around histones." Nat Rev Mol Cell Biol **7**(6): 437-47.

Saiki, Y., Yamazaki, Y., et al. (2000). "Human EVI9, a homologue of the mouse myeloid leukemia gene, is expressed in the hematopoietic progenitors and down-regulated during myeloid differentiation of HL60 cells." Genomics **70**(3): 387-91.

Sakata, J., Inoue, J., et al. (2004). "Involvement of V(D)J recombinase in the generation of intragenic deletions in the Rit1/Bcl11b tumor suppressor gene in gamma-ray-induced thymic lymphomas and in normal thymus of the mouse." Carcinogenesis **25**(6): 1069-75.

Satterwhite, E., Sonoki, T., et al. (2001). "The BCL11 gene family: involvement of BCL11A in lymphoid malignancies." Blood **98**(12): 3413-20.

Schultz, D. C., Friedman, J. R., et al. (2001). "Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD." Genes Dev **15**(4): 428-43.

Senawong, T., Peterson, V. J., et al. (2003). "Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression." J Biol Chem **278**(44): 43041-50.

Senawong, T., Peterson, V. J., et al. (2005). "BCL11A-dependent recruitment of SIRT1 to a promoter template in mammalian cells results in histone deacetylation and transcriptional repression." Arch Biochem Biophys **434**(2): 316-25.

Siddiqui, H., Solomon, D. A., et al. (2003). "Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition." Mol Cell Biol **23**(21): 7719-31.

Sikder, H., Huso, D. L., et al. (2003). "Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors." Cancer Cell **4**(4): 291-9.

Smith, J. S., Brachmann, C. B., et al. (2000). "A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family." Proc Natl Acad Sci U S A **97**(12): 6658-63.

Solari, F., Bateman, A., et al. (1999). "The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning." Development **126**(11): 2483-94.

Staal, F. J., Weerkamp, F., et al. (2001). "Transcriptional control of t lymphocyte differentiation." Stem Cells **19**(3): 165-79.

Su, X. Y., Busson, M., et al. (2004). "Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia." Genes Chromosomes Cancer **41**(3): 243-9.

Sun, L., Goodman, P. A., et al. (1999). "Expression of aberrantly spliced oncogenic ikaros isoforms in childhood acute lymphoblastic leukemia." J Clin Oncol **17**(12): 3753-66.

Sun, L., Liu, A., et al. (1996). "Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development." Embo J **15**(19): 5358-69.

Suzuki, M. and Yagi, N. (1994). "DNA recognition code of transcription factors in the helix-turn-helix, probe helix, hormone receptor, and zinc finger families." Proc Natl Acad Sci U S A **91**(26): 12357-61.

Takeda, K., Ichiki, T., et al. (2000). "Peroxisome proliferator-activated receptor gamma activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells." Circulation **102**(15): 1834-9.

Thiagalingam, S., Cheng, K. H., et al. (2003). "Histone deacetylases: unique players in shaping the epigenetic histone code." Ann N Y Acad Sci **983**: 84-100.

Tong, J. K., Hassig, C. A., et al. (1998). "Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex." Nature **395**(6705): 917-21.

Topark-Ngarm, A., Golonzhka, O., et al. (2006). "CTIP2 associates with the NuRD complex on the promoter of p57KIP2, a newly identified CTIP2 target gene." J Biol Chem **281**(43): 32272-83.

Tremblay, M., Herblot, S., et al. (2003). "Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL." J Biol Chem **278**(15): 12680-7.

Tsai, R. Y. and Reed, R. R. (1998). "Identification of DNA recognition sequences and protein interaction domains of the multiple-Zn-finger protein Roaz." Mol Cell Biol **18**(11): 6447-56.

Tzameli, I. and Zannis, V. I. (1996). "Binding specificity and modulation of the ApoA-I promoter activity by homo- and heterodimers of nuclear receptors." J Biol Chem **271**(14): 8402-15.

Van den Wyngaert, I., de Vries, W., et al. (2000). "Cloning and characterization of human histone deacetylase 8." FEBS Lett **478**(1-2): 77-83.

Vaquero, A., Scher, M., et al. (2004). "Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin." Mol Cell **16**(1): 93-105.

Vaziri, H., Dessain, S. K., et al. (2001). "hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase." Cell 1**07**(2): 149-59.

Verdel, A., Curtet, S., et al. (2000). "Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm." Curr Biol **10**(12): 747-9.

Verdone, L., Caserta, M., et al. (2005). "Role of histone acetylation in the control of gene expression." Biochem Cell Biol **83**(3): 344-53.

von Zelewsky, T., Palladino, F., et al. (2000). "The C. elegans Mi-2 chromatin-remodelling proteins function in vulval cell fate determination." Development **127**(24): 5277-84.

Voronova, A. F. and Lee, F. (1994). "The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation." Proc Natl Acad Sci U S A **91**(13): 5952-6.

Wakabayashi, Y., Watanabe, H., et al. (2003a). "Bcl11b is required for differentiation and survival of alphabeta T lymphocytes." Nat Immunol **4**(6): 533-9.

Wakabayashi, Y., Inoue, J., et al. (2003b). "Homozygous deletions and point mutations of the Rit1/Bcl11b gene in gamma-ray induced mouse thymic lymphomas." Biochem Biophys Res Commun **301**(2): 598-603.

Wang, L. H., Tsai, S. Y., et al. (1989). "COUP transcription factor is a member of the steroid receptor superfamily." Nature **340**(6229): 163-6.

Wang, Y., Wysocka, J., et al. (2004). "Linking covalent histone modifications to epigenetics: the rigidity and plasticity of the marks." Cold Spring Harb Symp Quant Biol **69**: 161-9.

Williams, C. J., Naito, T., et al. (2004). "The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development." Immunity **20**(6): 719-33.

Wolfer, A., Wilson, A., et al. (2002). "Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes." Immunity **16**(6): 869-79.

Wu, J. and Grunstein, M. (2000). "25 years after the nucleosome model: chromatin modifications." Trends Biochem Sci **25**(12): 619-23.

Xue, Y., Wong, J., et al. (1998). "NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities." Mol Cell **2**(6): 851-61.

Yang, W. M., Yao, Y. L., et al. (1997). "Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family." J Biol Chem **272**(44): 28001-7.

Yang, X. J., Ogryzko, V. V., et al. (1996). "A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A." Nature **382**(6589): 319-24.

Yang, Y., Hou, H., et al. (2005). "Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation." Embo J **24**(5): 1021-32.

Yao, Y. L. and Yang, W. M. (2003). "The metastasis-associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity." J Biol Chem **278**(43): 42560-8.

Yeung, F., Hoberg, J. E., et al. (2004). "Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase." Embo J **23**(12): 2369-80.

Yokota, Y. (2001). "Id and development." Oncogene 20(58): 8290-8.

Yokota, Y., Mansouri, A., et al. (1999). "Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2." Nature **397**(6721): 702-6.

Yoon, H. G., Chan, D. W., et al. (2003). "Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1." Embo J **22**(6): 1336-46.

You, A., Tong, J. K., et al. (2001). "CoREST is an integral component of the CoREST-human histone deacetylase complex." Proc Natl Acad Sci U S A **98**(4): 1454-8.

Zebedee, Z. and Hara, E. (2001). "Id proteins in cell cycle control and cellular senescence." Oncogene **20**(58): 8317-25.

Zhang, C. L., McKinsey, T. A., et al. (2002). "Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation." Mol Cell Biol **22**(20): 7302-12.

Zhang, Y., Iratni, R., et al. (1997). "Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex." Cell **89**(3): 357-64.

Zhang, Y., LeRoy, G., et al. (1998). "The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities." Cell **95**(2): 279-89.

Zhang, Y., Ng, H. H., et al. (1999). "Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation." Genes Dev **13**(15): 1924-35.

Zhang, Y., Sun, Z. W., et al. (1998). "SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex." Mol Cell **1**(7): 1021-31.

Zhao, X., Sternsdorf, T., et al. (2005). "Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications." Mol Cell Biol **25**(19): 8456-64.

Zhou, X., Marks, P. A., et al. (2001). "Cloning and characterization of a histone deacetylase, HDAC9." Proc Natl Acad Sci U S A **98**(19): 10572-7.

Zhou, X., Richon, V. M., et al. (2000). "Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras." Proc Natl Acad Sci U S A **97**(26): 14329-33.