AN ABSTRACT OF THE THESIS OF

Thanaset Senawong for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on March 3, 2004. Title: Transcriptional Repression Mediated by a Novel Family of C_2H_2 Zinc Finger Proteins.

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Abstract approved:

Mark E. Leid

Two novel and highly related C_2H_2 zinc finger proteins (CTIP1/BCL11A/EVI9 and CTIP2/BCL11B/Rit1) have been implicated in COUP-TF signaling, etiology of myeloid and lymphoid malignancies, and hematopoietic cell development. However, the precise cellular function(s) and the contribution of these proteins to neoplastic processes and hematopoietic cell development remain unknown. The goal of the studies described herein was to elucidate the molecular mechanisms underlying the transcriptional repression mediated by these proteins to understand their biological properties, and ultimately, their cellular function(s).

CTIP proteins repressed transcription of a reporter gene in a TSA-insensitive manner, suggesting that this repression mechanism(s) may not involve TSA-sensitive histone deacetylation catalyzed by member(s) of class I and II HDACs. One possible mechanism is that CTIP proteins may exert TSA-insensitive histone deacetylation catalyzed by TSA-insensitive

HDAC(s), such as SIRT1, to repress transcription. In deed, SIRT1 was found to interact with CTIP proteins both *in vitro* and in mammalian cells, and was recruited to the promoter template in a CTIP-dependent manner. The proline-rich regions of CTIP proteins and the sirtuin homology domain of SIRT1 were found to be essential for mediating CTIPs•SIRT1 interactions. Moreover, column chromatography revealed that SIRT1 and CTIP2 were components of a large complex in Jurkat cell nuclear extracts.

Based on the findings that SIRT1 associates with CTIP proteins in mammalian cells, SIRT1 may underlie the transcriptional repression activity of CTIP proteins. The following results support the hypothesis that SIRT1 may underlie the mechanism(s) of CTIP-mediated transcriptional repression. First, CTIP-mediated transcriptional repression was inhibited, at least partially, by nicotinamide, an inhibitor of the NAD^+-dependent, TSA-insensitive HDACs. Second, the decrease in levels of acetylated histones H3 and/or H4 at the promoter region of a reporter gene was observed upon overexpression of CTIP proteins, and this effect was inhibited, at least partially, by nicotinamide. Third, endogenous SIRT1 was recruited to the promoter template of a reporter gene in mammalian cells upon overexpression of CTIP proteins. Fourth, SIRT1 enhanced the transcriptional repression mediated by CTIP proteins and this enhancement required the catalytic activity of SIRT1. Finally, SIRT1 enhanced the

deacetylation of template-associated histones H3 and/or H4 in CTIP-transfected cells.

In summary, results described herein strongly suggest that CTIP-mediated transcriptional repression involves the recruitment of SIRT1 to the template, at which the TSA-insensitive, but nicotinamide-sensitive histone deacetylase catalyzes deacetylation of promoter-associated histones H3 and/or H4. These results contribute additional understanding to the molecular mechanisms underlying transcriptional activity of CTIP proteins, which might be helpful for identification and characterization of the target genes under the control of CTIP proteins in cells of hematopoietic system and/or the central nervous system.

Transcriptional Repression Mediated by a Novel Family of C_2H_2 Zinc Finger Proteins

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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*. All of the experiments in this chapter were performed by myself in the laboratory of Mark Leid with the following exception: Valerie Peterson performed protein fractionation using column chromatography to characterize the nature of CTIP2 complexes described in Fig. 2.9*B-F*. All remaining authors assisted with data interpretation and/or manuscript preparation.

All of the experiments described in Chapter 3 were performed by myself in the laboratory of Mark Leid. Valerie Peterson assisted in preparation of bacterial GST fusion protein lysates used in GST pulldown experiment.

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Chapter 1

Introduction

There are a number of similar but distinct zinc-binding motifs, such as C_2H_2 zinc fingers, C_2C_2 zinc-binding motifs (nuclear hormone receptors, GAL4, GATA-1, and TFIIS), C_2CH zinc-binding motifs (nucleocapsid, MetRS, and PKC CRD), RING finger (C_2C_2 and C_2CH), and LIM domain (C_2C_2 and C_2CH), found in several families of zinc-binding proteins (Klug and Schwabe, 1995). Some of these zinc-binding motifs are known to direct sequence-specific DNA binding, reinforcing the idea that zinc is ideally suited to folding small DNA-binding domains (DBDs). Among these zinc-binding motifs, the C_2H_2 zinc finger is the most prevalent protein motif in mammalian cells and defines the largest family of sequence-specific DNA-binding proteins (Lander et al., 2001; Tupler et al., 2001). The term zinc finger is used to describe a diverse set of protein motifs that have in common the property of binding zinc ions in order to stabilize the structure of a small, autonomously folded protein domain. The C_2H_2 zinc finger motif is characterized by conserved cysteines, histidines, and hydrophobic residues,

which stabilize the three dimensional structure consisting of a two-stranded antiparallel β-sheet and α-helix surrounding a central zinc ion (Wolfe et al., 2000). The proteins that contain C_2H_2 zinc finger not only use this domain for protein-DNA interactions but also for protein-protein and protein-RNA interactions (Mackay and Crossley, 1998; Shastry, 1996). Zinc finger proteins bind DNA with sufficient specificity and affinity to function independently (i.e., TFIIIA (Shastry, 1996) and NRSF/REST (Schoenherr and Anderson, 1995)), or can work cooperatively with other DNA binding proteins (i.e., Sp1 proteins (Lania et al., 1997)).

Besides the zinc finger motifs, C_2H_2 zinc finger proteins also contain certain other sequence motifs that define subgroups and may provide insights into the functions of the members of this large family of zinc finger proteins. The best characterized other sequence motifs commonly found in these zinc finger proteins include the poxvirus and zinc finger (POZ) domain (Bardwell and Treisman, 1994), which is also known as the BTB domain (Broad-Complex, Tramtrack, and Bric-a-brac), the Kruppel-associated box (KRAB) (Bellefroid et al., 1991; Losson, 1997), and the SCAN domain (Williams et al., 1995). The POZ domain is an evolutionary conserved protein-protein interaction domain found at the amino-terminal end of some C_2H_2-type zinc finger transcription factors and in some actin binding proteins (Albagli et al., 1995). As a transcriptional repression module, the POZ domain interacts with Sin3A, SMRT (silencing mediator for retinoid and

thyroid hormone receptors), and NCoR (nuclear receptor corepressor) (Grignani et al., 1998; Hong et al., 1997; Lin et al., 1998). The KRAB domain is a conserved amino acid sequence motif found at the amino-terminal end of proteins that contain multiple C_2H_2 zinc fingers at their carboxy termini (Bellefroid et al., 1991). The KRAB domain functions to repress transcription by recruiting a co-repressor KAP-1 (Schultz et al., 2001). The KAP-1 protein can bind to the heterochromatin protein 1 (HP1), which may then nucleate local heterochromatin formation, resulting in gene silencing. The SCAN domain is a conserved leucine-rich region (LeR) found in some zinc finger transcription factors. The name for this domain was derived from the first letters of the names of four proteins initially found to contain this domain (SRE-ZBP, CTfin51, AW-1 (ZNF174), and Number 18 cDNA or ZnF20) (Williams et al., 1995). The SCAN domain is capable of mediating homo- and heterodimerization between specific members of the SCAN domain family of zinc finger transcription factors (Schumacher et al., 2000).

1.1 A novel family of C_2H_2 zinc finger proteins

Recently, two novel and highly related C_2H_2 zinc finger proteins that lack the above characterized motifs were isolated and identified as chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting

proteins 1 and 2 (mouse CTIP1 and CTIP2 respectively) (Avram et al., 2000). CTIP1 protein is comprised of 776 amino acids with 4 zinc finger motifs, whereas CTIP2 contains 813 amino acid residues with 7 zinc finger motifs. These novel zinc finger proteins share 61% identity in their peptide sequences, and the zinc finger domains are 95% identical (see Fig. 1.1).

CTIP1 was subsequently and independently identified as *EVI9* (ecotropic viral integration site 9) by a proviral tagging strategy (Nakamura et al., 2000). Later, the human homolog of CTIP1 was isolated and identified as BCL11A (B Cell Leukemia 11A) due to its direct involvement in B-cell malignancy with t (2; 14)(p13; q32.3) chromosomal translocation (Satterwhite et al., 2001). The human CTIP2 was also named BCL11B due to its homology to BCL11A, although no evidence for its involvement in B-cell malignancy has been reported. Contrary to its name, BCL11B was not found to be expressed at detectable levels by Northern blot in any B-cell lines examined (Satterwhite et al., 2001), but preferentially expressed in normal T-lymphocytes (Bernard et al., 2001) and in malignant T-cell lines (Satterwhite et al., 2001). CTIP2 is also known as Rit1 (Radiation-induced tumor suppressor gene 1), which was identified as a potential tumor suppressor protein involved in γ-ray-induced mouse thymic lymphomas (Wakabayashi et al., 2003a). The human loci of CTIP1 and CTIP2 are located on chromosome 2p13 and 14q32.1, respectively (Satterwhite et al., 2001).

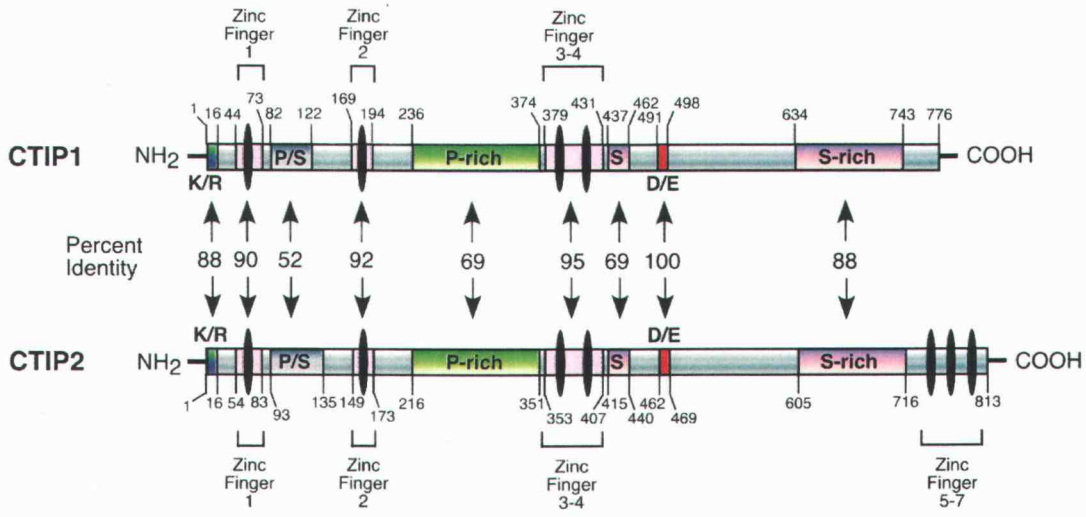


Figure 1.1. **Schematic diagram of CTIP1 and CTIP2 amino acid alignment.** The percentage of identity between each region of CTIP1 and CTIP2 is indicated. The overall characteristics of each domain are indicated. Zinc finger motifs are represented by black ovals.

1.1.1 Role of CTIP1 and CTIP2 in COUP-TF signaling

CTIP1 and CTIP2 were originally isolated as proteins that interacted with chicken ovalbumin upstream promoter transcription factors (COUP-TFs) (Avram et al., 2000). COUP-TFs are some of the most characterized orphan receptors of the steroid/thyroid hormone receptor superfamily and play important roles in the regulation of neurogenesis, organogenesis, and cellular differentiation during embryonic development (reviewed in (Pereira et al., 2000). Generally, COUP-TF proteins function as transcriptional repressors, and may utilize several mechanisms to repress transcription (Tsai and Tsai, 1997), however, there is growing evidence that COUP-TFs can function as transcriptional activators (Lin et al., 2000; Murray and Edgar, 2001).

Both CTIP1 and CTIP2 enhance COUP-TFII-mediated transcriptional repression in transfected cells (Avram et al., 2000), suggesting a role for these proteins in COUP-TF signaling pathway(s). CTIP1 and CTIP2 are co-expressed with at least one COUP-TF family member in several regions of the developing and adult mouse brain, suggesting that the interaction between the two families of protein may be functionally significant. However, COUP-TF family members are not expressed in cells of the immuno/hematopoietic system that express CTIP1 (B cells, dendritic cells) and CTIP2 (T cells, NK cells) at high levels (Leid et

al., manuscript in revision). This suggests two non-mutually exclusive possibilities: (1) CTIPs may work with other nuclear receptors that we have not yet identified and/or (2) CTIPs may work independently of nuclear receptors. Subsequent studies revealed that both CTIP1 and CTIP2 bound directly and specifically to a GC-rich sequence binding site, 5′-GGCCGGAGG-3′ (upper strand) (Avram et al., 2002). Moreover, both CTIPs were found to repress transcription of a reporter gene harboring this binding site in its promoter region in the absence of over-expressed COUP-TF proteins, suggesting that CTIP1 and CTIP2 may regulate transcription independently of COUP-TF proteins in some cell types and/or promoter contexts. Interestingly, both CTIP1/BCL11A and CTIP2/BCL11B repress transcription of a reporter gene independently of trichostatin A (TSA)-sensitive histone deacetylation (Avram et al., 2000; Avram et al., 2002), suggesting that this repression pathway may be different from that utilized by several other transcriptional repressors (Struhl, 1998).

1.1.2 Role of CTIP1 and CTIP2 in malignant transformation

Malignant transformation of B cells can occur at several steps of lymphocyte development from early B cell progenitors to mature B cells. There are three important recombination processes of the immunoglobulin

(IG) genes in normal B cells (Dyer and Oscier, 2002). Somatic recombination of the dispersed IG gene segments is firstly required to produce a functional IG gene. Secondly, somatic hypermutation (SHM) is required to produce antibodies with high affinity for a particular (or specific) antigen. Finally, class switch recombination (CSR) occurs shortly after SHM, which involves transposition of the completed VDJ gene to downstream constant region (C_H) segments. CSR occurs between highly repetitive switch regions, resulting in antibody molecules with different effector functions. All three recombination processes involve the introduction of double-stranded DNA breaks (Goossens et al., 1998), suggesting that errors in all three processes may contribute to the pathogenesis of B cell malignancies in various ways, but principally through the generation of chromosomal translocations (Goossens et al., 1998; Kuppers and Dalla-Favera, 2001).

Human BCL11A was isolated through its involvement in rare chromosomal translocation, t (2; 14)(p13; q32.3) in three cases of chronic lymphocytic leukemia (CLL) and one case of immunocytoma that progressed to leukemic phase (Satterwhite et al., 2001). The consequence of the translocation was over-expression of BCL11A. *BCL11A* is located near the *REL* gene on chromosome 2p13 and is co-amplified with *REL* in many cases of Hodgkin s disease and other B-cell, non-Hodgkin s lymphomas (Martin-Subero et al., 2002; Satterwhite et al., 2001). REL plays important roles in

regulation of proliferation, maturation, differentiation, and apoptosis of B lymphocytes via interaction with other members of the NF-κB family (Gerondakis et al., 1998; Liou et al., 1994; Tumang et al., 1998). Although human BCL11A is expressed in early, multipotent hemopoietic progenitors (Nakamura et al., 2000), the contribution of BCL11A amplification in the pathogenesis of lymphoid leukemias remains to be investigated.

Human CTIP2 has also been implicated in hematopoietic disorders. A translocation event involving the human CTIP2 locus, t (5; 14)(q35; q32), results in acute T lymphoblastic leukemia (T ALL) (Bernard et al., 2001). However, the contribution of CTIP2 to this neoplastic process has yet to be assessed. Human CTIP2 is highly expressed during normal and pathological T lymphoid differentiation, suggesting that deregulated expression of this gene may not be an important recurrent consequence of the translocation (Bernard et al., 2001).

1.1.3 Role of CTIP1 and CTIP2 in hematopoietic cell development

Many developmental stages are required for the differentiation of pluripotent hematopoietic stem cells to mature blood cells (Orkin, 2000). This differentiation process requires the interplay of both external signals such as cytokines and several transcription factors. Many transcription

factors are known to be essential for normal hematopoiesis. For example, AML1 is required for definitive hematopoiesis of all lineages (Okuda et al., 1996), whereas PU.1 is required for development of common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) (McKercher et al., 1996; Scott et al., 1994). Similarly, E2A (Bain et al., 1994; Zhuang et al., 1994), coupled with Ebf1 (Lin and Grosschedl, 1995), is required for commitment to the B cell pathway, whereas Pax5 is required for full commitment to the B cell pathway (Nutt et al., 1999; Rolink et al., 1999; Urbanek et al., 1994). In contrast, Gata3, Myb and Notch1 signaling are essential for the development of T cells (Allman et al., 2002; Ting et al., 1996).

BCL11A is expressed in various hematopoietic tissues including bone marrow, splenic B and T cells, monocytes, megakaryocytes (Nakamura et al., 2000), and in germinal center B cells (Lin and Grosschedl, 1995). The analysis of both BCL11A (Liu et al., 2003) and BCL11B (Wakabayashi et al., 2003a) null animals has demonstrated a role for each in hematopoiesis and postnatal development. BCL11A is essential for normal lymphoid development (Liu et al., 2003). The initiation of B lymphopoiesis in bone marrow is dependent on the functions of E2A and Ebf1 transcription factors (Schebesta et al., 2002). E2A and Ebf1 activate the expression of many B cell-specific genes, however, this activation is not sufficient to commit B cell progenitors to the B cell lineage in the absence of

Pax5, which restricts the further development of lymphoid progenitors to the B cell pathway (Nutt et al., 1999). BCL11A mutant mice failed to make $B220^+$ B cells and lacked expression of either Ebf1 or Pax5 (Liu et al., 2003), indicating that BCL11A may function upstream of Ebf1 and Pax5 in the B cell lineage commitment pathway. Moreover, BCL11A is also required for normal T cell development because mutant thymuses had abnormal ratio of $\alpha\beta$ to $\gamma\delta$ thymocytes and $CD4^+$ to $CD8^+$ thymocytes (Liu et al., 2003). Mice transplanted with BCL11A-deficient cells died from T cell leukemia derived from host, suggesting that BCL11A may also function as a non-autonomous T cell tumor suppressor gene (Liu et al., 2003).

In contrast, BCL11B is required for differentiation and survival of $\alpha\beta$ T lymphocytes (Wakabayashi et al., 2003b). The $BCL11B^{-/-}$ thymocytes demonstrated unsuccessful recombination of V_β to D_β and lacked the pre-T cell receptor (TCR) complex on the cell surface. Profound apoptosis in the thymus of neonatal $BCL11B^{-/-}$ mice was also observed, suggesting that thymocytes in $BCL11B^{-/-}$ mice are highly susceptible to apoptosis. However, loss of BCL11B does not affect the development of cells in B or $\gamma\delta$ T cell lineages (Wakabayashi et al., 2003b).

1.2 Mechanisms of transcriptional regulation in eukaryotic cells

Eukaryotic transcription is strongly influenced by the chromosomal context of the DNA template. During interphase of the cell cycle, genes are complexed with core histones and other chromosomal proteins in the form of chromatin. The basic repeating unit of chromatin is the nucleosome, consisting of two copies of each of four histones (H2A, H2B, H3, and H4) wrapped by 145-147 bp of DNA in left-handed super helical turns (Luger et al., 1997; Verreault, 2000). The shape of the nucleosome is similar to a flat disk with diameter of 11 nm and height of 6 nm, and the length of the DNA is ~34 nm circumference of the particle (Luger et al., 1997). The length of linker DNA varies from 8 bp to 114 bp per nucleosome. At metaphase of the cell cycle, chromatin fiber or solenoid (30 nm in diameter) is compactly folded into the chromosome that is comprised of two chromatids.

Histones are subject to a complex and dynamic set of covalent modification of transcription in eukaryotic cells. The histone tails (amino terminal part of histone molecules) are essential for nucleosome-nucleosome interaction (Luger et al., 1997), and for establishing transcriptionally repressive chromatin (heterochromatin; densely packed appearance in the nucleus) or transcriptionally active chromatin (euchromatin; relatively dispersed appearance in the nucleus) (Grunstein et al., 1995). The core histone tails are susceptible to various covalent

modifications (see Fig. 1.2), such as acetylation (Roth et al., 2001), phosphorylation (Cheung et al., 2000), methylation (Kaczynski et al., 2001; Lachner and Jenuwein, 2002), and ubiquitination (Jason et al., 2002). Among these covalent modifications, histone acetylation has been studied intensively regarding its function in transcriptional regulation.

Many histone acetyltransferases (HATs) have been identified. The TAF250 histone acetylase (Mizzen et al., 1996), a subunit of TFIID complex, is likely to be associated with essentially all promoters during transcription initiation. The p300/CBP histone acetylase is tightly associated with the Pol II holoenzyme (Nakajima et al., 1997), suggesting that this histone acetylase could be viewed as a more general component of the transcription machinery. The transcriptional co-activators ACTR (Chen et al., 1997) and SRC-1 (Spencer et al., 1997) associate various nuclear receptors in a hormone dependent manner. The P/CAF histone acetylase associates both with p300/CBP (Yang et al., 1996b) and with the nuclear receptor co-activators ACTR and SRC-1 (Chen et al., 1997; Chen et al., 1999; Spencer et al., 1997), thereby forming transcriptional regulatory protein complexes with multiple histone acetylases. These findings suggest that transcriptional regulation by histone acetylation is remarkably diverse.

Beyond histone acetylation, phosphorylation of histones H1 and H3 has long been implicated in chromosomal condensation during mitosis (Bradbury, 1992; Bui et al., 2004; Koshland and Strunnikov, 1996). Histone

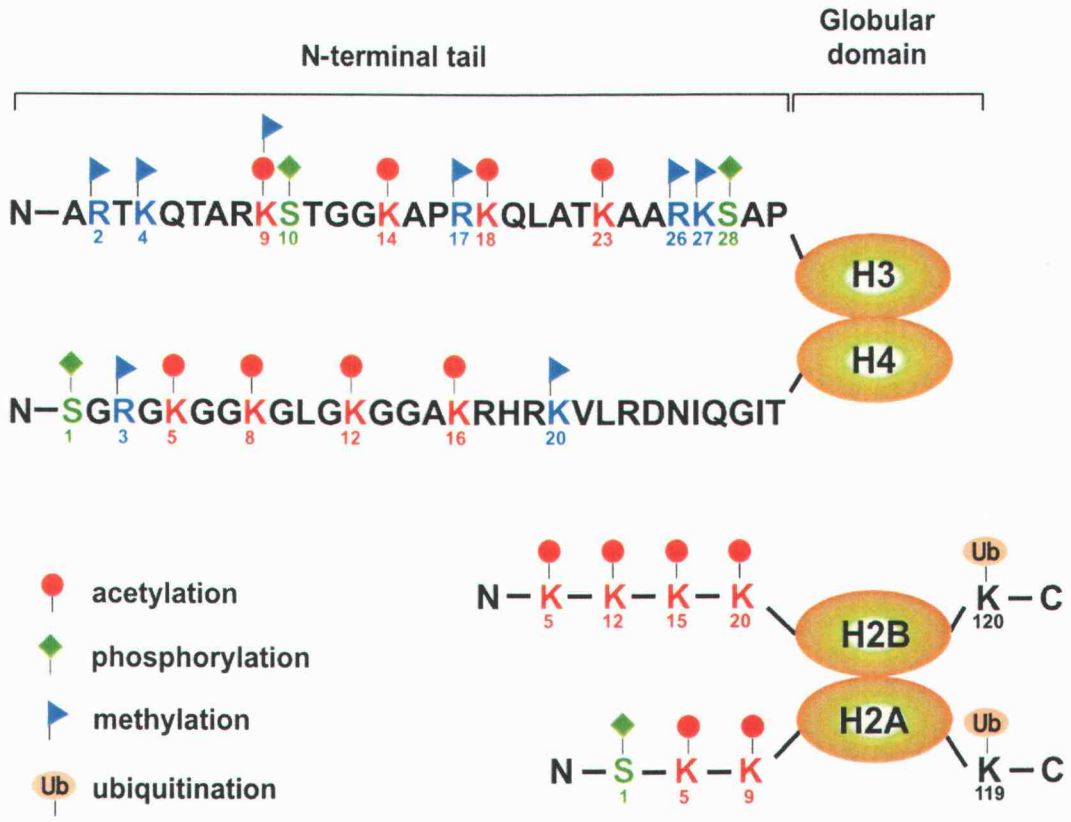


Figure 1.2. **Sites of post-translational modifications on the histone tails.**. The modifications shown include acetylation, methylation, phosphorylation, and ubiquitination. The lys 9 in the H3 tail can be either acetylated or methylated. The sites of covalent modifications shown above are based on information from the previously published report (Zhang and Reinberg, 2001).

methylation contributes to both transcriptional activation and repression (Zhang and Reinberg, 2001). PRMT1, a member of protein arginine methyltransferase (PRMTs), participates in transcriptional regulation through methylation of core histones. PRMT1-mediated methylation of H4-R3 facilitates subsequent acetylation of histone H4 by p300 (Wang et al., 2001). PRMT4/CARM1 (coactivator-associated arginine methyltransferase1) is a coactivator that participates in transcriptional regulation in the presence of the p160 family of coactivators. CARM1 and p300 have been shown to synergistically enhance transcription from the estrogen receptor (Koh et al., 2001). Unlike PRMT1 and PRMT4, PRMT5 has been shown to participate in transcriptional repression of *cyclin* E1 gene (Fabbrizio et al., 2002). Methylation of histone H3-K9 creates the binding surface for the heterochromatin-associated protein HP1 that bind and self-assemble into a supramolecular, chromatinized template, leading to transcriptional repression (Lachner et al., 2001).

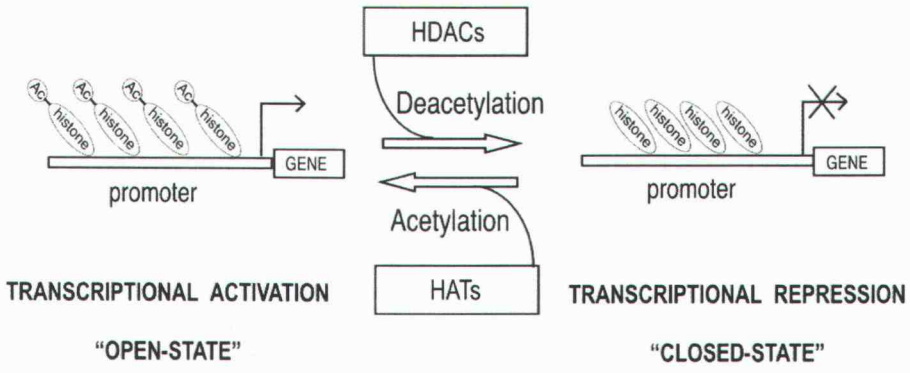
Similar to histone acetylation and phosphorylation, histone ubiquitination is a reversible modification, in which addition or removal of a group from a histone molecule results in opposing transcriptional effects. Histone ubiquitination regulates gene transcription in a positive and negative fashion, depending on its genomic and gene location. Histone H2B ubiquitination is required for telomeric and HML silencing but required for the activation of SAGA regulated genes (Zhang, 2003). The histone code

hypothesis predicts that a pre-existing modification affects subsequent modifications on histone tails, in which these modifications serve as marks for the recruitment of different proteins/protein complexes to regulate transcription, DNA replication, and chromosome segregation (Strahl and Allis, 2000).

1.3 Histone deacetylation and transcriptional repression

Transcriptional repression by sequence-specific transcription factors plays a crucial role in the regulation of diverse biological processes, including cell proliferation, development, and homeostasis (Mannervik et al., 1999). Nucleosomes act as general repressors of basal transcription by inhibition of transcriptional initiation and elongation by RNA pol II. The activation of transcriptionally repressed templates requires the actions of sequence-specific DNA-binding transcriptional activators, chromatin remodeling complexes, and histone acetyltransferases. In general, acetylation of core histone tails leads to opening of chromatin structure to allow transcription (see Fig. 1.3). Conversely, deacetylation of core histone tails by histone deacetylases (HDACs) facilitates formation of a compacted form of chromatin, making genes less accessible to transcriptional activators and/or the general transcription machinery (Struhl, 1998).

A



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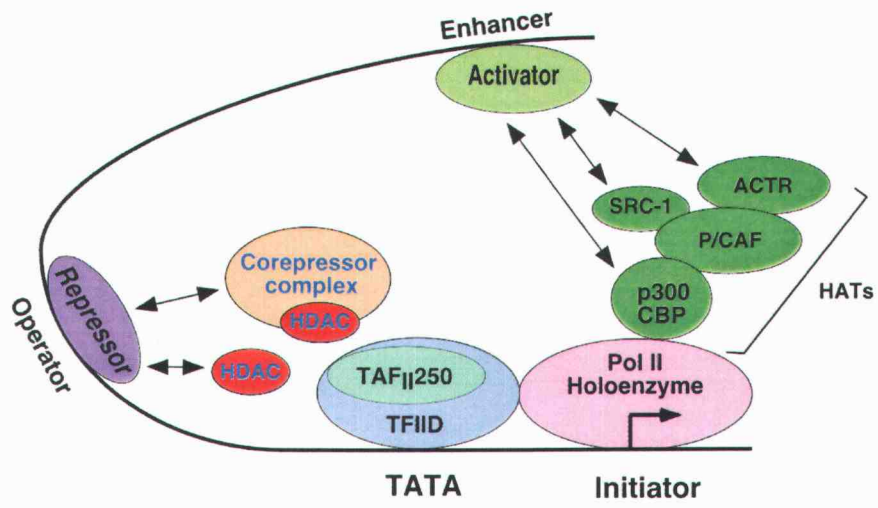

Figure 1.3. **Histone acetylation and deacetylation regulate transcription.** *A*, Histone acetylation by histone acetyltransferases (HATs) leads to transcriptional activation, whereas histone deacetylation by histone deacetylases (HDACs) leads to transcriptional repression. *B*, HDACs and HATs are associated with the Pol II transcription machinery. Relationship of HATs (green ovals) and HDACs (red ovals) with components of the basic transcriptional machinery (TFIID and Pol II holoenzyme), gene-specific activator (yellow-green oval) and gene-specific repressor (purple oval), is shown. Biochemically defined interactions are indicated by direct contact of the relevant ovals or by solid arrows.

Steady-state levels of acetylation in the core histone result from the balance between histone acetylation and histone deacetylation (Strahl and Allis, 2000; Struhl, 1998). In general, increased levels of histone acetylation are associated with transcriptional activation, whereas increased deacetylation levels are associated with repression (Grunstein, 1997; Struhl, 1998).

1.4 Histone deacetylases (HDACs)

Mammalian HDACs have been identified and divided into three classes based on their similarity to yeast enzymes. Class I HDACs including HDAC 1-3, 8, and possibly HDAC 11, are similar to yeast Rpd3, whereas class II HDACs including HDAC 4-7, 9, and 10, are similar to yeast enzyme Hda1 (De Ruijter et al., 2002; Fischer et al., 2002; Gao et al., 2002; Gray and Ekstrom, 2001). All known members of class I and class II HDACs are sensitive to inhibition by TSA to varying degrees (De Ruijter et al., 2002; Fischer et al., 2002; Fischle et al., 2001; Gao et al., 2002; Gray and Ekstrom, 2001; Zhou et al., 2001). Although Class I HDACs are currently believed to be expressed in most cell types, the expression of class II HDACs is more restricted, suggesting that the latter may be involved in cellular differentiation and developmental processes (Buggy et al., 2000; Galasinski et al., 2002). Class I HDACs have been associated with classical

transcriptional co-repressors including Sin3, NcoR/SMRT and methyl CpG-binding proteins (Wade, 2001). HDAC complexes are recruited to the nucleosomal template via specific interaction with DNA-binding repressor proteins such as MAD (Hassig et al., 1997; Laherty et al., 1997), YY1 (Yang et al., 1996a), Ume6 (Kadosh and Struhl, 1997), REST/NRSF (Roopra et al., 2000), and BTEB3 (Kaczynski et al., 2001), or with their associated co-repressors such as NcoR/SMRT (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), and Sin3A (Huang et al., 2000).

A third class of HDACs, represented by the Sir2-like proteins, are structurally and catalytically different from class I and II HDACs (De Ruijter et al., 2002; Gray and Ekstrom, 2001). Yeast Sir2 and its mammalian homologs (class III HDACs) are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases (Imai et al., 2000), which distinguishes them from class I and II HDACs. Currently, seven human homologs of class III HDACs, identified as Sir2-like proteins (Sirtuins; SIRT1-7), have been isolated (Frye, 1999; Frye, 2000). According to protein sequence homology, SIRT1 and its mouse ortholog Sir2α are the closest homologs to yeast Sir2 (Frye, 1999; Gray and Ekstrom, 2001). All mammalian class III HDACs have a sirtuin core domain or sirtuin homology domain, which contains a series of sequence motifs conserved in organisms ranging from bacteria to human (Frye, 1999; Frye, 2000). Various in vitro inhibitors of SIRT isozymes have been described, however, the catalytic activity of class III HDACs is not

inhibited by TSA, but may be inhibited by nicotinamide (NAM), a product of SIRT-mediated deacetylation reaction (Bitterman et al., 2002; Grozinger et al., 2001; Imai et al., 2000). The substrate specificity of mammalian class III HDACs has not been studied intensively, however, the mouse SIRT1 homolog (Sir2α) has been demonstrated to deacetylate lysine 9 and 14 of histone H3 and lysine 16 of histone H4 in the context of synthetic acetylated peptides (Imai et al., 2000). Although yeast Sir2 is involved in mediating the transcriptional silencing of the silent mating type (MAT) loci HML and HMR (Ivy et al., 1986), the contribution of mammalian homologs of yeast Sir2 to transcriptional repression remains to be explored. Recently, a Sir2α knockout study in mouse embryonic stem cells has demonstrated that the absence of Sir2α protein has no effect on global gene silencing (McBurney et al., 2003). However, the possibility that member(s) of mammalian class III HDACs may involve in transcriptional repression of specific genes cannot be excluded.

SIRT1 has been shown to repress p53-mediated transcriptional activation in mammalian cells (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001)., providing evidence that SIRT1, at least in part, may play a role in transcriptional repression of specific genes. In addition, SIRT1 has also been demonstrated to deacetylate p53 and attenuate p53-mediated functions (Luo et al., 2001; Vaziri et al., 2001), and to antagonize PML/p53-induced cellular senescence (Langley et al., 2002). Similarly, SIRT1 has

been found to interact with the bHLH repressors HES1 and HEY2 and involve in transcriptional repression mediated by these proteins (Takata and Ishikawa, 2003). Recently, SIRT1 has been shown to deacetylate and repress the transcriptional activity of the Forkhead transcription factors (Motta et al., 2004). Unlike SIRT1, SIRT2 is a cytoplasmic protein and has been implicated in the control of mitotic exit in the cell cycle (Dryden et al., 2003), suggesting a role for SIRT2 as a regulator of mitotic progression. The biological function(s) of the other SIRT family members remains to be explored.

1.5 Research objectives

Understanding the biological properties of CTIP1 and CTIP2 may ultimately lead to a more thorough understanding of their cellular function(s). Toward the goal of elucidating potential mechanism(s) of transcriptional repression mediated by these proteins, biochemical and molecular biological techniques described herein were employed to study the molecular mechanism(s) that may underlie the biological function(s) of these proteins in mammalian cells. The specific aims are detailed in the following paragraphs.

Chapter 2 describes studies that examine the involvement of the histone deacetylase SIRT1 in transcriptional repression mediated by CTIP2. The interaction between SIRT1 and CTIP2 was determined both in vitro and in mammalian cells. CTIP2 and SIRT1 truncation mutants were generated to identify the amino acid regions that are required for CTIP2•SIRT1 interaction. The Gal4 reporter gene assay was conducted to assess CTIP2•SIRT1-mediated transcriptional repression. The changes in levels of acetylated histones H3 and H4 at the promoter region of a reporter gene were also determined by chromatin immunoprecipitation (ChIP) assay. Column chromatography was conducted to analyze the CTIP2•SIRT1 protein complex(es).

Chapter 3 describes studies identifying the role of SIRT1 in CTIP1-mediated transcriptional repression. Co-immunoprecipitation assay was performed to determine CTIP1•SIRT1 interaction in mammalian cells. The GST-pulldown experiments were conducted to identify the interaction interface(s) of both CTIP1 and SIRT1 that are required for direct interaction between these proteins. ChIP assays were also performed in order to determine the presence of SIRT1 and the levels of acetylated histones H3 and H4 at the promoter region of a reporter gene in CTIP1-transfected cells.

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Chapter 2

Involvement of the Histone Deacetylase SIRT1 in COUP-TF-interacting Protein 2-mediated Transcriptional Repression

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2.1 Abstract

Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins 1 and 2 (CTIP1 and CTIP2) enhance transcriptional repression mediated by COUP-TF II and have been implicated in hematopoietic cell development and malignancies. CTIP1 and CTIP2 are also sequence-specific DNA binding proteins that repress transcription through direct, COUP-TF-independent binding to a GC-rich response element. CTIP1- and CTIP2-mediated transcriptional repression is insensitive to trichostatin A, an inhibitor of known class I and II histone deacetylases. However, chromatin immunoprecipitation assays revealed that expression of CTIP2 in mammalian cells resulted in deacetylation of histones H3 and/or H4 that were associated with the promoter region of a reporter gene. CTIP2-mediated transcriptional repression, as well as deacetylation of promoter-associated histones H3/H4 in CTIP2-transfected cells, was reversed by nicotinamide, an inhibitor of class III histone deacetylases such as the mammalian homologs of yeast Silent Information Regulator 2 (Sir2). The human homolog of yeast Sir2, SIRT1, was found to interact directly with CTIP2 and was recruited to the promoter template in a CTIP2-dependent manner. Moreover, SIRT1 enhanced the deacetylation of template-associated histones H3/H4 in CTIP2-transfected cells, and stimulated CTIP2-dependent transcriptional repression. Finally, endogenous

SIRT1 and CTIP2 co-purified from Jurkat cell nuclear extracts in the context of a large (1-2 mDa) complex. These findings implicate SIRT1 as a histone H3/H4 deacetylase in mammalian cells and in transcriptional repression mediated by CTIP2.

2.2 Introduction

CTIP1 (EVI9 or BCL11A) and CTIP2 (BCL11B) are two related C_2H_2 zinc finger proteins that were originally isolated and identified as COUP-TF-interacting protein (Avram et al., 2000). CTIP1 (Avram et al., 2000) and CTIP2 (Avram et al, unpublished results) both enhance COUP-TFII-mediated transcriptional repression in transfected cells independently of trichostatin A (TSA)-sensitive histone deacetylation. Both CTIPs are expressed in hematopoietic cells of lymphoid origin (Shepherd et al., manuscript in preparation), which is of interest because lymphoid-derived cells are devoid of transcripts encoding COUP-TF family members (Shepherd et al., manuscript in preparation). Thus, it is likely that CTIPs either function with other nuclear receptors in cells of lymphoid origin or act as COUP-TF-independent transcription factors in these cells. The latter appears to be true as CTIP1 and CTIP2 have been demonstrated to repress expression of a reporter gene through direct binding to a recently

identified binding site, 5′-GGCCGGAGG-3′ (upper strand) (Avram et al., 2002). This repression was observed in the absence of cotransfected COUP-TF proteins and was insensitive to reversal by TSA (Avram et al., 2002). These findings indicate that CTIP proteins may regulate transcription independently of COUP-TF proteins and TSA-sensitive HDACs in some cell types and/or promoter contexts.

CTIP1 and CTIP2 have been implicated in the etiology of both myeloid and lymphoid malignancies. Overexpression of the CTIP1 gene following proviral integration in murine hematopoietic cells results in the generation of myeloid leukemia (Nakamura et al., 2000). Similarly, dysregulation of the human CTIP1 locus (2p13) either by amplification or translocation appears to result in B cell chronic lymphocytic leukemia and immunocytoma (Satterwhite et al., 2001). The human CTIP2 locus (14q32) has been associated with a translocation event, t(5;14)(q35;q32), which results in acute T lymphoblastic leukemia (Bernard et al., 2001). More recently, CTIP2 loss of function mutations were found to contribute to mouse lymphoma genesis, leading the authors to speculate that CTIP2 may function as a tumor suppressor protein (Wakabayashi et al., 2003a). The analysis of both CTIP1- (Liu et al., 2003) and CTIP2- (Wakabayashi et al., 2003b) null animals has demonstrated a role for each in hematopoiesis and postnatal development. Although human CTIP1 and CTIP2 are expressed in early, multipotent hemopoietic progenitors, neither the physiological

function(s) nor the contribution of these proteins to neoplastic processes in these cells is known (Bernard et al., 2001; Saiki et al., 2000). Accordingly, it is important to elucidate the mechanism(s) underlying transcriptional repression mediated by CTIP proteins in order to understand the biological properties of these proteins, and ultimately, their cellular function(s).

A number of mammalian HDACs have been identified and divided into three classes based on their similarity to yeast enzymes. Mammalian class I HDACs, such as HDAC 1-3, 8, and possibly 11, are similar to yeast Rpd3, whereas class II HDACs (HDAC 4-7, 9, and 10) are most similar to the yeast enzyme Hda1 (De Ruijter et al., 2002; Fischer et al., 2002; Gao et al., 2002; Gray and Ekstrom, 2001). All known members of class I and II HDAC families are sensitive to inhibition by TSA to varying degrees (De Ruijter et al., 2002; Fischer et al., 2002; Fischle et al., 2001; Gao et al., 2002; Gray and Ekstrom, 2001; Zhou et al., 2001). Histone deacetylation is believed to promote the repressed transcriptional state by facilitating formation of a compacted form of chromatin, thereby making genes less accessible to transcriptional activators and/or the general transcription machinery (Struhl, 1998). Moreover, TSA-sensitive histone deacetylation is required for the initial steps of heterochromatin formation leading to gene silencing (Zhang and Reinberg, 2001). HDAC complexes are recruited to the nucleosomal template via specific interaction with DNA-binding repressor proteins such as Mad (Hassig et al., 1997; Laherty et al., 1997),

YY1 (Yang et al., 1996), Ume6 (Kadosh and Struhl, 1997), REST/NRSF (Roopra et al., 2000), and BTEB3 (Kaczynski et al., 2001), or with their associated corepressors such as N-CoR/SMRT (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997) and Sin3A (Huang et al., 2000).

A third class of HDACs whose members are structurally and catalytically distinct from class I and class II enzymes is represented by the Sir2-like proteins (reviewed in Refs. (De Ruijter et al., 2002; Gray and Ekstrom, 2001). Yeast Sir2 and its mammalian homologs, are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases (Imai et al., 2000), which distinguishes them from class I and II HDACs. To date, seven human homologs of class III HDACs, known as Sirtuins (SIRT1-7), have been isolated (Frye, 1999; Frye, 2000). Based on protein sequence homology, SIRT1 and its mouse ortholog Sir2α are the closest homologs to yeast Sir2 (Frye, 1999; Gray and Ekstrom, 2001). Although the substrate specificity of mammalian sirtuins has not been rigorously investigated, mouse Sir2α has been demonstrated to deacetylate lysine 9 and 14 of histone H3 and lysine 16 of H4 in the context of synthetic, acetylated peptides (Imai et al., 2000). SIRT1 has also been demonstrated to deacetylate p53 and attenuate p53-mediated functions (Luo et al., 2001; Vaziri et al., 2001), and to antagonize PML/p53-induced cellular senescence (Langley et al., 2002). In addition, SIRT1 has been shown to repress p53-mediated transcriptional activation in mammalian cells

(Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), providing evidence that SIRT1, at least in part, may play a role in transcriptional repression of specific genes. Although various *in vitro* inhibitors of SIRT isozymes have been described, the catalytic activity of class III HDACs is not inhibited by TSA but may be inhibited by nicotinamide, a product of the SIRT-mediated deacetylation reaction (Bitterman et al., 2002; Grozinger et al., 2001; Imai et al., 2000).

Although CTIPs repress transcription in a TSA-insensitive manner (Avram et al., 2000; Avram et al., 2002), we found that transient transfection of CTIP2 resulted in deacetylation of histone H3 and/or H4 that were associated with the promoter region of a target gene. Subsequently, we found that nicotinamide reversed both CTIP2-mediated transcriptional repression and deacetylation of promoter-associated histone H3/H4 in CTIP2-transfected cells. These findings led us to investigate the possibility that a class III HDAC may be involved in CTIP-mediated transcriptional repression. CTIP2 and the class III HDAC SIRT1 co-immunoprecipitated from extracts of transfected, co-transfected and untransfected cells, and the two proteins were shown to interact directly in vitro. Moreover, SIRT1 potentiated both transcriptional repression and histone H3/H4 deacetylation in cells transfected with CTIP2. These findings suggest a role for the NAD^+-dependent histone deacetylase SIRT1 in the transcriptional repression activity of CTIP2 in mammalian cells.

2.3 Materials and Methods

2.3.1 Constructs

The $(17\text{-mer})_4$-tk-CAT reporter construct was a kind gift from Dr. Ming-jer Tsai (Baylor College of Medicine). Flag-CTIP2 construct was prepared by PCR amplification of the CTIP2 open reading frame (Avram et al., 2000) with appropriate primers and insertion into pcDNA3(+) (Invitrogen). The Gal4 DBD-CTIP2 construct was prepared by PCR amplification with appropriate primers followed by insertion into a commercially available vector (pM; Clontech). Myc-SIRT1, Myc-SIRT1 H363Y and GST-SIRT1 constructs (Langley et al., 2002) were kind gifts from Dr. T. Kouzarides (University of Cambridge, Cambridge, UK). All vectors encoding GST fusion proteins were prepared by PCR amplification of appropriate templates followed by insertion into pGEX-2T (Amersham Pharmacia Biotech). 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). All constructs were verified by complete DNA sequencing.

2.3.2 Antibodies

The rabbit anti-SIRT1 antibody was described previously (Langley et al., 2002). Purified rabbit anti-Sir2α (which cross-reacts with human SIRT1; data not shown), and anti-acetylated-histone H3 and -histone H4 were obtained from Upstate. Mouse anti-Flag and -Myc monoclonal antibodies were purchased from Sigma and Oncogene, respectively. The rat anti-CTIP2 monoclonal antibody (25B6) was raised against a recombinant GST fusion protein by Dr. Michael Marusich (Monoclonal Antibody Facility, Institute for Neuroscience, University of Oregon, Eugene, Oregon). Epitope mapping has revealed that the 25B6 anti-CTIP2 monoclonal antibody recognizes an antigen within the 150 amino acids located at the extreme amino terminus of the protein (CTIP2 1-150; unpublished results)

2.3.3 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed on transfected cells essentially as described by the Dean laboratory (Luo et al., 1998) with slight modifications. HEK293 cells were co-transfected at 60% confluency (10 cm plates) with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter, 1.5-10 μg of Gal4-CTIP2, 0.5 μg of Myc-SIRT1, and/or the parent control vectors using the calcium

phosphate method. After 48 h, cells were washed twice with phosphate buffered saline (PBS) and cross-linked with 1% formaldehyde in PBS at room temperature. Cells were washed twice with ice-cold PBS buffer and collected in harvesting buffer (100 mM Tris-HCl, pH 9.4 containing 10 mM DTT). The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.1 containing 1% SDS, 10 mM EDTA, and a protease inhibitor cocktail). The sonicated lysates were then cleared by centrifugation and diluted 2.5-fold with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1 containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and a protease inhibitor cocktail). One tenth of the diluted lysate was reserved as an input sample to determine total amount of reporter plasmid in transfected cells for subsequent normalization procedures. Two equal aliquots of the remaining lysate were used for immunoprecipitation with and without the addition of antibodies against K9-, K16- di-acetylated histone H3, and K5-, K8-, K12-, K16-tetra-acetylated histone H4 (Upstate; 5 μg of each antibody per immunoprecipitation reaction). Immune complexes were recovered with Protein A sepharose (Amersham Pharmacia) and washed under stringent conditions. Chromatin complexes were eluted with the freshly prepared elution buffer (0.1 M $NaHCO_3$ containing 1% SDS). The eluates and the above input samples were subjected to an overnight reversal of cross-links at 65°C, followed by a treatment with Proteinase K at 45°C for 1 h. DNA was recovered by using a Nucleospin PCR Extraction Kit (Clontech) and

amplified using a forward primer (5′-GGCATCAGAGCAGATTGTACT-3′) upstream of the multimerized 17-mer and a reverse primer (5′-CCTTAGCTCCTGAAAATCTCG-3′) downstream of the tk promoter but upstream of the transcriptional start site. The resulting PCR product (327 bp) was analyzed by agarose gel electrophoresis and ethidium bromide staining. Experiments were performed three to five times.

2.3.4 Transfection and reporter

HEK293 cells were transfected and harvested as described above. Where indicated, TSA (100 ng/mL) and nicotinamide (10 mM) treatments were initiated 24 h after transfection, and cells were harvested 24 h later. A β-galactosidase expression vector (pCMV-Sport-βGal, Life Technologies) was cotransfected as an internal control, and β-galactosidase activity was used to normalized CAT activity as described (Dowell et al., 1997a).

2.3.5 Coimmunoprecipitation

HEK293 cells were transfected as described above with 10 μg each of expression vectors encoding Flag-CTIP2 and/or Myc-SIRT1. Forty-eight hours after transfection the cells were lysed in NET-N buffer (20 mM Tris-HCl, pH 8 containing 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM

EDTA, and a protease inhibitor cocktail) by agitation at 4°C for 30 min. After a brief sonication, lysates were cleared by centrifugation, and immunoprecipitated as described previously (Nevrivy et al., 2000) using the antibodies described above. Nuclear extracts from Jurkat cells were prepared using standard techniques (Dignam et al., 1983), and immunoprecipitated (500 μg of protein per reaction) with purified anti-CTIP2 monoclonal (2 μg) or anti-Sir2α (0.5 - 2.5 μg) antibodies. All immunoprecipitates were analyzed by immunoblotting with appropriate antibodies.

2.3.6 GST pulldown experiment

GST pulldown experiments were conducted as described previously (Dowell et al., 1997b). Briefly, equivalent amounts of GST or GST-SIRT1 fusion proteins were bound to glutathione-sepharose (Pharmacia) and incubated with [^{35}S]methionine-labeled proteins (CTIP2 or CTIP2 truncation mutants) prepared using TNT transcription-translation system (Promega). The reactions were washed five times with binding buffer (10 mM Na-HEPES containing 10% glycerol, 1 mM EDTA, 1 mM DTT, 150 mM NaCl and 0.05% NP-40) and bound proteins were eluted and resolved on denaturing SDS-PAGE gels for analysis by autoradiography.

2.3.7 Column chromatography

Nuclear extracts were prepared from 30 L of Jurkat cells (~1.5 x 10^{10} cells; provided by the National Cell Culture Center, Minneapolis) using the Dignam method (Dignam et al., 1983) with the following minor modifications: Buffer C contained 720 mM NaCl instead of 420 mM, and Buffer D was replaced with Buffer 100 (20 mM HEPES, pH 7.9; 100 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT and 10% glycerol). CTIP2 immunoreactivity was found only in the nuclear fraction using this protocol (data not shown). Jurkat nuclear extract (100 mg protein) was applied to a 1.5 x 8 cm phosphocellulose P11 column (Whatman) that had been equilibrated in Buffer 100 and running at 80 mL/hr. The column was washed with Buffer 100 until the A_{280} returned to baseline and then eluted in a stepwise fashion with Buffer 100 containing 300, 600, and 1000 mM NaCl. The A_{280} of the eluate was allowed to return to baseline at each step. CTIP2 and SIRT1 immunoreactivities were analyzed in the step fractions by immunoblotting with appropriate antibodies and chemiluminescence detection. The 600 mM step off the phosphocellulose column (16 mg of protein), was dialysed against Buffer 100 and loaded onto a 2.5 mL DEAE-Biogel A column (Biorad) that had been equilibrated with Buffer 100. After collecting the flow-through fraction, the column was eluted with Buffer 100 containing 200, 400, and 1000 mM NaCl in a stepwise fashion, and the steps were analyzed by immunoblotting as described above. The 200 mM

step off the DEAE column, which contained both CTIP2 and SIRT1 immunoreactivity, was concentrated to 1 mL (3.1 mg of protein) using a Milipore Ultrafree centrifugal filter device (30 kDa nominal molecular weight limit), and loaded onto a 1.6 x 90 cm Superose 6 size exclusion column (Pharmacia) that was running at a flow rate of 0.4 mL/min. The Superose 6 column had been previously equilibrated with Buffer 100 and calibrated with protein standards (thyroglobulin 669 kDa; catalase, 232 kDa; bovine serum albumin, 67 kDa; blue dextran, 2000 kDa; all from Pharmacia). Fractions (5.0 min., 2.0 mL) were analyzed for the presence of CTIP2 and SIRT1 by immunoblotting.

2.4 Results

2.4.1 CTIP2-mediated transcriptional repression is partially reversed by nicotinamide, an inhibitor of NAD^+-dependent deacetylases

Previous studies indicated that CTIP1-mediated transcriptional repression was independent of TSA-sensitive histone deacetylation (Avram et al., 2000; Avram et al., 2002). In the present study, transcriptional repression mediated by a Gal4-CTIP2 fusion protein was found to be similarly insensitive to reversal with TSA in HEK293 cells (Fig. 2.1A, compare *lanes* 4 and 5 with 7 and 8). Considered together, these findings

suggest that neither CTIP1- nor CTIP2-mediated transcriptional repression involves recruitment of class I or class II TSA-sensitive HDACs to the template. However, nicotinamide (NAM), an inhibitor of NAD^+-dependent, class III HDACs, such as the SIRT family of proteins (Frye, 1999; Frye, 2000), was found to inhibit CTIP2-mediated transcriptional repression, at least partially (~70% reversal of repression; Fig. 2.1A, compare lanes 7 and 9 with lane 4).

These data indicated a possible role of a nicotinamide-sensitive HDAC(s) in the transcriptional repression mechanism of CTIP2 in mammalian cells. If true, transfection of Gal4-CTIP2 would be expected to result in recruitment of a nicotinamide-sensitive HDAC(s) to the template and deacetylation of template-associated histones. This hypothesis was tested by conducting chromatin immunoprecipitation (ChIP) studies in transiently transfected cells. Although the nature and/or extent of chromatin formation on transiently transfected templates likely differs from that of

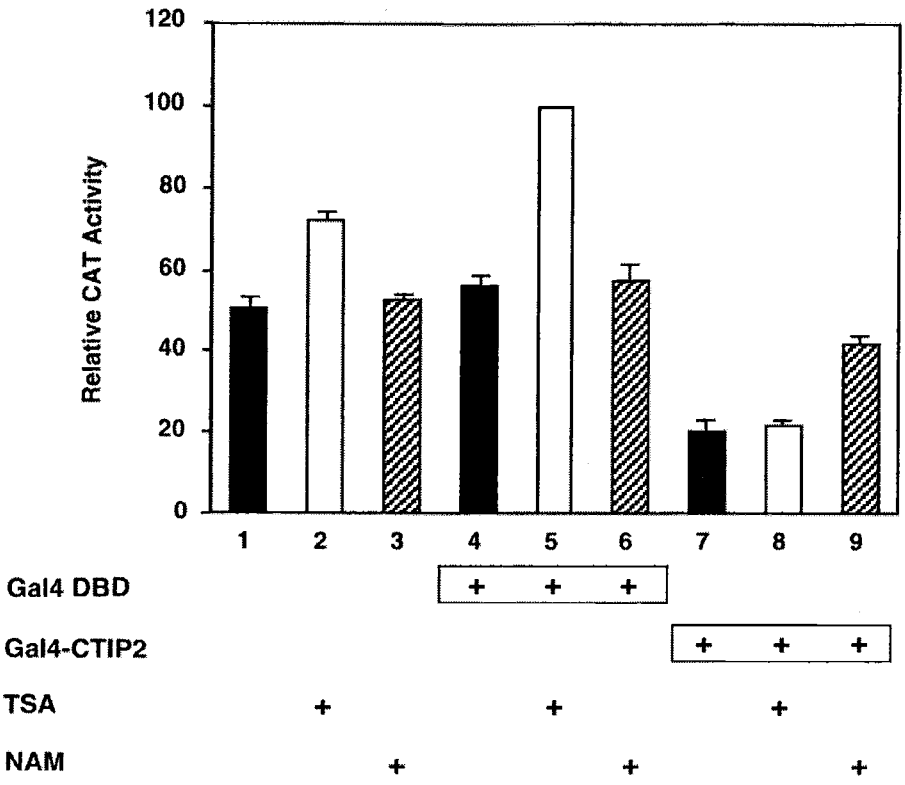


Figure 2.1. **CTIP2-mediated transcriptional repression is partially reversed by nicotinamide, an inhibitor of NAD^+-dependent deacetylases.** HEK293 cells were transiently transfected with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with 1.5 μg of expression vectors encoding either Gal4-CTIP2 or Gal4 DBD as indicated. Twenty-four hours after transfection, cells were treated or not (solid bars) with histone deacetylase inhibitors, TSA (100 ng/ml; open bars) and nicotinamide (NAM, 10 mM; hatched bars), for 24 h before collection. Transfection efficiency was normalized by β-galactosidase activity produced by a cotransfected β-galactosidase expression vector. CAT activity determined in the presence of Gal4 DBD and TSA (lane 5) was taken to be maximal and that against which all other determined CAT activities were compared. The results presented represent the mean (± S.E.M.) of three independent experimental determinations.

chromosomal genes, Dean and colleagues have previously validated ChIP studies in transiently transfected cells and used this approach to demonstrate HDAC-dependent and -independent transcriptional repression mediated by the Rb protein (Luo et al., 1998). Thus, a similar approach was employed in the present study toward the goal of determining if histone deacetylation may underlie CTIP2-mediated transcriptional repression in mammalian cells. Alterations in the levels of acetylated histone H3/H4 associated with the template (a multimerized 17mer-tk-CAT construct) were not observed at the lowest amount of Gal4-CTIP2 transfected (upper panel of Fig. 2.2, compare lanes 10 with 12). However, transfection of increasing amounts of Gal4-CTIP2 dramatically reduced the level of acetylated histone H3/H4 associated with the template (upper panel of Fig. 2.2, compare lanes 10, 12, 14, 16), and this was unaffected by treatment of the transfected cells with TSA (lanes 10-14 of the middle panel of Fig. 2.2). However, the deacetylation of template-associated histones observed in CTIP2-transfected cells was partially reversed by treatment of the cells with nicotinamide (compare lanes 10-14 of the top and bottom panels of Fig. 2.2). Although inhibition of the deacetylation of promoter-associated histone H3 and/or H4 by treatment of the CTIP2-transfected cells with 10 mM nicotinamide was obvious, complete reversal was not observed (compare lanes 10 and 16 of the bottom panel of Fig. 2.2). This

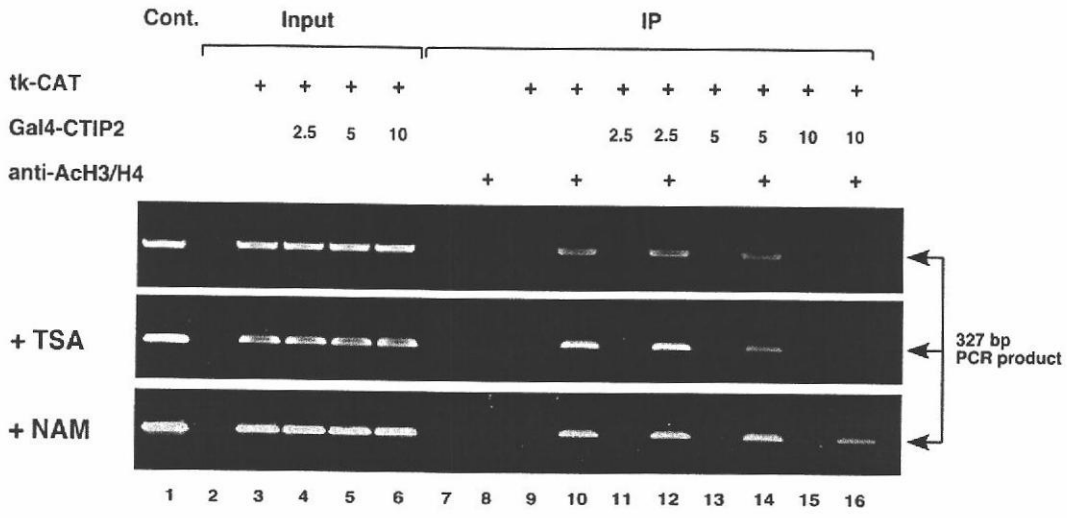


Figure 2.2. **Nicotinamide-sensitive histone deacetylation underlies CTIP2-mediated transcriptional repression.** HEK293 cells were transfected with 5 μg of $(17\text{-mer})_4$-tk-CAT reporter and increasing amounts (2.5, 5.0, and 10 μg) of an expression vector encoding Gal4-CTIP2 as indicated. The treatments with TSA and NAM were carried out as described above. Transfection efficiency was normalized by total amount of the transfected $(17\text{-mer})_4$-tk-CAT reporter as determined by PCR amplification (input lanes 3-6; 2.5% of total). Lanes 7-16 represent template amplification reactions from samples immunoprecipitated with or without antibodies specific for acetylated histone H3/H4 as indicated. Amplification reactions were separated on a 1% agarose gel that was stained with ethidium bromide to visualize DNA products. The indicated band is the expected, 327 bp amplification product from the reporter gene template. Lane 1 corresponds to a positive control in which the reporter plasmid was used as the template. Results are representative of three independent experiments.

may be due to the relative lack of efficacy of nicotinamide as an inhibitor of NAD^+-dependent deacetylases and/or insufficient intracellular levels of nicotinamide in treated cells. Concentrations of nicotinamide above 10 mM appeared to result in cellular toxicity, thus, precluding the use of very high levels of nicotinamide. Nonetheless, these findings, which are consistent with transcriptional repression studies (Fig. 2.1), further demonstrate that nicotinamide-sensitive histone deacetylation may underlie the mechanistic basis of CTIP2-mediated transcriptional repression in transiently transfected HEK293 cells.

2.4.2 CTIP2 interacts with and recruits SIRT1 to the promoter template in mammalian cells

The above results indicate that a member(s) of class III HDACs may be associated with and mediates the transcriptional repression activity of CTIP2 in mammalian cells. To investigate this, co-immunoprecipitation experiments were conducted using extracts prepared from HEK293 cells transiently transfected with an expression vector encoding Flag epitope-tagged CTIP2 (Flag-CTIP2). Flag-CTIP2 was immunoprecipitated with endogenous SIRT1 by anti-SIRT1 antibody (Fig. 2.3A, lane 6) but not by pre-immune serum (lane 5). This finding demonstrates that transfected CTIP2 directly or indirectly interacts with endogenous SIRT1 in HEK293 cells.

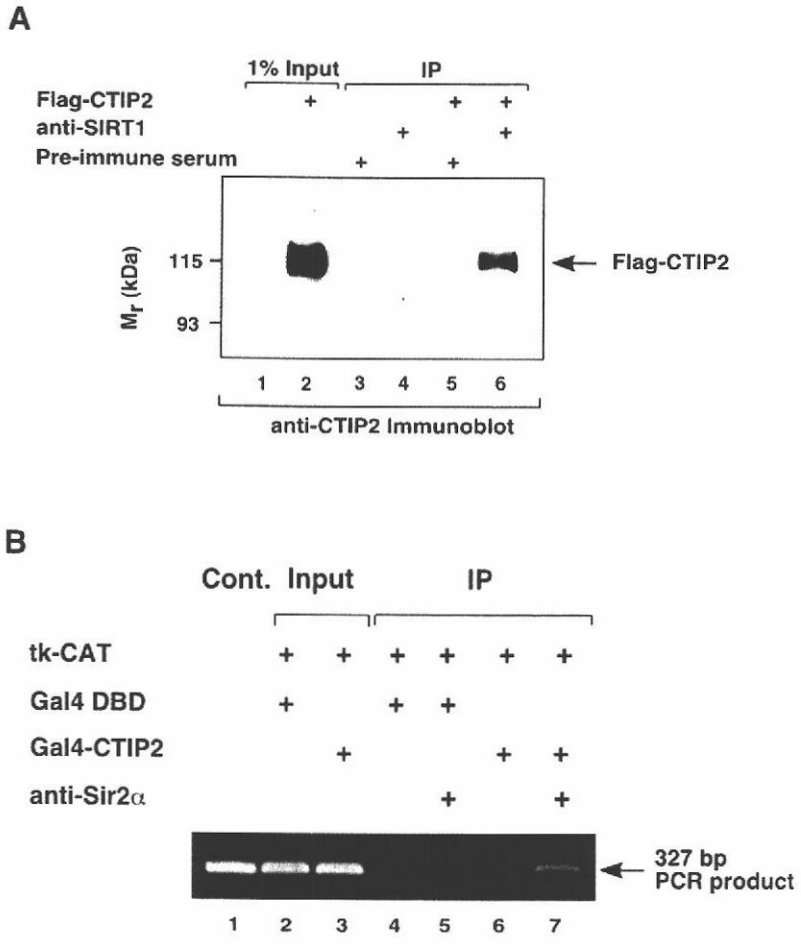


Figure 2.3. **CTIP2 interacts with and recruits SIRT1 to the promoter template in mammalian cells.** *A*, Flag-CTIP2 coimmunoprecipitates with endogenous SIRT1 from HEK293 cell lysates. Whole cell extracts from HEK293 cells, untransfected and transiently transfected with expression vectors encoding Flag-CTIP2, were immunoprecipitated with either anti-SIRT1 or pre-immune sera, and the immunocomplexes were analyzed by immunoblotting with anti-CTIP2 antibody. The position of Flag-CTIP2 is indicated. *B*, Endogenous SIRT1 is recruited to promoter template of the reporter gene upon expression of Gal4-CTIP2. HEK293 cells were transfected with 5 μg of $(17\text{-mer})_4$-tk-CAT reporter and 10 μg of expression vectors encoding either Gal4-CTIP2 or Gal4 DBD. Transfection efficiency was normalized as described in the legend of Fig. 2.2 (input lanes 2 and 3; 2.5% of total). Lanes 4-7 represent template amplification reactions from samples immunoprecipitated with or without antibody directed against SIRT1. Results are representative of three independent experiments.

ChIP experiments were conducted to determine if endogenous SIRT1 was recruited to the promoter template in cells transfected with a Gal4-CTIP2 expression vector. SIRT1 was not associated with the template in cells transfected with Gal4 DBD (Fig. 2.3B, lane 5). However, recruitment of endogenous SIRT1 to the template was apparent in cells expressing Gal4-CTIP2 (Fig. 2.3B, lane 7). These data demonstrate CTIP2-dependent recruitment of endogenous SIRT1 to the promoter template in HEK293 cells, and further suggest a role for this histone deacetylase in the transcriptional repression mechanism of CTIP2.

2.4.3 SIRT1 enhances CTIP2-mediated transcriptional repression

Reporter gene assays were carried out in transiently transfected HEK293 cells to determine if SIRT1 enhances CTIP2-mediated transcriptional repression. First, the interaction of the co-overexpressed proteins was verified by co-immunoprecipitation experiments using Flag-CTIP2 and Myc-SIRT1. Flag-CTIP2 was immunoprecipitated by the anti-Myc antibody but only when Myc-SIRT1 was co-expressed (compare lanes 5 and 6 of Fig. 2.4*A*). Similarly, Flag-CTIP2 was immunoprecipitated with a Myc-tagged, catalytically inactive SIRT1 point mutant, Myc-SIRT1 H363Y (Langley et al., 2002; Luo et al., 2001), to an extent similar to that of the wild-type protein (lane 6 of Fig. 2.4B*)*. These findings,

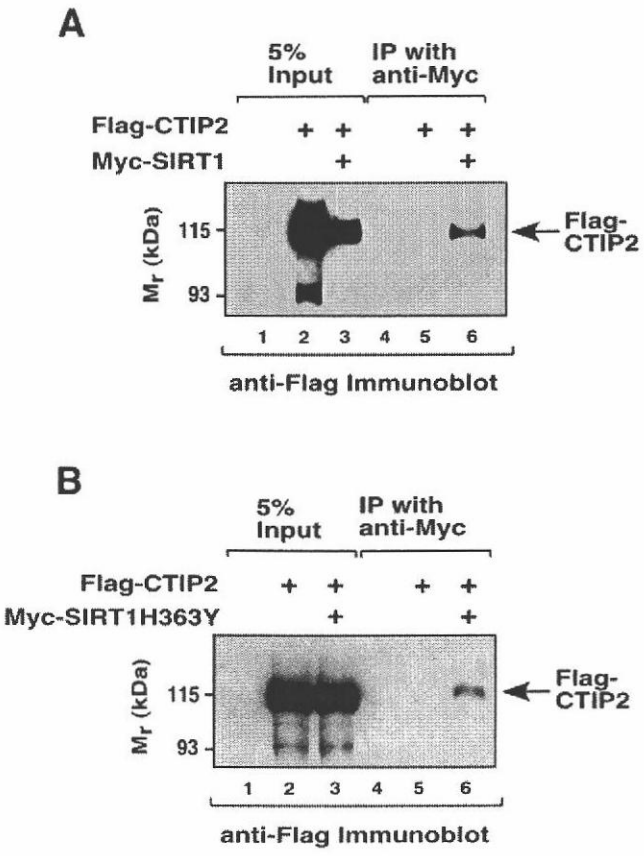


Figure 2.4. **CTIP2 interacts with SIRT1 in HEK 293 cells.** *A*, Flag-CTIP2 coimmunoprecipitates with Myc-SIRT1. Whole cell extracts from HEK293 cells, untransfected and transiently transfected with the indicated expression vectors, were immunoprecipitated with anti-Myc monoclonal antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by western blotting with anti-Flag monoclonal antibody that detects Flag-CTIP2. The position of Flag-CTIP2 is indicated. *B,* Flag-CTIP2 coimmunoprecipitates with the catalytically inactive Myc-SIRT1 H363Y. Transfections, immunoprecipitations, and immunoblots were conducted as described under *A*.

which are consistent with results presented in Figs. 2.3*A* and *B*, confirm that transfected CTIP2 interacts with both wild-type SIRT1 and a catalytically inactive form, SIRT1 H363Y, in mammalian cells. The functional consequence of these interactions was investigated in cells transiently co-transfected with expression vectors encoding Gal4-CTIP2, the $(17\text{-mer})_4$-tk-CAT reporter, and either Myc-SIRT1 WT or SIRT1 H363Y. Both wild-type SIRT1 and SIRT1 H363Y repressed reporter gene expression in a concentration-dependent manner in the absence of Gal4-CTIP2 (Fig. 2.5, lanes 3-5 and 7-9, respectively). Although the mechanistic basis for recruitment of SIRT1 to the template under these conditions is unknown, these findings, which are consistent with previous reports (Langley et al., 2002), demonstrate that the catalytic activity of the enzyme is not required for repression of the basal level of reporter gene expression. In contrast, the catalytic activity of SIRT1 was required for enhancement of CTIP2-mediated transcriptional repression. Co-transfection of wild-type SIRT1 stimulated transcriptional repression mediated by Gal4-CTIP2 in a concentration-dependent (Fig. 2.5, lanes 10-13) but TSA-insensitive (data not shown) manner, whereas SIRT1 H363Y did not enhance CTIP2-mediated repression (lanes 14-17). These findings demonstrate that the co-transfected SIRT1 stimulates the transcriptional repression activity of CTIP2 and this requires the catalytic activity of the enzyme.

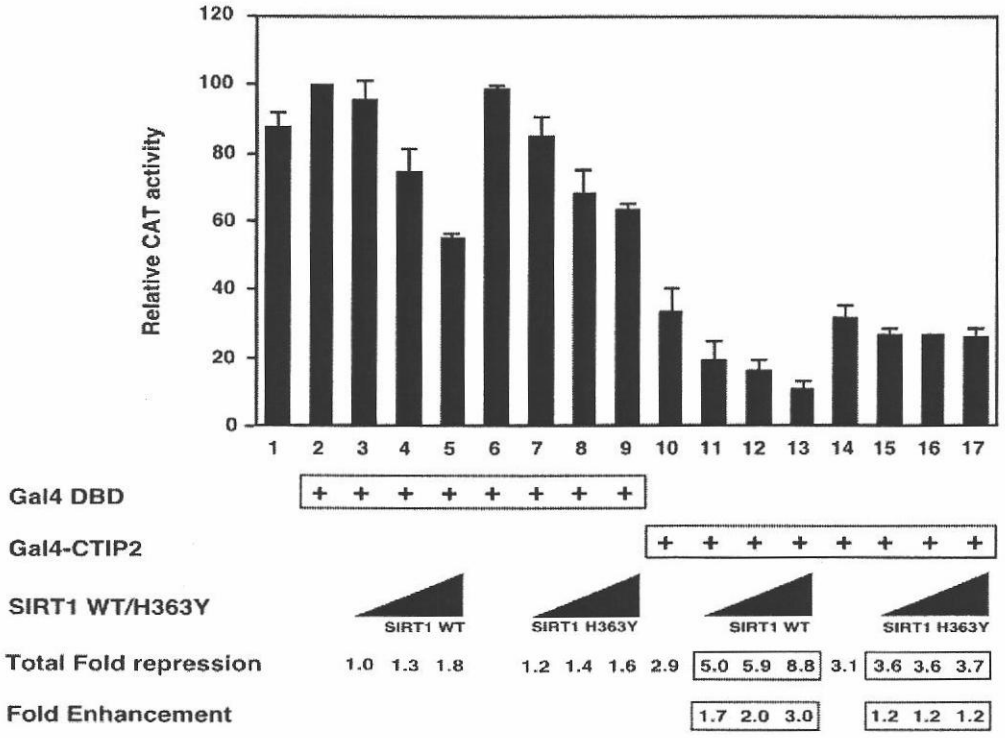


Figure 2.5. **SIRT1 enhances CTIP2-mediated transcriptional repression.** Wild-type SIRT1, but not SIRT1 H363Y, stimulates CTIP2-mediated transcriptional repression. HEK293 cells were transiently transfected with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with 1.5 μg of expression vectors encoding either Gal4-CTIP2 or Gal4 DBD, and increasing amounts (0.125, 0.25, and 0.5 μg) of expression vectors encoding either SIRT1 WT or SIRT1 H363Y, as indicated. Transfection efficiency was normalized as described in the legend of Fig. 2.1. The activity of the CAT reporter in the presence of Gal4 DBD alone (lane 2) was taken to be maximal and that against which all other determined CAT activities were compared. The results presented represent the mean (± S.E.M.) of three independent experimental determinations.

ChIP assays were conducted to determine if histone deacetylation may underlie the molecular basis for the observed stimulation of CTIP2-mediated transcriptional repression by SIRT1. The amounts of transfected expression vectors used in these studies were titrated downward to levels at which neither CTIP2 (upper panel of Fig. 2.6, lane 12; 1.5 μg transfected) nor SIRT1 (upper panel, lane 14; 0.5 μg transfected) independently affected the acetylation level of promoter-associated histone H3/H4 to an appreciable degree. This was necessary as both Gal4-CTIP2 (Fig. 2.2) and SIRT1 (data not shown) individually decreased acetylation of histone H3/H4 when transfected at higher levels. Under the experimental conditions employed, co-transfection of Gal4-CTIP2 and SIRT1 resulted in a large decrease in the level of acetylated histone H3/H4 that was associated with the reporter gene template and this effect was clearly greater than that observed by transfection of either expression vector individually (compare lanes 10, 12, 14, and 16 of the upper panel of Fig. 2.6). The catalytically inactive point mutant, SIRT1 H363Y, did not enhance deacetylation of promoter-associated histones H3/H4 in cells transfected with Gal4-CTIP2 (compare lanes 10, 12, 14, and 16 of the bottom panel of Fig. 2.6). These findings suggest that SIRT1-catalyzed histone deacetylation may underlie, at least in part, the ability of the enzyme to stimulate CTIP2-mediated transcriptional repression.

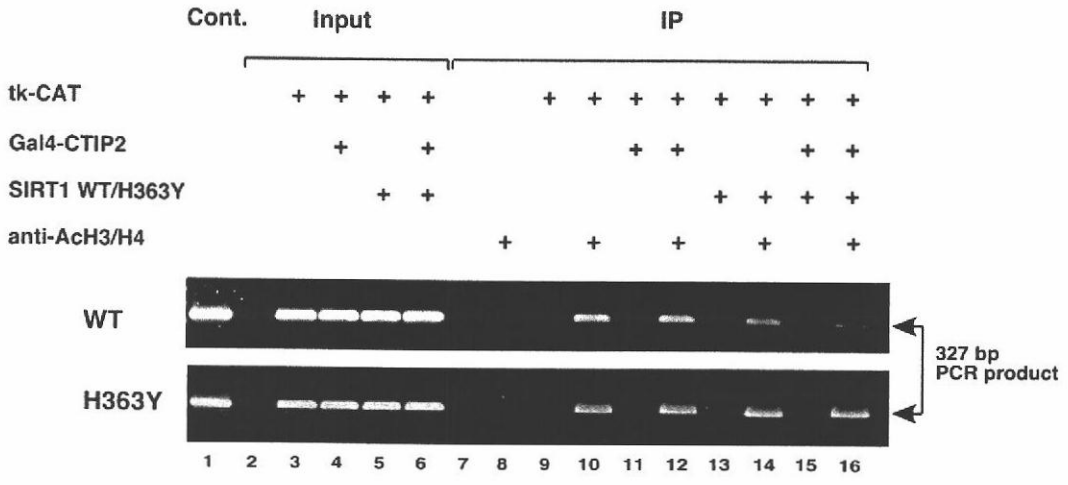


Figure 2.6. **SIRT1 stimulates deacetylation of template-associated histones H3 and/or H4 in CTIP2-transfected cells.** HEK293 cells were transfected with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with expression vectors encoding Gal4-CTIP2 (1.5 μg) and SIRT1 WT or SIRT1 H363Y (0.5 μg) as indicated. Acetylated histones H3 and H4 were determined by a ChIP assay as described in experimental procedures. Transfection efficiency was normalized as described in the legend of Fig. 2.2. Input lanes (3-6) correspond to amplification reactions conducted using 3.75% (upper panel) and 1.0% (lower panel) of the lysates used for IP reactions. Lanes 7-16 represent template amplification reactions from samples immunoprecipitated with or without anti-acetylated histone H3/H4 antibodies as indicated. Results are representative of three independent experiments.

2.4.4 CTIP2 interacts directly with SIRT1 *in vitro*

The co-immunoprecipitation experiments presented above (Figs. 2.3A, 2.4A, and 2.4B) indicated that CTIP2 and SIRT1 associate with similar complexes in transfected cells. However, these studies are incapable of distinguishing between direct physical interaction and indirect association resulting from interaction with a common, intermediary protein(s) within a complex of proteins. Thus, in vitro GST pulldown experiments were conducted to determine if CTIP2 interacts directly with SIRT1. Full-length CTIP2 (CTIP2 1-813) was found to interact with full-length SIRT1 fused to GST (Fig. 2.7, lane 3), but not with GST alone (lane 2). These results demonstrate that CTIP2 and SIRT1 participate in a direct, physical interaction. The use of a series of CTIP2 deletion mutants (see Fig. 2.7) revealed that the SIRT1 interaction interface is contained within CTIP2 amino acids 171-350 (Fig. 2.7, lane 3 of panel E). All CTIP2 deletion mutants containing this region strongly interacted with GST-SIRT1 (Fig. 2.7, lane 3 of panels A, B, D, and E) but mutants lacking it interacted weakly or not at all (Fig. 2.7, lane 3 of panels C and F-I). Thus, CTIP2 171-350, a region that is relatively rich in proline, but devoid of C_2H_2 zinc finger motifs (Avram et al., 2000), appears to be primarily responsible for interaction with SIRT1 in vitro.

The CTIP2 interaction interface of SIRT1 was similarly mapped by deletion mutagenesis (Fig. 2.8*A*). Based on the crystal structure of

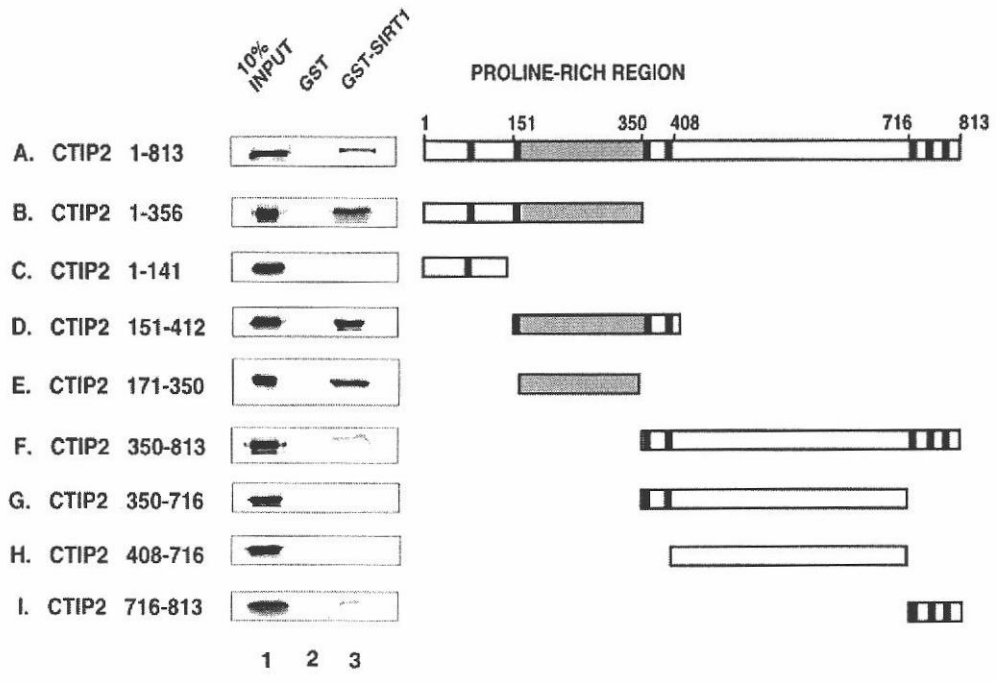


Figure 2.7. **The proline-rich region of CTIP2 mediates the interaction with SIRT1 *in vitro*.** In vitro translated and [^{35}S]Met-labeled full-length CTIP2 and truncation CTIP2 mutants were incubated with equivalent amounts of bacterially expressed GST (lane 2) or GST-SIRT1 fusion protein (lane 3). After extensive washing, [^{35}S]Met-labeled CTIP2 associated with the affinity resin was determined by SDS-PAGE and autoradiography. Input [^{35}S]Met-labeled proteins are shown in lane 1. CTIP2 truncation mutants used in these studies (panels B-I) are schematically represented on the right with zinc finger motifs denoted by vertical bars. Results are representative of 3-5 independent experiments.

SIRT2 (Finnin et al., 2001), we generated three deletion mutants of SIRT1 (see Fig. 2.8A): the amino terminal region, the centrally located sirtuin homology domain, and the carboxyl terminus, all of which were fused to GST for use in in vitro pulldown experiments. CTIP2 171-350 weakly interacted with the amino terminal region of SIRT1 (residues 1-214; Fig. 2.8B, lane 4) but did not interact with the carboxyl terminus (residues 541-747; lane 6). However, CTIP2 171-350 interacted strongly with the SIRT1 sirtuin homology domain (residues 214-541; Fig. 2.8B, lane 5). Two additional deletion mutants within the sirtuin homology domain were constructed to map the CTIP2 interaction interface more precisely, SIRT1 214-441 and SIRT1 441-541 (see Fig. 2.8A). CTIP2 171-350 was found to interact primarily with SIRT1 214-441 (Fig. 2.8B, lane 7), and less so with SIRT1 441-541 (lane 8). Thus, these results suggest that CTIP2 interacts primarily with a portion of the SIRT1 sirtuin homology domain that overlaps, at least partially, with the catalytic domain of the enzyme. Collectively, these results suggest that CTIP2 and SIRT1 interact directly in vitro and this interaction requires the proline-rich region of CTIP2 (amino acids 171-350) and residues within the amino terminal part of the sirtuin homology domain of SIRT1.

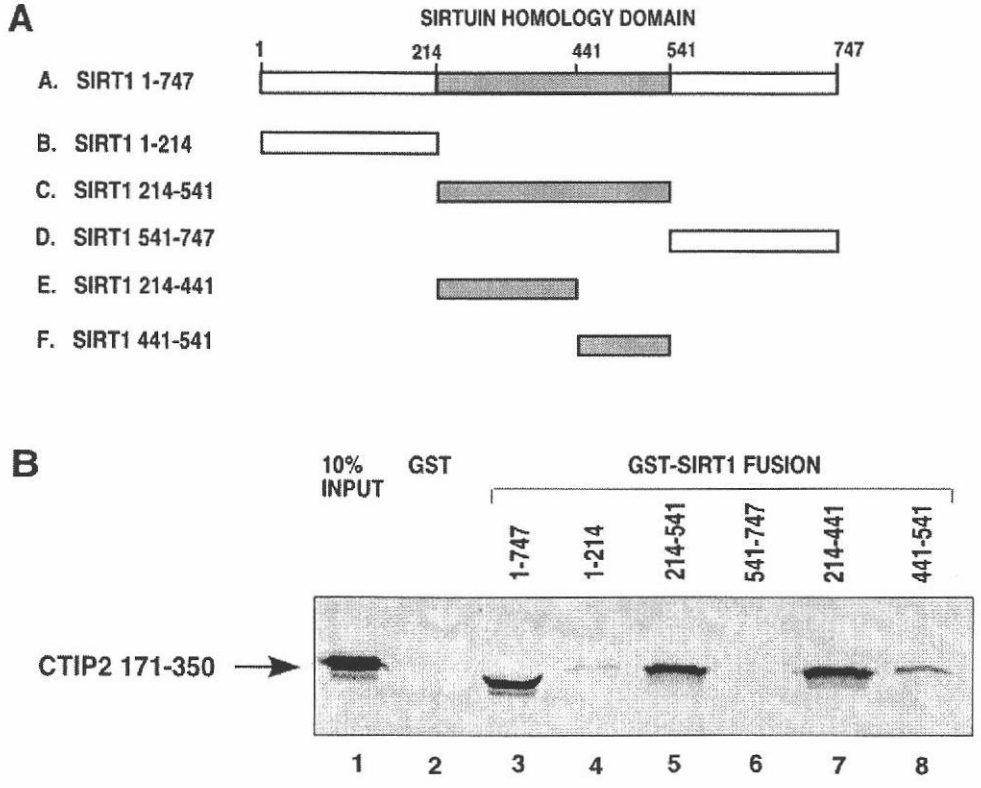


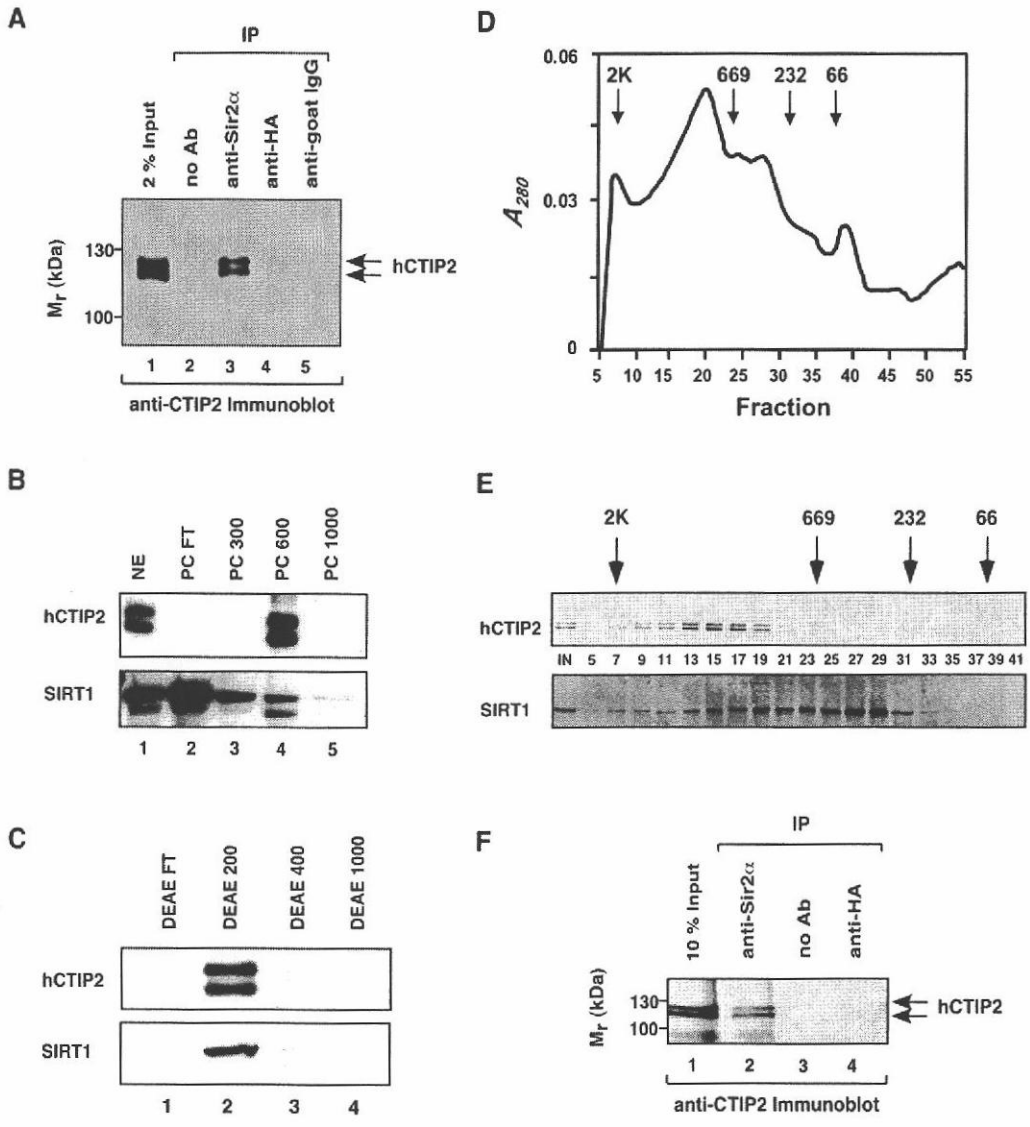
Figure 2.8. **The sirtuin homology domain of SIRT1 mediates the interaction with CTIP2 *in vitro*** *A*, A schematic representation of full-length SIRT1 and SIRT1 truncation mutants used to generate GST-SIRT1 fusion proteins for in vitro pulldown experiments (see below). *B*, [^{35}S]Met-labeled CTIP2 171-350 (the minimal SIRT1-interaction domain or proline-rich region) was incubated with equivalent amounts of GST (lane 2) or GST-SIRT1 fusion proteins (lanes 3-8). The position of bound [^{35}S]Met-labeled CTIP2 171-350 is indicated by an arrow on the left. Lane 1 corresponds to 10% of the [^{35}S]Met-labeled CTIP2 171-350 that was incubated with GST or GST-SIRT1 fusion proteins. Results are representative of 3-5 independent experiments.

2.4.5 CTIP2 and SIRT1 are components of a large complex in Jurkat cell nuclear extracts

The above co-immunoprecipitation studies were performed using transiently transfected cells overexpressing CTIP2. However, it is important to verify that endogenous CTIP2 and SIRT1 interact in the cellular context, i.e., in the absence of overexpression. To test this possibility, nuclear extracts (NE) were prepared from untransfected Jurkat cells, which express both CTIP2 (Fig. 2.9A, lane 1) and SIRT1 (data not shown) endogenously. Endogenous human CTIP2 was co-immunoprecipitated with SIRT1 by both the purified anti-Sir2α antibody (Fig. 2.9A, lane 3), and anti-SIRT1 antiserum (data not shown), but not by an irrelevant antibody (anti-HA, lane 4) or control IgG (lane 5). Similar results were obtained when the precipitating and detecting antibodies were reversed (data not shown). These findings demonstrate that endogenous CTIP2 and SIRT1 physically associate with similar complexes in the nuclei of mammalian cells when expressed at physiological levels.

Jurkat cell nuclear extracts were then fractionated to characterize the nature of CTIP2 complexes. First, Jurkat cell NE was applied to a P11 phosphocellulose column, which was then eluted in a step-wise fashion with increasing salt. CTIP2 immunoreactivity was eluted in the 600 mM step (Fig. 2.9B, lane 4 of the upper panel), although lesser amounts of CTIP2 could be detected in the 1000 mM step with longer exposures of the blot

Figure 2.9. **CTIP2 and SIRT1 are components of a large complex in Jurkat cell nuclear extracts.** *A,* Endogenous CTIP2 and SIRT1 interact in Jurkat cell nuclear extracts. Nuclear extracts prepared from Jurkat cells were immunoprecipitated with no antibody, anti-Sir2α or irrelevant antibodies (anti-HA or goat-IgG). The immunocomplexes were then analyzed by western blotting with anti-CTIP2 antibody. The positions of two forms of endogenous human CTIP2 are indicated, which may correspond to two CTIP2 transcripts present in Jurkat cells as previously reported (Bernard et al., 2001). *B,* Fractionation of CTIP2 and SIRT1 immunoreactivity on a P11 phosphocellulose column. Jurkat cell nuclear extract was applied to a phosphocellulose column, eluted, and eluates from this column were analyzed by immunoblotting as described in experimental procedures. Each lane of the gel contained 10 μg of protein except lane 5 in which 2.5 μg of protein was loaded. Shown is an autoradiograph from the chemiluminescent detection of CTIP2 (upper panel) and SIRT1 (lower panel). *C,* Fractionation of PC600 on a DEAE column. The 600 mM step off the phosphocellulose column (PC600) was applied to a DEAE Biogel A column and eluted stepwise with increasing salt. Steps (6-8 μg of protein per lane) were analyzed for the presence of CTIP2 and SIRT1 immunoreactivity as described above. *D-E,* Fractionation of DEAE200 on Superose 6 size-exclusion column. The 200 mM step off the DEAE column (DEAE200) was applied to Superose 6 column and the column was eluted isocratically in Buffer 100. The chromatographic elution profile of this column is shown in *D*, while the upper and lower panels of *E* respectively correspond to immunodetection of CTIP2 and SIRT1 in the fractions (fraction numbers are indicated between the CTIP2 and SIRT1 immunoblots; equal volumes from each fraction were analyzed). The positions of elution of calibrating proteins are indicated by downward arrows in both *D* and *E*. *F,* Co-immunoprecipitation of SIRT1 and CTIP2 in the Superose 6 purified fractions. Fractions 14-18 from the Superose 6 column shown in *D-E* were pooled, immunoprecipitated with an antibody that recognizes SIRT1, and the immunoprecipitate was analyzed for the presence of CTIP2 as described under *A*.


A
IP
2 % Input
no Ab
anti-Sir2α
anti-HA
anti-goat IgG
M_r (kDa)
130
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hCTIP2
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B
NE
PC FT
PC 300
PC 600
PC 1000
hCTIP2
SIRT1
1 2 3 4 5
C
DEAE FT
DEAE 200
DEAE 400
DEAE 1000
hCTIP2
SIRT1
1 2 3 4
D
0.06
0.03
0
A280
2K
669
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5 10 15 20 25 30 35 40 45 50 55
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no Ab
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Fig. 2.9

(data not shown). In contrast, the majority of the SIRT1 immunoreactivity appeared in the flow-through of the phosphocellulose column (Fig. 2.9B, lane 2 of the lower panel). These findings suggest that most of SIRT1 in Jurkat cell nuclei does not exist within CTIP2 complexes, at least when subjected to the chromatographic conditions utilized in this study. This would be consistent with the notion that SIRT1 plays multiple cellular roles, many of which appear to be independent of CTIP2. Nonetheless, a small amount of SIRT1 did co-chromatograph with CTIP2 within the 600 mM step off the phosphocellulose fraction and this material was then dialyzed and applied to a DEAE column to fractionate the CTIP2 immunoreactivity further. Both CTIP2 (upper panel) and SIRT1 (lower panel) immunoreactivity eluted in the 200 mM step off the DEAE column (Fig. 2.9C, lane 2). The DEAE-purified material was then applied to a Superose 6 sizing column in order to determine the relative molecular masses of CTIP2 and SIRT1 complexes. CTIP2 was eluted from the Superose 6 column as a sharp peak with a relative mass approximately between 1 and 2 mDa (Fig. 2.9D and upper panel of Fig. 2.9E, fractions 7-19). In contrast, SIRT1 was eluted as a broad peak beginning at 2 mDa and ending at approximately 232 kDa (lower panel of Fig. 2.9E, fractions 7-31). These size-exclusion chromatography results demonstrate that: (1) native CTIP2 migrated at least 10-fold larger than the predicted size of the monomeric protein (95.5 and 88.5 kDa, respectively, for the CTIP2 splice variants), (2)

SIRT1 appeared to be present in heterogenous complex(es), some of which corresponded to CTIP2 immunoreactivity (lower panel of Fig. 2.9E, fractions 7-19), others that clearly did not (fractions 21-31), and all of which migrated much larger than the predicted size of the monomeric SIRT1 (81.7 kDa). Fractions containing both CTIP2 and SIRT1 immunoreactivity were pooled and immunoprecipitated with the SIRT1 antibody. The immunoprecipitate was analyzed by immunoblotting with the CTIP2 antibody to verify that CTIP2 and SIRT1 co-existed within a similar complex in this partially purified preparation. Indeed, CTIP2 was found to co-immunoprecipitate with the SIRT1 (Fig. 2.9F, lane 2), indicating the CTIP2-SIRT1 interaction that was observed in Jurkat cell nuclear extracts (Fig. 2.9A) was maintained through a high-salt extraction and three sequential chromatographic steps.

2.5 Discussion

The combinatorial, covalent modification of histones, including acetylation, methylation, phosphorylation, and ubiquitination has been proposed to underlie the mechanistic basis of dynamic transcriptional regulation from yeast to man. In general, histone acetylation, phosphorylation and ubiquitination are believed to promote decondensation of chromatin and have been implicated in the mode of action of

transcriptional activators (Jenuwein and Allis, 2001). The transcriptional outcome of histone methylation, including mono-, di-, and tri-methylation of lysine residues located primarily in the tails of histones H3 and H4, is more complex and can either promote activation (Santos-Rosa et al., 2002) or repression (Lachner and Jenuwein, 2002; Lachner et al., 2001) in a context-dependent manner. In contrast, many transcriptional repressors, including unliganded nuclear receptors and members of numerous other transcription factor families, have been shown to recruit TSA-sensitive HDACs to the template, resulting in condensation of chromatin and transcriptional silencing (Struhl, 1998). In most of these cases, the HDACs recruited to the template of RNA polymerase II-transcribed genes by transcriptional repressors have been shown or suspected to be either class I or II HDACs (Hermanson et al., 2002).

We have previously observed that CTIP1 (Avram et al., 2000), a member of a novel family of C_2H_2 zinc finger proteins, repressed transcription of a reporter gene in a manner that was only minimally sensitive to reversal by TSA. Similar findings were reported herein for CTIP2 (Fig. 2.1). However, CTIP2-mediated transcriptional repression was found to be inhibited, at least partially, by nicotinamide (NAM), an inhibitor of the NAD^+-dependent, class III HDACs of the SIRT family. Consistent with these findings, expression of CTIP2 in HEK293 cells resulted in recruitment of SIRT1 to the promoter template, and deacetylation of

template-associated histones H3/H4 in a manner that was unaffected by TSA but reversed by NAM. Moreover, co-expression of SIRT1, but not a catalytically inactive point mutant, was found to enhance CTIP2-mediated transcriptional repression in transiently transfected cells. The possibility that SIRT1 plays a role in CTIP2-mediated transcriptional repression was given strong support by the finding that the two proteins interacted directly and co-immunoprecipitated from extracts prepared from both co-transfected and singly transfected HEK293 cells, as well as non-transfected Jurkat cells. Finally, SIRT1 partially co-purified with CTIP2 over three chromatographic steps, and the apparent mass of this SIRT1-containing, CTIP2 complex was estimated to be between 1 and 2 mDa, indicating the possible existence of several other component proteins.

To our knowledge, the results of ChIP assays conducted herein provide the first cellular evidence that SIRT1 is capable of deacetylating histones H3 and H4, histones that play a key role in transcriptional regulatory events (Guarente, 2000; Zhang and Reinberg, 2001). Although the chromatin organization of transiently transfected DNA templates may differ from that of their chromosomal counterparts (Smith and Hager, 1997), the effect of TSA on basal CAT activity (Fig. 2.1), and the observed decrease in template-associated, acetylated histone H3/H4 as determined by ChIP analyses (Figs. 2.2 and 2.6) indicated that the transiently transfected reporter template adopted some form of chromatin structure.

This finding is also supported by previous reports (Cereghini and Yaniv, 1984; Luo et al., 1998; Reeves et al., 1985).

The proline-rich region of CTIP2, which harbors an autonomous transcriptional repression activity (Avram, et. al., submitted), was found to interact with the centrally located sirtuin homology domain of SIRT1 (see Figs. 2.8*A* and *B*). Because the sirtuin homology domain is highly conserved among all SIRT proteins, it is conceivable that other members of sirtuin family may also serve as an alternative CTIP2-interacting partner(s). If this is true, one may envision that other SIRT proteins may play a role(s) in CTIP2-mediated transcriptional repression, perhaps in different cellular and/or promoter contexts. Similarly, the proline-rich region of CTIP2 is also present and highly conserved in CTIP1 (69% identity over 138 amino acids) and may also be expected to interact with SIRT proteins. Indeed, we have also observed that CTIP1 interacts directly with SIRT1 in vitro and in transfected cells (unpublished results).

Other C_2H_2 zinc finger proteins, such as the Kruppel-associated box (KRAB) protein Kox1, have also been reported to repress transcription in a TSA-insensitive manner (de Haan et al., 2000; Lorenz et al., 2001). Transcriptional repression by KRAB zinc finger proteins requires interaction with KRAB domain binding protein, KAP-1, which in turn may recruit members of heterochromatin protein 1 (HP1) family to the template (Lechner et al., 2000; Ryan et al., 1999). These findings suggest a role for

HP1 proteins and heterochromatin formation in transcriptional repression mediated by KRAB•KAP-1 complexes. While none of these studies addressed the potential role of TSA-insensitive histone deacetylation, possibly catalyzed by a SIRT family member, in transcriptional repression and/or heterochromatinization mediated by KRAB•KAP-1 complexes, deacetylation of H3-K9 is required at the initial step of heterochromatin formation (Zhang and Reinberg, 2001). This is believed to be followed by SUV39H-catalyzed methylation of H3-K9, which provides a binding surface for HP1 proteins that bind and self-assemble into a supramolecular, heterochromatinized template (Lachner et al., 2001). Therefore, it is of interest that we have also observed a potential role for HP1 proteins in CTIP-mediated transcriptional repression (Rohr et al., 2003). In this context, CTIPs may serve to recruit SIRT1 (or other SIRT family members) to a particular genomic locus, either by direct DNA binding or via tethering to a COUP-TF family member. Once recruited to the template, SIRT1 may catalyze histone deacetylation, ultimately leading to HP1 binding, heterochromatin formation, and gene silencing. Verification of this model will require identification of all of the component proteins of the CTIP repressor complex(es) bound to the promoter regions of target genes.

In summary, the present results strongly suggest that CTIP2-mediated transcriptional repression involves the recruitment of SIRT1 to the template, at which the TSA-insensitive, NAD^+-dependent histone deacetylase

catalyzes deacetylation of promoter-associated histones H3 and/or H4. One outcome of this deacetylation may simply be chromatin condensation and short-term silencing. Alternatively or additionally, these deacetylation events may ultimately result in formation of heterochromatin, contributing to a persistent, silenced state. In either case, recruitment of SIRT1 to the DNA template by CTIP2 would be expected to result in gene silencing. This feature might be helpful for identification and characterization of the target genes under the control of CTIP proteins in cells of the hematopoietic system and/or the developing central nervous system.

2.6 Abbreviations

The abbreviations used are BCL, B cell leukemia; bp, base pair; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CTIP 1 and 2, COUP-TF-interacting proteins 1 and 2; GST, glutathione S-transferase; HA, hemagglutinin; HDAC, histone deacetylase; HEK293, human embryonic kidney 293 cells; IgG, immunoglobulin G; kDa, kilodalton; mDa, megadalton; NAM, nicotinamide; Sir2, silent information regulator 2; SIRT1, Sir2-like protein 1 or sirtuin 1; TSA, trichostatin A; WT, wild type.

2.7 Acknowledgements

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Chapter 3

BCL11A Recruits the Histone Deacetylase SIRT1 to Repress Transcription

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3.1 Abstract

The B cell leukemia 11A protein (BCL11A/EVI9/CTIP1) has been implicated in hematopoietic cell development and malignancies, however, its contribution to these cellular processes remains unknown. BCL11A is a transcriptional repressor that binds to a GC-rich motif or is recruited to the template via interaction with COUP-TF orphan nuclear receptors. In both cases, BCL11A-mediated transcriptional repression is not reversed by trichostatin A, suggesting the lack of involvement of class I or II histone deacetylases. Nonetheless, chromatin immunoprecipitation assays revealed that expression of BCL11A in mammalian cells resulted in deacetylation of histones H3 and/or H4 that were associated with the promoter region of a reporter gene. Here we show that BCL11A interacts with and recruits SIRT1 to the promoter template to repress transcription of a reporter gene. Moreover, BCL11A-mediated transcriptional repression, as well as deacetylation of promoter-associated histones H3/H4 in BCL11A-transfected cells, was reversed by nicotinamide, an inhibitor of class III histone deacetylases, such as SIRT1. Finally, SIRT1 enhanced the deacetylation of template-associated histones H3/H4 in BCL11A-transfected cells, and stimulated BCL11A-dependent transcriptional repression. These findings define a role for SIRT1 in transcriptional repression mediated by BCL11A.

3.2 Introduction

BCL11A was originally identified as a protein that interacted with and stimulated the transcriptional repression activity of chicken ovalbumin transcription factor II (COUP-TFII), and was named COUP-TF-interacting protein 1 (CTIP1) (Avram et al., 2000) BCL11A was also independently identified by Copeland's group as EVI9 (Ecotropic Viral Integration site 9), the locus of which was demonstrated to be a site of proviral integration resulting in acute myeloid leukemia in BXH2 mice (Nakamura et al., 2000). Subsequently, the human locus of BCL11A was shown to be involved in a translocation event, t(2; 14)(p13; q32.3), that appears to underlie some forms of chronic lymphocytic leukemia (CLL) and immunocytoma. However, the contribution of BCL11A to neoplastic processes in hematopoietic cells of murine or human origin remains unclear.

Although BCL11A has been shown to interact directly with COUP-TF1 (Avram et al., 2000) and BCL6 (Nakamura et al., 2000), BCL11A also binds directly to a GC-rich motif and represses transcription of a downstream reporter gene in the absence of overexpressed COUP-TF family members or BCL6 (Avram et al., 2002), suggesting COUP-TF- and BCL6-independent mechanisms of BCL11A-mediated transcriptional repression may be operant in some cell types.

The analysis of BCL11A-null mice has demonstrated the role for BCL11A in both hematopoiesis and postnatal development (Liu et al., 2003). BCL11A and its paralog BCL11B/CTIP2 are similar in sequence, DNA binding specificity, and interacting partners (i.e., COUP-TF proteins). However, the lymphoidal defects resulting disruption of each locus differ. BCL11A is essential for B cell development (Liu et al., 2003), whereas BCL11B is required for αβ T cell development (Wakabayashi et al., 2003).

Previous studies have revealed that both BCL11A and BCL11B mediate transcriptional repression of a reporter gene in a trichostatin A (TSA)-insensitive manner (Avram et al., 2000; Avram et al., 2002; Senawong et al., 2003). More recently, we have demonstrated that BCL11B interacts with and recruits the class III HDAC, SIRT1, to repress transcription of a reporter gene (Senawong et al., 2003). This finding implicates SIRT1 in the transcriptional activity of BCL11B in mammalian cells, which at least in part, explains TSA-insensitive manner of BCL11B-mediated transcriptional repression. The similarities in both amino acid sequences and physical properties of BCL11A and BCL11B prompted us to speculate that the histone deacetylase SIRT1 may also underlie the mechanism of BCL11A-mediated transcriptional repression. Six lines of evidence indicate that SIRT1 is involved in BCL11A-mediated transcriptional repression in transfected cells: (1) overexpression of BCL11A resulted in deacetylation of histone H3 and/or H4 that were

associated with the promoter region of a target gene, (2) both the deacetylation of histone H3/H4 in BCL11A-transfected cells and BCL11A-mediated transcriptional repression were found to be partially reversed by nicotinamide, an inhibitor SIRT1, (3) endogenous SIRT1 was specifically recruited to the reporter gene template by overexpressed BCL11A, (4) SIRT1, but not a catalytically inactive mutant, stimulated transcriptional repression mediated by BCL11A, (5) endogenous BCL11A and SIRT1 were found to coimmunoprecipitate from nuclear extracts prepared from untransfected 70z/3 cells, and (6) BCL11A and SIRT1 were found to participate in a direct, physical interaction in vitro. Collectively, these findings implicate the histone deacetylase SIRT1 in the transcriptional repression activity of BCL11A in mammalian cells.

3.3 Materials and Methods

3.3.1 Constructs

The $(17\text{-mer})_4$-tk-CAT reporter construct was a kind gift from Dr. Ming-jer Tsai (Baylor College of Medicine). Flag-BCL11A construct was prepared by PCR amplification of the BCL11A open reading frame (Avram et al., 2000) with appropriate primers and insertion into pcDNA3(+) (Invitrogen). The Gal4 DBD-BCL11A construct was prepared by PCR

amplification with appropriate primers followed by insertion into a commercially available vector (pM; Clontech). Myc-SIRT1, Myc-SIRT1 H363Y and GST-SIRT1 constructs (Langley et al., 2002) were kind gifts from Dr. T. Kouzarides (University of Cambridge, Cambridge, UK). All vectors encoding GST fusion proteins were prepared by PCR amplification of appropriate templates followed by insertion into pGEX-2T (Amersham Pharmacia Biotech). 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). All constructs were verified by complete DNA sequencing.

3.3.2 Antibodies

Purified rabbit anti-Sir2α, mouse anti-SIRT1 and rabbit anti-acetylated-histone H3 and -histone H4 antibodies were obtained from Upstate. Mouse anti-Flag and -Myc monoclonal antibodies were purchased from Sigma and Oncogene, respectively. Mouse anti-Gal4 was obtained from Santa Cruz Biotechnology. The mouse anti-BCL11A monoclonal antibody was prepared and raised against a recombinant GST fusion protein by Dr. Michael Marusich (Monoclonal Antibody Facility, Institute for Neuroscience, University of Oregon, Eugene, Oregon).

3.3.3 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed on transfected cells essentially as described previously (Senawong et al., 2003) with slight modifications. HEK293 cells were co-transfected at 60% confluency (10 cm plates) with 3 μg of the $(17\text{-mer})_4$-tk-CAT reporter, 5-20 μg of Gal4-BCL11A, 0.5 μg of Myc-SIRT1, and/or the parent control vectors using the calcium phosphate method. After 48 h, cells were washed twice with phosphate buffered saline (PBS) and cross-linked with 1% formaldehyde in PBS at room temperature. Cells were washed twice with ice-cold PBS buffer and collected in harvesting buffer (100 mM Tris-HCl, pH 9.4 containing 10 mM DTT). The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.1 containing 1% SDS, 10 mM EDTA, and a protease inhibitor cocktail). The sonicated lysates were then cleared by centrifugation and diluted 2.5-3.75 fold with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1 containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and a protease inhibitor cocktail). One tenth of the diluted lysate was reserved as an input sample to determine total amount of reporter plasmid in transfected cells for subsequent normalization procedures. Two equal aliquots of the remaining lysate were used for immunoprecipitation with and without the addition of antibodies against K9-, K16- di-acetylated histone H3, and K5-, K8-, K12-, K16-tetra-acetylated histone H4 (Upstate; 5 μg of each antibody per immunoprecipitation reaction). Immune complexes were recovered with

Protein A/ Protein G sepharose (Amersham Pharmacia) and washed under stringent conditions. Chromatin complexes were eluted with the freshly prepared elution buffer (0.1 M $NaHCO_3$ containing 1% SDS). The eluates and the above input samples were subjected to an overnight reversal of cross-links at 65°C, followed by a treatment with Proteinase K at 45°C for 1-2 h. DNA was recovered by using QIAquick PCR Purification Kit (QIAGEN) and amplified using a forward primer (5′-GGCATCAGAGCAGATTGTACT-3′) upstream of the multimerized 17-mer and a reverse primer (5′-CCTTAGCTCCTGAAAATCTCG-3′) downstream of the *tk* promoter but upstream of the transcriptional start site. The resulting PCR product (327 bp) was analyzed by agarose gel electrophoresis and ethidium bromide staining. Experiments were performed three to five times.

3.3.4 Transfection and reporter

HEK293 cells were transfected and harvested as described above. Where indicated, TSA (100 ng/mL) and nicotinamide (15 mM) treatments were initiated 24 h after transfection, and cells were harvested 24 h later. A β-galactosidase expression vector (pCMV-Sport-βGal, Life Technologies) was cotransfected as an internal control, and β-galactosidase activity was used to normalized CAT activity as described (Dowell et al., 1997a).

3.3.5 Coimmunoprecipitation

HEK293 cells were transfected as described above with 15 μg each of expression vectors encoding Flag-CTIP2 and/or Myc-SIRT1. Forty-eight hours after transfection the cells were lysed in NET-N buffer (20 mM Tris-HCl, pH 8 containing 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, and a protease inhibitor cocktail) by agitation at 4°C for 30 min. After a brief sonication, lysates were cleared by centrifugation, and immunoprecipitated as described previously (Nevrivy et al., 2000) using the antibodies described above. Whole cell extracts from pre-B lymphocytes were prepared by using NET-N buffer as described above, and immunoprecipitated (10 mg of total protein per reaction) with purified anti-BCL11A monoclonal (10-15 μg) or anti-Sir2α (0.5 - 2.5 μg) antibodies. All immunoprecipitates were analyzed by immunoblotting with appropriate antibodies.

3.3.6 GST pulldown experiment

GST pulldown experiments were conducted as described previously (Dowell et al., 1997b). Briefly, equivalent amounts of GST or GST-SIRT1 fusion proteins were bound to glutathione-sepharose (Pharmacia) and incubated with [^{35}S]methionine-labeled proteins (BCL11A

or BCL11A truncation mutants) prepared using TNT transcription-translation system (Promega). The reactions were washed five times with binding buffer (10 mM Na-HEPES containing 10% glycerol, 1 mM EDTA, 1 mM DTT, 150 mM NaCl and 0.05% NP-40) and bound proteins were eluted and resolved on denaturing SDS-PAGE gels for analysis by autoradiography.

3.4 Results

3.4.1 BCL11A represses transcription in a nicotinamide-sensitive manner

Previous reports demonstrated that BCL11A/CTIP1 repressed transcription of a reporter gene in a TSA-insensitive manner, suggesting that BCL11A-mediated transcriptional repression does not involve the recruitment of class I or class II TSA-sensitive HDACs to the template. However, we cannot exclude the possibility that histone deacetylation mediated by TSA-insensitive HDACs may underlie the transcriptional repression activity of BCL11A. To further explore the precise mechanism(s), we determined whether TSA-insensitive HDAC(s) is involved in BCL11A-mediated transcriptional repression. The Gal4 reporter assay in the presence of nicotinamide (NAM), an inhibitor of NAD^+-dependent, TSA-insensitive, class III HDACs, was performed. The results demonstrated that transcriptional repression mediated by a Gal4-BCL11A

fusion protein was partially reversed in the presence of 15 nM nicotinamide in HEK293 cells (Fig. 3.1, compare lanes 4 and 6 with lane 1). Consistent to previous studies (Avram et al., 2000; Avram et al., 2002), TSA, at 100 ng/ml, has no effect on transcription repression mediated by BCL11A (Fig. 3.1, compare lanes 1 and 2 with 4 and 5).

The above results suggest that a nicotinamide-sensitive HDAC(s) may involve in BCL11A-mediated transcriptional repression. To investigate whether histone deacetylation by nicotinamide-sensitive HDAC(s) underlies the repression mechanism(s), the level of acetylated histones associated with the reporter template was determined. The amount of acetylated histones H3 and/or H4 at the promoter region was found to be decreased in BCL11A-transfected cells (upper panel of Fig. 3.2, compare lanes 4 with 6), and this was unaffected by treatment of the transfected cells with TSA (Fig. 3.2, compare lanes 3-6 of the top and middle panels). However, the deacetylation of promoter-associated histone H3 and/or H4 in BCL11A-transfected cells, was inhibited by nicotinamide (Fig. 3.2, compare lanes 3-6 of the top and bottom panels). Based on the above results, which are similar to that of BCL11B/CTIP2 (Senawong et al., 2003), we hypothesized that BCL11A/CTIP1 may also interact with and recruit SIRT1, a member of nicotinamide-sensitive histone deacetylases, to promoter template to repress transcription.

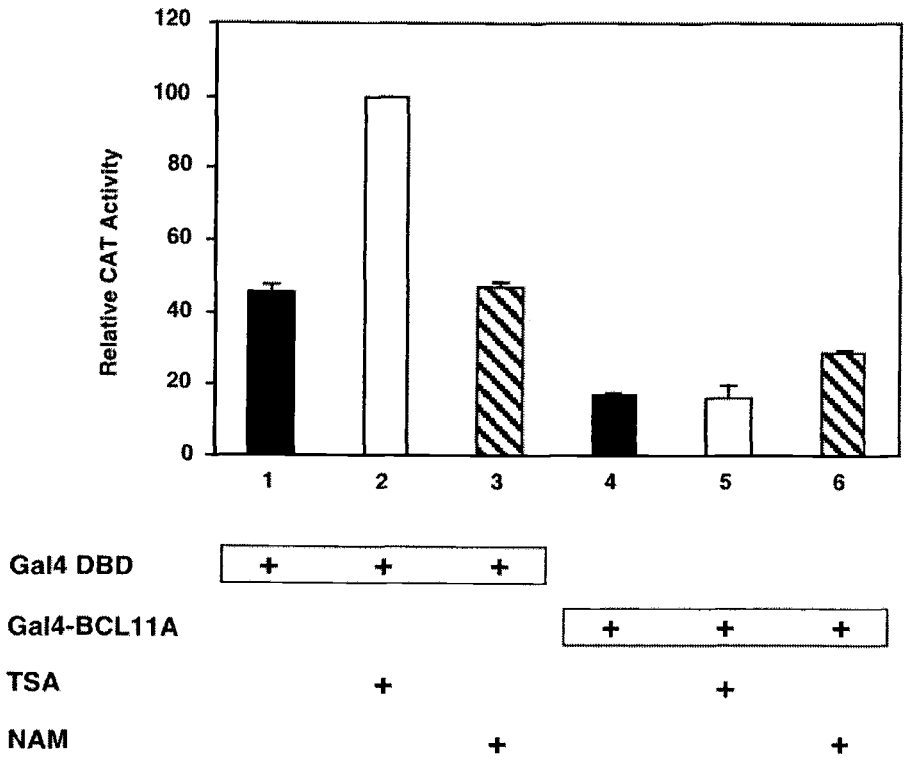

Figure 3.1. **BCL11A represses transcription in a nicotinamide-sensitive manner.** HEK293 cells were transiently transfected with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with 5 μg of expression vectors encoding either Gal4-BCL11A or Gal4 DBD as indicated. Twenty-four hours after transfection, cells were treated or not (solid bars) with histone deacetylase inhibitors, TSA (100 ng/ml; open bars) and nicotinamide (NAM; 15 mM; hatched bars), for 24 h before collection. Transfection efficiency was normalized by β-galactosidase activity produced by a cotransfected β-galactosidase expression vector. CAT activity determined in the presence of Gal4 DBD and TSA (lane 2) was taken to be maximal and that against which all other determined CAT activities were compared. The results presented represent the mean (± S.E.M.) of three independent experimental determinations.

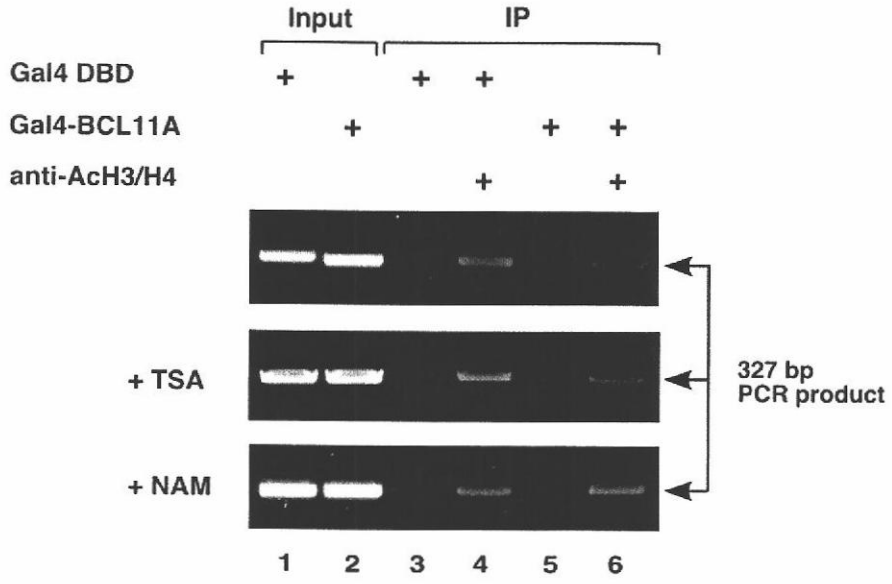

Figure 3.2. **Nicotinamide-sensitive histone deacetylation underlies BCL11A-mediated transcriptional repression.** HEK293 cells were transfected with 3 μg of $(17\text{-mer})_4$-tk-CAT reporter and 20 μg of an expression vector encoding Gal4-BCL11A. The treatments with TSA and NAM were carried out as described above. Transfection efficiency was normalized by total amount of the transfected $(17\text{-mer})_4$-tk-CAT reporter as determined by PCR amplification (input lanes 1 and 2; 5% of total). Lanes 3-6 represent template amplification reactions from samples immunoprecipitated with or without antibodies specific for acetylated histones H3 and H4 as indicated. Amplification reactions were separated on a 1% agarose gel that was stained with ethidium bromide to visualize DNA products. The indicated band is the expected, 327 bp amplification product from the reporter gene template. Results are representative of three independent experiments.

3.4.2 BCL11A interacts with and recruits SIRT1 to the promoter template

To investigate whether BCL11A associated with SIRT1, a member of nicotinamide sensitive HDACs, co-immunoprecipitation assays were performed. First, the interaction of co-overexpressed proteins was determined using Flag-BCL11A and Myc-SIRT1. Myc-SIRT1 was immunoprecipitated by anti-Flag antibody but only when Flag-BCL11A was co-expressed in HEK293 cells (Fig. 3.3*A*, compare lanes 5 with 6). Next, we determined if overexpressed BCL11A associated with endogenous SIRT1 in HEK293 cells, which we used as a model system for BCL11A-mediated transcriptional repression. We found that endogenous SIRT1 was immunoprecipitated with Flag-BCL11A by anti-Flag antibody but only in the extracts prepared from BCL11A-transfected HEK293 cells (Fig. 3.3*B*, compare lanes 3 with 4). These findings suggest that BCL11A interacts directly or indirectly with SIRT1 in HEK293 cells.

However, the above co-immunoprecipitation studies were performed using transiently transfected cells expressing BCL11A. It is important to verify that endogenous BCL11A and SIRT1 interact in the cellular context without overexpression of the proteins. To verify this, cell

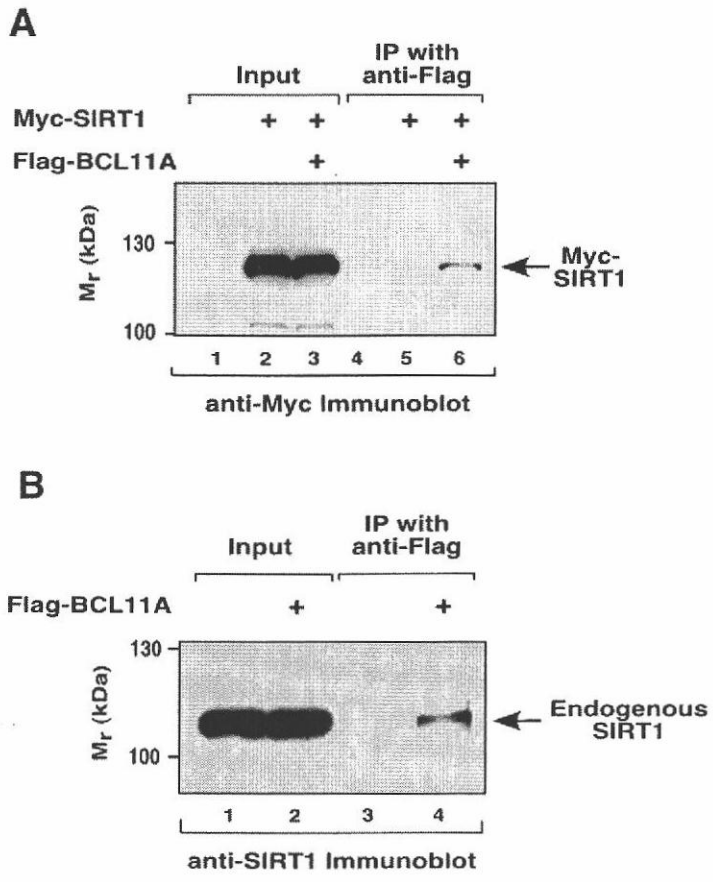

Figure 3.3. **BCL11A interacts with SIRT1 in HEK 293 cells.** *A*, Myc-SIRT1 coimmunoprecipitates with Flag-BCL11A. Whole cell extracts from HEK293 cells, untransfected and transiently transfected with the indicated expression vectors, were immunoprecipitated with anti-Flag monoclonal antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by western blotting with anti-Myc monoclonal antibody that detects Myc-SIRT1. The position of Myc-SIRT1 is indicated. *B*, Endogenous SIRT1 co-immunoprecipitates with Flag-BCL11A from HEK293 cell lysates. Whole cell extracts from HEK293 cells, untransfected and transiently transfected with expression vectors encoding Flag-BCL11A, were immunoprecipitated with anti-Flag monoclonal antibody, and the immunocomplexes were analyzed by immunoblotting with anti-SIRT1 antibody. The position of endogenous SIRT1 is indicated.

extracts were prepared from untransfected mouse pre-B lymphocytes (70z/3 cells), which express both mouse BCL11A/CTIP1 (data not shown) and mouse SIRT1 homolog, Sir2α (Fig. 3.4*A*, lane 1). Endogenous Sir2α was co- immunoprecipitated with BCL11A by anti-BCL11A (Fig. 3.4*A*, lane 3), but not by an irrelevant antibody (mouse anti-Gal4, lane 4). Similar results were obtained when precipitating and detecting antibodies were reversed (data not shown). These findings suggest that endogenous BCL11A and Sir2α (mouse SIRT1 homolog) physically associate with similar complex(es) in mammalian cells when expressed at physiological levels.

Based on the observed ability of BCL11A to interact with SIRT1, we further investigated if endogenous SIRT1 was recruited to the promoter template by using ChIP assays in HEK293 cells transfected with a Gal4-BCL11A expression vector. Endogenous SIRT1 was recruited to the promoter template in cells expressing Gal4-BCL11A (Fig. 3.4*B*, lane 6). These results demonstrate BCL11A-dependent recruitment of endogenous SIRT1 to the promoter template in HEK293 cells, suggesting a role for this histone deacetylase in the transcriptional repression mechanism of BCL11A.

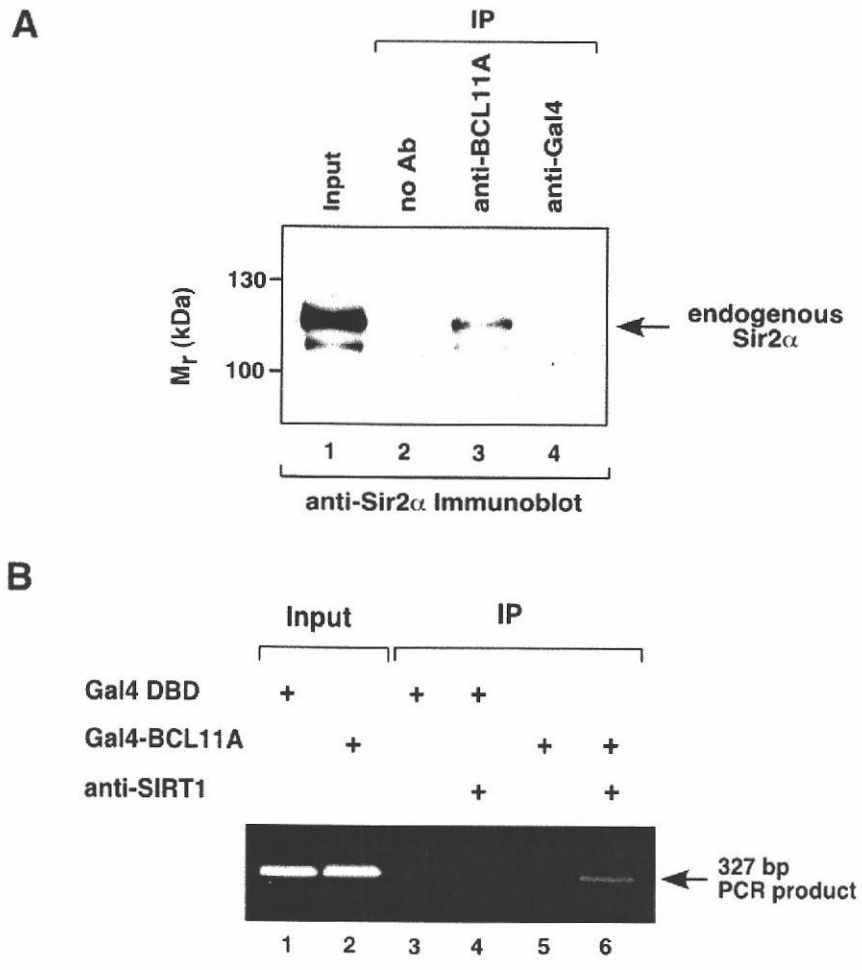

Figure 3.4. **BCL11A interacts with Sir2α in mouse pre-B lymphocytes and recruits SIRT1 to the promoter template in HEK 293 cells.** A, Endogenous Sir2α, a mouse SIRT1 homolog, and BCL11A interact in pre-B lymphocyte whole cell extracts. The cell extracts were immunoprecipitated with no antibody, anti-BCL11A or irrelevant antibody (anti-Gal4). The immunocomplexes were then analyzed by western blotting with anti-Sir2α antibody. The position of endogenous Sir2α is indicated, which corresponds to ~120 KDa as previously reported (McBurney et al., 2003). B, Endogenous SIRT1 is recruited to promoter template of the reporter gene upon expression of Gal4-BCL11A. HEK293 cells were transfected with 5 μg of $(17\text{-mer})_4$-tk-CAT reporter and 20 μg of expression vectors encoding either Gal4-BCL11A or Gal4 DBD. Transfection efficiency was normalized as described in the legend of Fig. 3.2 (input lanes 1 and 2; 5% of total). Lanes 3-6 represent template amplification reactions from samples immunoprecipitated with or without antibody directed against SIRT1. Results are representative of three independent experiments.

3.4.3 BCL11A interacts directly with SIRT1 *in vitro*

The above results demonstrate that BCL11A and SIRT1 associate with similar complex(es) in mammalian cells, however, we cannot distinguish if their interactions are direct physical contact or connected by intermediary protein(s). Therefore, we determined whether BCL11A interact directly with SIRT1 by *in vitro* GST pull-down assays. Full-length, in vitro translated BCL11A was found to interact with full-length SIRT1 fused to GST (Fig. 3.5, lane 3 of panel A), but not with GST alone (lane 2). This finding suggests that BCL11A and SIRT1 participate in a direct, physical interaction. The SIRT1 interaction interface of BCL11A was mapped by using a series of BCL11A deletion mutants (see Fig. 3.5) and was found to contain within BCL11A amino acids 194-378 (Fig. 3.5, lane 3 of *panel* D), which is relatively rich in Proline residues (Avram et al., 2000). This proline-rich region is 69 % identical to that of CTIP2, which is required for SIRT1•CTIP2 interaction (Senawong et al., 2003). All BCL11A deletion mutants containing this region strongly interacted with GST-SIRT1 (Fig. 3.5, lane 3 of panels C and D) but mutants lacking it interacted weakly or not at all (Fig. 3.5, lane 3 of panels B, E and F). In summary, a proline-rich region, BCL11A 194-378, appears to be primarily responsible for interaction with SIRT1 *in vitro*.

The BCL11A interaction interface of SIRT1 was similarly mapped by using a series of SIRT1 deletion mutants (see Fig.3.6*A*) fused to GST.

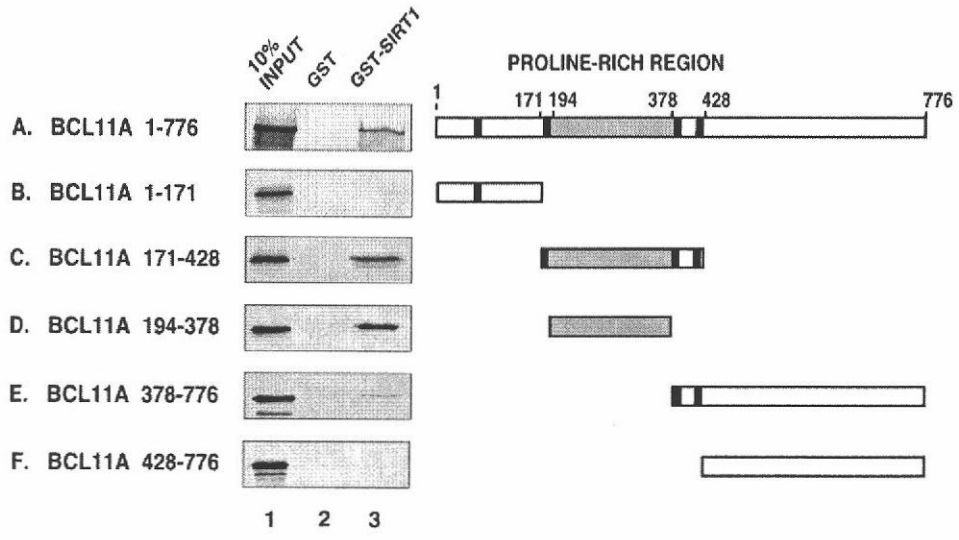

Figure 3.5 **The proline-rich region of BCL11A mediates the interaction with SIRT1 *in vitro*.** In vitro translated and [^{35}S]Met-labeled full-length BCL11A and truncation BCL11A mutants were incubated with equivalent amounts of bacterially expressed GST (lane 2) or GST-SIRT1 fusion protein (lane 3). After extensive washing, [^{35}S]Met-labeled BCL11A associated with the affinity resin was determined by SDS-PAGE and autoradiography. Input [^{35}S]Met-labeled proteins are shown in *lane* 1. BCL11A truncation mutants used in these studies (panels B-F) are schematically represented on the right with zinc finger motifs denoted by vertical bars.

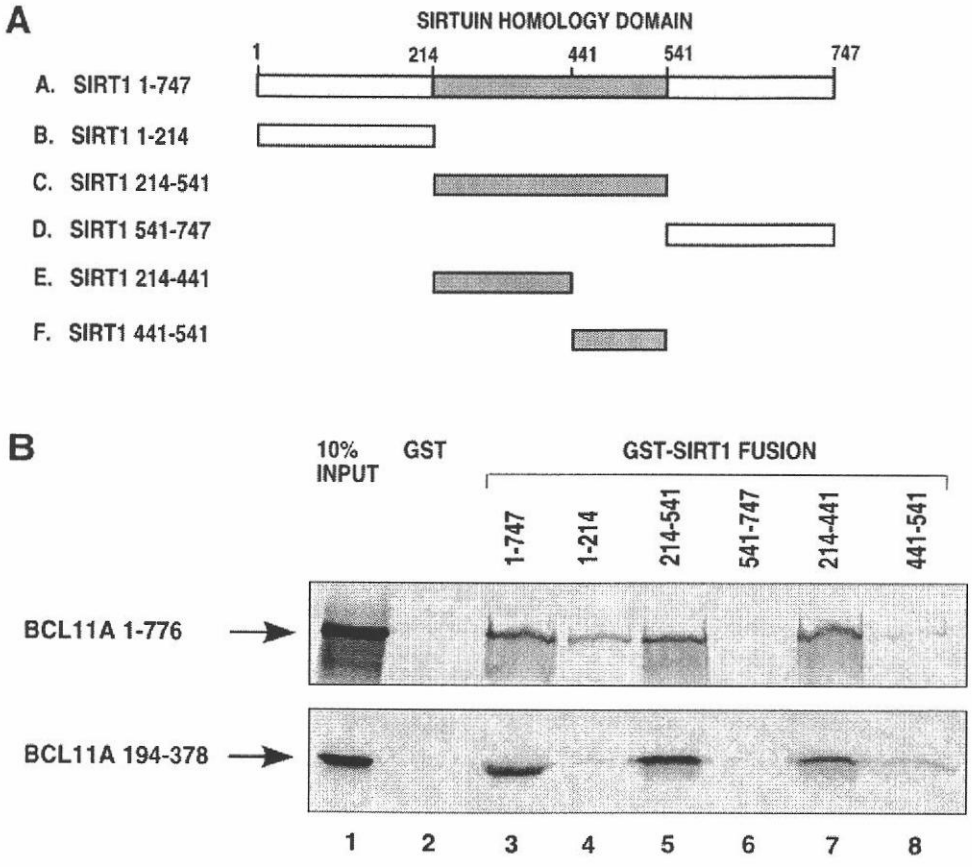

Figure 3.6 **The sirtuin homology domain of SIRT1 mediates the interaction with BCL11A *in vitro.*** *A*, A schematic representation of full-length SIRT1 and SIRT1 truncation mutants used to generate GST-SIRT1 fusion proteins for in vitro pull down experiments (see below). *B*, Both [^{35}S]Met-labeled full-length BCL11A (upper panel) and BCL111A 194-378 (*lower panel*; the minimal SIRT1-interaction domain) were incubated with equivalent amounts of GST (lane 2) or GST-SIRT1 fusion proteins (lanes 3-8). The position of bound [^{35}S]Met-labeled full-length BCL11A and BCL11A 194-378 are indicated by arrows on the left. Lane 1 corresponds to 10% of the [^{35}S]Met-labeled full-length BCL11A and BCL11A 194-378 that were incubated with GST or GST-SIRT1 fusion proteins. Shown in *A* and *C* are representative experiments that were replicated 3-5 times.

Both in vitro translated, full-length BCL11A and BCL11A 194-378 interacted strongly with the sirtuin homology domain of SIRT1 (Fig. 3.6*B*, lane 5), very weak with SIRT1 1-214 (lane 4), but not at all with SIRT1 541-747 (lane 6). Moreover, BCL11A was found to interact primarily with amino terminal part of sirtuin homology domain, SIRT1 214-441(Fig. 3.6*B*, lane 7), and less so with the carboxyl terminal part, SIRT1 441-541 (lane 8). These findings suggest that BCL11A interacts directly with SIRT1 *in vitro*, and the interaction requires residues within the amino terminal part of sirtuin homology domain of SIRT1 and the proline-rich region of BCL11A (amino acids 194-378).

3.4.4 SIRT1 enhances BCL11A-mediated transcriptional repression

Based on the ability of BCL11A to interact and recruit SIRT1 to the promoter template, we hypothesized that transcriptional repression by BCL11A is at least in part mediated through SIRT1. To assess a potential role for SIRT1 in BCL11A-mediated transcriptional repression, reporter gene assays were carried out in transiently transfected HEK293 cells. In the absence of BCL11A, both wild-type SIRT1 and a catalytically inactive point mutant, SIRT1 H363Y, repressed reporter gene expression in a concentration-dependent manner (Fig. 3.7, lanes 1-4 and 5-8, respectively), suggesting that the catalytic activity of SIRT1 is not required for basal

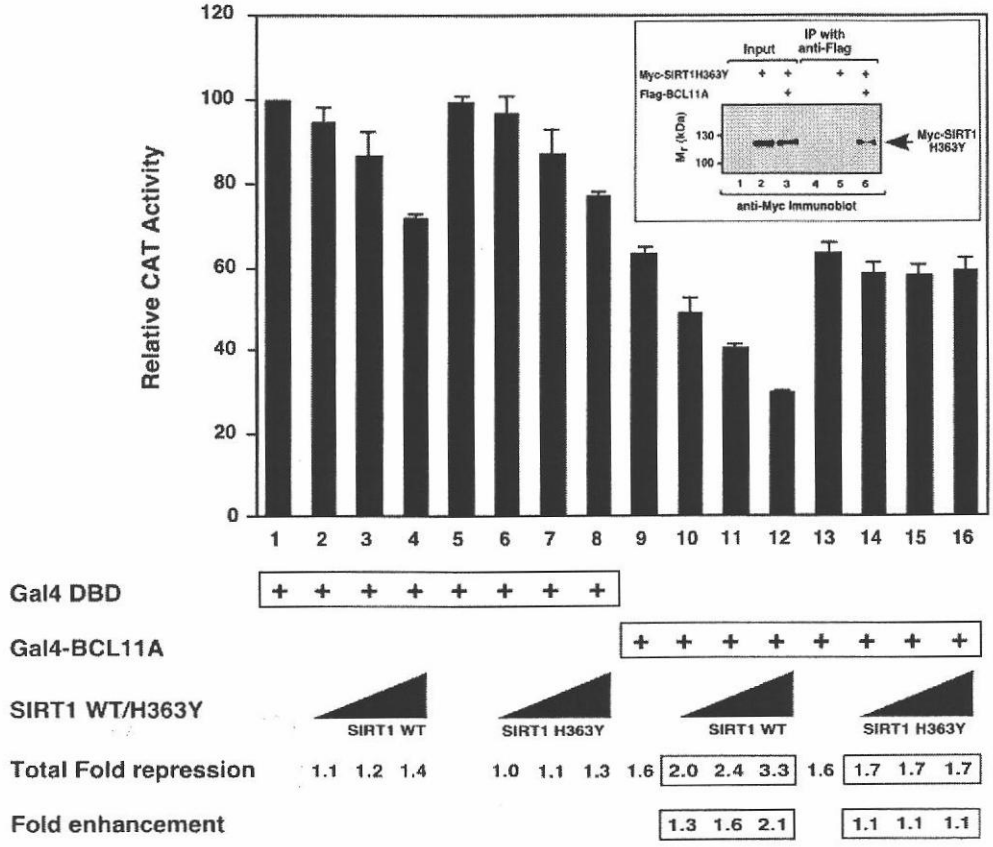

Figure 3.7. **SIRT1 enhances BCL11A-mediated transcriptional repression.** Wild-type SIRT1, but not SIRT1 H363Y, stimulates CTIP2-mediated transcriptional repression. HEK293 cells were transiently transfected with 5 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with 5 μg of expression vectors encoding either Gal4-BCL11A or Gal4 DBD, and increasing amounts (0.125, 0.25, and 0.5 μg) of expression vectors encoding either SIRT1 WT or SIRT1 H363Y, as indicated. Transfection efficiency was normalized as described in the legend of Fig. 3.1. The activity of the CAT reporter in the presence of Gal4 DBD alone (lane 1) was taken to be maximal and that against which all other determined CAT activities were compared. The results presented represent the mean (± S.E.M.) of three independent experimental determinations. *Insert*, the catalytically inactive Myc-SIRT1 H363Y coimmunoprecipitates with Flag-BCL11A. Transfections, immunoprecipitations, and immunoblots were conducted as described in Fig. 3.3*A*.

transcriptional repression of the reporter gene. These findings are consistent with previous reports (Langley et al., 2002; Senawong et al., 2003), however, the mechanism(s) for the observed repression mediated by both wild-type SIRT1 and SIRT1 H363Y under the experimental conditions performed is unknown. In contrast, the catalytic activity of SIRT1 was required for enhancement of BCL11A-mediated transcriptional repression. Wild-type SIRT1 enhanced transcriptional repression mediated by BCL11A in a concentration-dependent manner (Fig. 3.7, lanes 9-12). A catalytically inactive form, SIRT1 H363Y, did not enhance BCL11A-mediated repression (Fig. 3.7, lanes 13-16), even though it was capable of interacting with BCL11A in HEK293 cells (insert Fig. 3.7, lane 6). These findings demonstrate that SIRT1 exerts its catalytic activity to stimulate transcriptional repression activity of BCL11A.

Based on the above results, ChIP assays were conducted to determine whether histone deacetylation by SIRT1 underlie the molecular basis for BCL11A-mediated transcriptional repression. Under the experimental conditions employed, co-transfection of Gal4-BCL11A and SIRT1 resulted in a decrease in the level of acetylated histones H3 and/or H4 associated at the promoter region when compared with that observed by transfection of either expression vector individually (Fig. 3.8, compare lanes 6, 8, 10, and 12 of the upper panel). Co-transfection of Gal4-BCL11A and SIRT1 H363Y resulted in no change in the level of acetylated histone

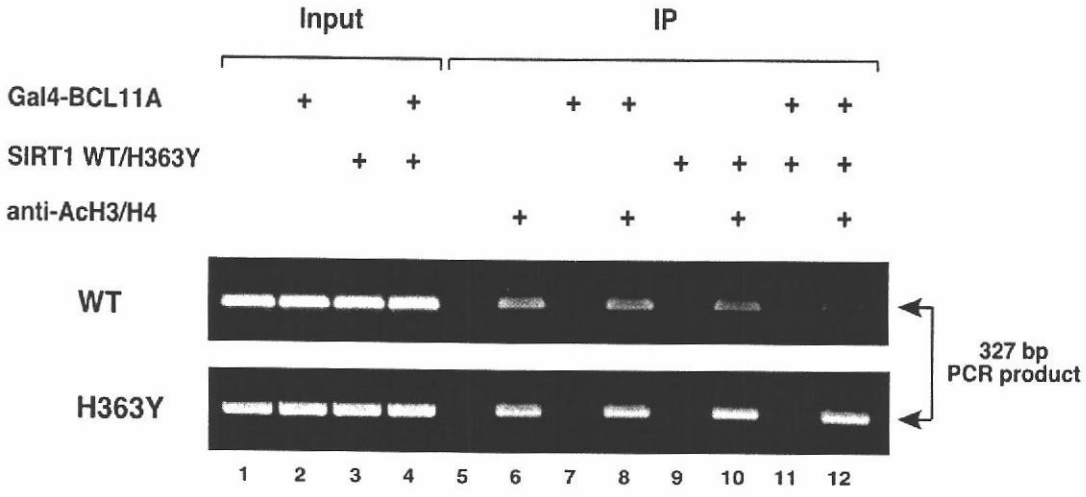

Figure 3.8. **SIRT1 stimulates deacetylation of template-associated histones H3 and/or H4 in BCL11A-transfected cells.** HEK293 cells were transfected with 3 μg of the $(17\text{-mer})_4$-tk-CAT reporter along with expression vectors encoding Gal4-BCL11A (10 μg) and SIRT1 WT or SIRT1 H363Y (0.5 μg) as indicated. Acetylated histones H3 and H4 were determined by a ChIP assay as described in experimental procedures. Transfection efficiency was normalized as described in the legend of Fig. 3.2. Input lanes (1-4) correspond to amplification reactions conducted using 3.75% (upper panel) and 5% (lower panel) of the lysates used for IP reactions. Lanes 5-12 represent template amplification reactions from samples immunoprecipitated with or without anti-acetylated histone H3/H4 antibodies as indicated. Results are representative of three independent experiments.

H3/H4 that was associated with the reporter gene template (Fig. 3.8, compare lanes 6, 8, 10, and 12 of the bottom panel). These findings suggest that SIRT1-catalyzed histone deacetylation may underlie, at least in part, the ability of the enzyme to stimulate both BCL11A- and BCL11B-mediated transcriptional repressions.

3.5 Discussion

Transcriptional regulation of eukaryotic gene expression in response to developmental or environmental signals is a complex multi-step process that requires the combinatorial action of many cellular factors. Transcriptional repression by the recruitments of TSA-sensitive HDACs have been characterized for many transcription factors including nuclear hormone receptors (Jones et al., 1998), Rb (Luo et al., 1998), YY1 (Yang et al., 1996), CBF1 (Hsieh et al., 1999), and Ikaros (Koipally et al., 1999). In most cases, these repressors recruit TSA-sensitive HDACs by binding the co-represssor mSin3 or NcoR/SMRT, however, Rb and YY1 recruit HDAC to the template via direct interaction with HDACs (Luo et al., 1998).

Recently, we have provided the evidence that TSA-insensitive HDAC, is recruited to the promoter template to repress transcription of a reporter gene (Senawong et al., 2003). This finding implicates the NAD^+-dependent,

TSA-insensitive HDAC, SIRT1, in transcriptional repression mediated by CTIP2, a member of novel C_2H_2-zinc finger protein family. In this study, we demonstrate that BCL11A (CTIP1/EVI9), a close paralog of CTIP2 (BCL11B/Rit1), can also interact directly and recruit SIRT1 to the promoter template to repress transcription. Similar to that of CTIP2, the proline-rich region of BCL11A/CTIP1 was found to interact strongly with sirtuin homology domain of SIRT1 (see Fig. 3.6*B*), indicating that the interactions between SIRT1 and this novel family of C_2H_2-zinc finger proteins are highly conserved. Vice versa, the sirtuin homology domain is highly conserved among all SIRT proteins, thus it remains possible that other members of the sirtuin family may also interact with both BCL11A and BCL11B, perhaps in different cellular context(s). However, the physiological consequence(s) of these interactions other than transcriptional function remains to be explored.

The findings that overexpression of BCL11A following proviral integration results in generation of myeloid leukemia and that BCL11A induces anchorage independence of NIH 3T3 cells (Nakamura et al., 2000), implicate BCL11A as a dominant oncogene. However, the contribution of BCL11A to neoplastic processes in hematopoietic cells remains unclear. Like BCL11A, SIRT1 can interact directly with p53 and overexpression of SIRT1 inhibits p53 transcriptional activity and p53-dependent apoptosis in response to DNA damage and oxidative stresses (Langley et al., 2002; Luo

et al., 2001). In response to DNA damage and other cellular stresses, p53 is activated to trigger apoptosis and cell-cycle arrest (Appella and Anderson, 2001; Brooks and Gu, 2003). Based on our findings that BCL11A interacts directly and recruits SIRT1 to repress transcription, one can imagine that SIRT1 may link p53 and BCL11A to oncogenesis of hematopoietic cells.

Although, the physiological target gene(s) of BCL11A is unknown, its direct interactions with BCL6 (Nakamura et al., 2000) and COUP-TF proteins (Avram et al., 2000) suggest that BCL11A may involve in the transcriptional repressions mediated by BCL6 and COUP-TF proteins. However, the relative contributions of BCL6 or COUP-TF-dependent and -independent pathways to the overall transcriptional regulatory activities of BCL11A in mammalian cells are unknown. BCL6 encodes a transcriptional repressor required for the development of germinal centers (GCs) and implicated in the pathogenesis of GC-derived B cell lymphoma. (Dalla-Favera et al., 1994; Dent et al., 1999). Recently, BCL6 has been shown to control the expression of the B7-1/CD80 costimulatory receptor in GC B cells, leading the authors to speculate that BCL6 may directly control the ability of B cell to interact with T cells during normal GC development (Niu et al., 2003). Lui et al. demonstrated that BCL11A is required for B cell development and unexpectedly discovered that the absence of BCL11A resulted in abnormal ratios of $\alpha\beta$ to $\gamma\delta$ thymocytes and CD4 to CD8

thymocytes (Liu et al., 2003). This indicates that BCL11A is involved in the differentiation of these thymocyte subsets. BCL11A is expressed in $CD4^-$ $CD8^-$ cells but not $CD4^+CD8^+$cells, suggesting that BCL11A may be involved in early thymocyte development (Liu et al., 2003). These findings support a model that BCL6 and BCL11A function in a common signaling pathway(s).

Our finding that treatment of BCL11A-transfected cells with nicotinamide, an inhibitor of TSA-insensitive class III HDACs, does not fully relieve BCL11A-mediated transcriptional repression suggests that additional corepressor(s) may be required for the repression mechanism(s) of BCL11A. Recruitment of HP1 proteins to the promoter template results in transcriptional repression either by HP1 self-assembly into a supramolecular, heterochromatinized template (Lachner et al., 2001), or by interaction of HP1 with a component of TFIID, $TAF_{II}130$, precluding the nucleation of the preinitiation complex formation (Vassallo and Tanese, 2002). Although, a potential role for HP1 proteins in CTIP2-mediated transcriptional repression has been reported (Rohr et al., 2003), whether or not HP1 proteins may play a role in BCL11A-mediated transcriptional repression is currently being investigated in this laboratory. However, it remains possible that BCL11A and CTIP2 interact with different co-repressor(s)/co-activator(s) under different conditions and future experiments may better clarify these possibilities.

The results described in this study and the previous study (Senawong et al., 2003) strongly suggest that the NAD+-dependent, nicotinamide-sensitive histone deacetylase SIRT1 contributes, at least in part, to the transcriptional repression activities of both BCL11A (CTIP1) and CTIP2(BCL11B). Beyond transcriptional repression, another possibility would be that both BCL11A and BCL11B could themselves be substrates of SIRT1, which could thereby modulate their function(s). This feature will further help to identify and characterize their target genes in order to elucidate the biological functions of these proteins in cells of the hematopoietic and central nervous systems.

3.6 Abbreviations

The abbreviations used are BCL, B cell leukemia; bp, base pair; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CTIP 1 and 2, COUP-TF-interacting proteins 1 and 2; GST, glutathione S-transferase; HA, hemagglutinin; HDAC, histone deacetylase; HEK293, human embryonic kidney 293 cells; IgG, immunoglobulin G; kDa, kilodalton; NAM, nicotinamide; Sir2, silent information regulator 2; SIRT1, Sir2-like protein 1 or sirtuin 1; TSA, trichostatin A; WT, wild type.

3.7 Acknowledgements

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Chapter 4

Conclusions

CTIP proteins are two novel and highly related C_2H_2 zinc finger proteins that have been implicated in COUP-TF signaling (Avram et al., 2000), etiology of myeloid (Nakamura et al., 2000) and lymphoid (Bernard et al., 2001; Satterwhite et al., 2001) malignancies, and hematopoietic cell development (Liu et al., 2003; Wakabayashi et al., 2003). However, the precise cellular function(s) and the contribution of these proteins to neoplastic processes and hematopoietic cell development remain to be investigated. CTIP proteins have been shown to possess transcriptional repression activity (Avram et al., 2000; Avram et al., 2002) that may utilize a different mechanism(s) from that utilized by some C_2H_2 zinc finger transcription factors and several nuclear receptors (Struhl, 1998).

A greater understanding the molecular mechanism(s) underlying the CTIP-mediated transcriptional repression would contribute additional knowledge toward the goal of elucidating the cellular function(s) of these proteins. The studies described herein were designed to contribute additional understanding of the molecular mechanisms underlying the transcriptional repression activity of CTIP proteins through the functional characterization of a potential CTIP-interacting protein(s).

Previous studies demonstrated that CTIP proteins repressed transcription of a reporter gene in a TSA-insensitive manner (Avram et al., 2000; Avram et al., 2002), suggesting that this repression mechanism(s) may not involve TSA-sensitive histone deacetylation catalyzed by member(s) of class I and II HDACs. Several potential repression mechanisms that may underlie the CTIP-mediated transcriptional repression have been proposed (Avram et al., 2002). One of those potential mechanisms is that CTIP proteins may exert TSA-insensitive histone deacetylation catalyzed by TSA-insensitive HDAC(s), such as SIRT1, to repress transcription.

In this study, SIRT1 was demonstrated to interact with CTIP proteins both in vitro and in mammalian cells. The SIRT1•CTIPs interactions were strongly supported by the findings that endogenous SIRT1 and CTIP proteins interacted in the cellular context, *i.*e. in the absence of overexpression. Moreover, column chromatography revealed that SIRT1 and CTIP2 were components of a large complex in Jurkat cell nuclear extracts. The SIRT1 interaction interfaces of both CTIP1 and CTIP2 were identified and contained within the proline-rich regions of these proteins. These results may be relevant to the findings that these highly conserved regions harbor an autonomous transcriptional repression activity when fused to the GAL4 DNA binding domain (Avram D and Leid M, unpublished results). The CTIP interaction interface of SIRT1 was contained within the

amino-terminal part of sirtuin homology domain that overlaps, at least partially, with the catalytic domain of the enzyme.

In light of results demonstrating that SIRT1 associates with CTIP proteins in mammalian cells, SIRT1 may underlie the transcriptional repression activity of CTIP proteins. In order to determine if SIRT1 underlies the mechanism(s) of CTIP-mediated transcriptional repression, sequential experiments were conducted. First, GAL4 reporter gene assays demonstrated that CTIP-mediated transcriptional repression was inhibited, at least partially, by nicotinamide, an inhibitor of the NAD^+-dependent, TSA-insensitive HDACs. Second, ChIP assays demonstrated that TSA-insensitive, but nicotinamide-sensitive histone deacetylation might underlie transcriptional activity of CTIP proteins due to the observed decrease in levels of acetylated histones H3 and/or H4 at the promoter region of a reporter gene upon overexpression of CTIP proteins. Third, ChIP assays revealed that endogenous SIRT1 was recruited to the promoter template of a reporter gene in mammalian cells upon overexpression of CTIP proteins. Fourth, GAL4 reporter gene assays demonstrated that SIRT1 enhanced the transcriptional repression mediated by CTIP proteins and this enhancement required the catalytic activity of SIRT1. Finally, ChIP assays demonstrated that SIRT1 enhanced the deacetylation of template-associated histones H3 and/or H4 in CTIP-transfected cells. These results support the hypothesis that the observed TSA-insensitive manner of CTIP-mediated transcriptional

repression was due to the ability of CTIP proteins to recruit the TSA-insensitive HDACs to repress transcription.

In summary, these results strongly suggest that CTIP-mediated transcriptional repression involves the recruitment of SIRT1 to the template, at which the TSA-insensitive, NAD^+-dependent histone deacetylase catalyzes deacetylation of promoter-associated histones H3 and/or H4. One outcome of this deacetylation may simply be chromatin condensation, leading to transcriptional repression. Alternatively and/or additionally, this deacetylation may ultimately result in formation of heterochromatin, contributing to a persistent, silenced state. This feature might be helpful for identification and characterization of the target genes under the control of CTIP proteins in cells of hematopoietic system and/or the developing central nervous system.

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