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Title: Deciphering the Molecular Mechanisms of the Transcriptional Regulation Mediated by Orphan Nuclear Receptor COUP-TFI and C2H2 Zinc Finger Protein Ctip2

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COUP-TFI, an orphan nuclear receptor of the steroid/thyroid hormone receptor superfamily, plays important roles in homeostasis and the CNS development, including differentiation, patterning, axonal projection, cell migration, cortical arealization and the temporal specification of neural stem cells. A number of COUP-interacting proteins have been described previously, and the majority of these proteins were identified and/or characterized by yeast two-hybrid systems. None of these COUP-interacting proteins, to our knowledge, have been demonstrated to be recruited to the promoter of target genes in a COUP-dependent manner, and a systematic study of COUP-TFI complexes in mammalian cells has not been conducted.

In our study, we have identified a number of COUP-TFI-interacting proteins in HeLa S3 cells by a tandem affinity purification procedure. We found that COUP-TFI associated with transcriptional regulatory proteins, including the nuclear receptor corepressor (NCoR) and TIF1β, and a proapoptotic protein DBC1. In vitro experiments revealed that COUP-TFI interacted directly with NCoR but in a manner different from that of other nuclear receptors. DBC1 stabilized the interaction between COUP-TFI and NCoR by interacting directly with both proteins. The gene encoding the anti-apoptotic protein TNFAIP8 (TNFα-induced protein 8) was identified as being repressed by COUP-TFI in a manner that required several of the component proteins of the COUP-TFI complex. Our studies also highlight a central role for COUP-TFI in the induction of the TNFAIP8 promoter by TNFα.

Ctip2, a C2H2 zinc finger transcription factor, plays crucial roles in the development of the CNS, and in the immune and cutaneous systems, and is essential for post-natal life. Germline deletion of Ctip2 results in arrest of early T cell development with the complete absence of αβ T cells. In contrast, deletion of Ctip2 at the $CD4^+$ $CD8^+$ double-positive (DP) stage allows development of DP cells but not transition to the $CD4^+$ or $CD8^+$ single positive (SP) stage. Thus, Ctip2 plays an important role in at least two stages of T cell development: a very early stage prior to the development of the DP cell and also in the DP → $CD4^+$ or $CD8^+$ single positive (SP) transition.

In the effort to understand the influence of cell signaling pathways on the transcriptional regulatory activity of Ctip2 in thymocytes, we adapted an ex vivo system in which the combination of phorbol ester PMA and calcium ionophore A23187 (P/A) triggered differentiation of primary mouse thymocytes. We have discovered a dynamically regulated pathway in stimulated mouse DP cells involving sequential and linked post-translational modifications (PTMs) of Ctip2, as follows: Phosphorylation $\rightarrow$ Dephosphorylation $\rightarrow$ SUMOylation. SUMOylation of Ctip2 is dynamic and is tightly regulated by the phosphorylation status of Ctip2. Our data also suggest that SUMOylation of Ctip2 may serve as a molecular switch that converts Ctip2 from a repressor to an activator of the promoter of Id2 gene, a newly identified Ctip2-targeted gene. We have also demonstrated the molecular basis for the output of this switch: P/A treatment promotes interaction of Ctip2 with the transcriptional coactivator p300 on the Id2 promoter.

These results described herein provide a framework for understanding the mechanisms underlying the transcription regulatory activities of COUP-TFI and Ctip2, and how these two transcription factors are regulated by the TNFα signaling pathway and the T cell signaling pathway, respectively. These studies may contribute to a better understanding of the molecular and cellular basis for COUP-TFI and Ctip2 function in vivo.

Deciphering the Molecular Mechanisms of the Transcriptional Regulation Mediated by Orphan Nuclear Receptor COUP-TFI and C2H2 Zinc Finger Protein Ctip2

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Lingjuan Zhang, Author

CONTRIBUTION OF AUTHORS

Dr. Mark Leid designed research. Lingjuan Zhang performed research. Lingjuan Zhang and Dr. Mark Leid analyzed and wrote the manuscripts.

Chapter 2: All experiments were performed by Lingjuan Zhang, except that mass spectrometry identification of COUP-interacting proteins was performed by Philip R. Gafken (the Proteomics Facility, Fred Hutchinson Cancer Research Center, Seattle, Washington). Xiao Liu provided technical assistance in generation of stable cell line, preparation of cell lysate for ChIP analyses and RNA extraction. Dr. Chrissa Kioussi generously provided the NCoR antibody.

Chapter 3: Most of the experiments were performed by Lingjuan Zhang. Dr. Acharawan Khamsiritrakul performed the experiments described in Fig. 3.4B and 3.4C. Xiao Liu contributes to generation of the SUMO-mutant constructs as well as the constitutive SUMO-Ctip2 constructs, and he also assisted in the experiments described in Fig. 3.3 and 3.8. Dr. Theresa Filtz and Dr. Walter Vogel provided valuable suggestions for this project.

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CHAPTER 1

GENERAL INTRODUCTION

Regulation of gene expression is the primary regulatory process that is used by cells, tissues and organisms to tightly control the complex programming of cellular metabolism, and development of tissue and organs (Greive and von Hippel, 2005). It is not surprising, therefore, that elaborate cellular mechanisms have evolved to regulate the first step in gene expression — transcription, to determine which genes are turned on, and when. At the chemical level, transcription means copying DNA templates that correspond to genes into RNA transcripts. During this process, monomeric ribonucleotides bind specifically and sequentially to the DNA template while RNA polymerases advance processively along the DNA template and catalyze the formation of covalently linked RNA chain. The function of RNA polymerases is regulated by transcription factors that operate directly and specifically on the regulatory DNA sequences surrounding the promoter of specific gene (Kadonaga, 1998). The complex interplay between transcription regulators, general transcription factors and chromatin structure enables the establishment of a barrier to gene activation, ensuring that a gene can only be transcribed at specific stage of the cell cycle when appropriate signals have been sent to the nucleus (Hill and Treisman, 1995).

1. Regulation of gene expression by transcription regulators — in the human genome, there are more than 2000 genes encoded for transcription factors (Venter et al., 2001), which positively or negatively regulate gene transcription as activators and repressors, respectively. Both types of control proteins typically contain one or more DNA binding domains (DBDs) which tether them to the promoter of the genes that they regulate, as well as a functional domain that is responsible for activation or repression (Jones and Kadonaga, 2000).

Numerous mechanisms through which transcription activators function have been discovered. First, the recruitment of proteins with histone-modifying and -remodelling activities, such as histone acetyltransferases (HATs), including HAT1 protein, GCN5 protein, the TAFII250 subunit of TFIID, p300/CBP, P/CAF, and the SRC-1 family of coactivators (Roth et al., 2001). These HATs catalyze the acetylation of core histones, particularly H3 and H4, which causes a reduction in the affinity of histone–DNA interactions and thus leads to increased access of transcription initiation machinery to the repressed chromatin template (Roth et al., 2001). A second mechanism through which transcription regulators facilitate activation involves a direct interaction with the general transcription machinery components, including TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF and TFIIH, and even RNA polymerase itself, The third mechanism involves the recruitment of coactivators that facilitate transcription activation in response to specific activator (Muratani and Tansey, 2003). The net effect of these interactions is to remodel the chromatin structure at the locus, recruit RNA polymerase to the transcription start site and transcribe the gene. Transcription repressors, on the other hand, antagonize many of the same steps: recruiting histone deacetylases (HDACs) to deacetylate histones, blocking the association of the general transcription machinery with the target locus and interacting with transcription corepressors (Gray and Ekstrom, 2001).

2. Epigenetic gene regulation — Epigenetics refers to the alteration of cellular phenotype without altering the genotype. The modulation of epigenetic mechanisms enables the changes of chromatin accessibility to transcriptional regulation locally and globally via modification of the DNA and by modification of surrounding histones. These changes may remain through cell divisions for

the rest of the cell's life and may also last for multiple generations (Biel et al., 2005).

The fundamental repeating unit of eukaryotic chromatin is the nucleosome core particle, in which approimately 147 DNA base pairs are wrapped around the histone octamer consisting of two copies each of the four core histones: H2A, H2B, H3 and H4 (Lorch et al., 1999). The highly conserved amino termini of histones (called histone tails), are flexible chains at the surface of the nucleosome, and are frequent targets for post-translational modifications, including acetylation, methylation, ubiquitylation, phosphorylation and SUMOylation (Spotswood and Turner, 2002).

2.1. Histone Acetylation — Acetylation is the most highly studied and the best understood histone modification, and it takes place at conserved lysine residues (J. A. Johnson, 1999). Acetylation is often associated with "active" transcription (Biel et al., 2005). First, acetylation neutralize the positive charge of histone tails and thus leads to a decrease of the affinity of histone to the negatively charged phosphates of the DNA backbone, resulting in increased DNA accessibility to the transcription initiation machinery. Lysine acetylation of histone may also function as a docking site to recruit other activating chromatin modifying enzymes. Many proteins that help activate transcription, including the transcription complex SWI/SNF, were found to bind acetyl-lysine specifically through their bromo domains (Lomvardas and Thanos, 2001). HATs and HDACs activities are responsible for the control of histone acetylation levels (Kurdistani and Grunstein, 2003).

2.2. Histone Methylation — Similar to acetylation, histone methylation

can modulate histone interaction with DNA, and also acts as docking modules for related factors (Rice and Allis, 2001). However, methylation at different histone residues may have different biological effects. For example, histone methylation at H3K9, H3-K27 or H4-K20 is often associated with transcriptionally silent chromatin, whereas transcriptionally active euchromatin is methylated at H3-K4, H3-K36 or H3-K79 (Biel et al., 2005). Specific levels of histone methylation are controlled by histone methyltransferases (HMT) (Kouzarides, 2002)

3. Regulation of transcription factors by signal transduction pathways — to control transcription accurately, it takes more than just an elaborate series of interactions. The transcription regulators themselves have to be present at the right time, at the right place and in the right amounts, and their activity has to be fine-tuned to generate appropriate levels of transcription for each gene. Recently, therefore, two very broad field of research, signal transduction and control of gene expression, have merged to become a pivotal arena for molecular and cellular biology (Brivanlou and Darnell, 2002).

By sensing extracellular signals, cells initiate a series of signaling cascades, which then transduce to the nucleus and eventually lead to changes of gene expression, which involve: (1) a cascade of transcriptional control of transcription factor genes themselve, and (2) post-translational modifications of already formed transcription factors. In recent years it has become evident that post-translational modifications (PTMs), including phosphorylation, acetylation, methylation, ubiquitination and SUMOylation, play essential roles in modulating activity of transcription regulators (Hill and Treisman, 1995).

3.1. Phosphorylation and protein function — Protein phosphorylation is the most common type of post-translational modification used in signal transduction. It affects every basic cellular process, including growth, metabolism, proliferation, differentiation, organelle trafficking, motility, membrane transport, immunity, muscle contraction, learning and memory. Protein kinases catalyze the transfer of the γ-phosphate from ATP to Ser, Thr or Tyr residues of protein targets in eukaryotes (Ubersax and Ferrell, 2007).

One of the most studied and also the most understood pathways is initiated by activation of growth factor receptors (Hazzalin and Mahadevan, 2002), which harbor intrinsic tyrosine-kinase activity. Upon ligand binding, growth factor receptors autophosphorylate themselves, which subsequently activate many downstream kinase cascades, including the phosphatidylinositol 3-kinase pathway, the protein kinase C pathway, the pathways that regulate small GTPases, such as Rho, Rac and Cdc42, and the extracellular-signal-regulated kinase / mitogen-activated protein kinase (ERK/MAPK) pathway (Hazzalin and Mahadevan, 2002). Activated kinases, such as PKCs and ERK1/2 can enter the nucleus and phosphorylate Ser or Thr residues of a variety of resident transcription factors that bind to the upstream regulatory elements of target genes. For example, it has been shown that epidermal growth factor (EGF) by activating EGF receptor, induces full activation of ERK1/2 within 1minute of treatment (Hazzalin et al., 1997). Activated ERK1/2 then translocates from the cytosol to the nucleus, and phosphorylates Elk1(Ets-like protein-1), a member of the Ets family of transcription activators, at Ser383 and Ser389 residues, leading to increase of its transcription activation activity. Phosphorylation not only enhances Elk1's DNA binding (Yang et al., 1999), but also leads to the recruitment of the

CBP/p300 coactivator complex through direct protein-protein interaction between Elk1 and p300, to target promoter of Elk1 (Li et al., 2003).

In some other cases, dephosphorylation also plays a crucial role in regulating transcription factor function. One example is the calcium signaling and NFAT activation. NFAT (nuclear factors in activated T cells) molecules are heavily phosphorylated in resting T cells and localize primarily in the cytosol (Okamura et al., 2000). Upon activation of T cell receptors, internal Ca^{2+} concentration increases, leading to activation of calcineurin, a phosphatase that dephosphorylates NFAT. Dephosphorylated NFAT then translocates into the nucleus, resulting in activation of genes responsible for generating immune responses (Rao et al., 1997).

3.2. SUMOylation and protein function — Recognition of reversible, covalent modification of proteins by small ubiquitin-related modifier (SUMO) has occurred only relatively recently. Yeast SUMO (*SMT3*) was identified as a suppressor the centromeric protein Mif2 in 1995 (Meluh and Koshland, 1995), but the mechanism of this suppression remained obscure. Subsequently, mammalian SUMO was identified in yeast two-hybrid screens as an interaction partner for the DNA repair proteins RAD51/RAD52 (Shen et al., 1996), the proapoptotic protein Fas (Okura et al., 1996), and the acute promyelocytic leukemia protein PML (Boddy et al., 1996). However, the functional consequences of protein SUMOylation did not come into focus until two groups independently described a subcellular compartmental translocation that was induced by SUMOylation (Mahajan et al., 1997; Matunis et al., 1996). Both groups found that the unmodified Ran-GTPase activating protein, RanGAP1, was cytosolic but the SUMOylated species translocated to the nucleus by

virtue of its interaction with a nucleoporin protein, RanBP2 (Mahajan et al., 1997). The latter finding was important for three reasons: **(1)** SUMO was demonstrated to be a covalent, reversible modifier of proteins, **(2)** SUMOylation conferred a change in protein function (subcellular localization), and **(3)** these findings implied that RanBP2 may interact preferentially with SUMOylated RanGAP1, implying the first existence of SUMO-interaction motifs (SIM), which was later confirmed (see below).

The human genome encodes four SUMO proteins, SUMO1-4, which bear little sequence similarity to ubiquitin. SUMO2 and SUMO3 are more highly related to each other than to SUMO1 or SUMO4, and this difference is also reflected in the specificity of SUMOylated targets, as SUMO1 and SUMO2/3 appear to conjugate to different substrates (Geiss-Friedlander and Melchior, 2007). The specificity of SUMO4, and its ability to be conjugated to substrates in vivo, remain open questions (Guo et al., 2004; Owerbach et al., 2005).

All SUMO proteins are expressed as immature forms that require proteolytic processing prior to conjugation. This processing is carried out by SUMO-specific isopeptidases of the sentrin-specific protease (SENP) family (Geiss-Friedlander and Melchior, 2007). The mature form of SUMO proteins is then activated by the E1 activating enzyme, a heterodimer composed of AOS1 and UBA2 (Desterro et al., 1999; Gong et al., 1999; Johnson and Blobel, 1997; Okuma et al., 1999). SUMO is then transferred from AOS1/UBA2 E1 activating complex to the E2 conjugating enzyme UBC9 (Desterro et al., 1999; Johnson and Blobel, 1997; Lee et al., 1998; Saitoh et al., 1998), from which it is transferred to substrates with or without catalytic facilitation by SUMO E3 ligases (Geiss-Friedlander and Melchior, 2007). While only one E2 enzyme

exists in the SUMO pathway (UBC9), a multitude of E3 ligases have been described, including those harboring a SP-RING motif, and those similar to the RanBP2 and polycomb protein families (Geiss-Friedlander and Melchior, 2007). In contrast to ubquitination of proteins, SUMOylation usually involves the addition of a single SUMO moiety to a target protein (Geiss-Friedlander and Melchior, 2007), with the exception of SUMO2/3, which may form polySUMO chains on substrates (Mukhopadhyay et al., 2006; Tatham et al., 2001). Removal of SUMO from conjugated proteins is catalyzed by the SENP family of proteins (SENP1-3 and SENP5-7; (Di Bacco et al., 2006; Gong and Yeh, 2006), which are also required for production of the mature forms of SUMO (see above).

SUMOylation may affect protein localization, stability and/or activity, most likely through altered intra- or intermolecular interactions (Geiss-Friedlander and Melchior, 2007). These interactions are mediated by recognition of the SUMO motif by proteins harboring a SIM (see above), which generally consists of a stretch of acidic amino acids and/or serine residues preceded or followed by a patch of hydrophobic amino acids (Hecker et al., 2006; Song et al., 2004). Although protein SUMOylation was originally implicated in transcriptional repression or extinguishing activation by several factors, including p300 (Girdwood et al., 2003), HDAC1 (David et al., 2002), KAP1 (Ivanov et al., 2007) and PPARγ (Pascual et al., 2005), a number of more recent reports have described a role for SUMOylation in transcriptional activation. For example, SUMOylation of Ikaros (Gomez-del Arco et al., 2005), NFAT1 (Terui et al., 2004), Oct4 (Wei et al., 2007), and JunB (Garaude et al., 2008) promotes transcriptional activation by these factors.

4. COUP-TFI

4.1. Nuclear Receptor — Nuclear receptors (NRs) belonging to steroid/thyroid hormone receptor superfamily are ligand-dependent transcription factors that regulate critical processes in diverse aspects of growth, development and homeostasis (Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003). Extensive sequence and functional analyses of nuclear receptors revealed a common modular structure, characterized by several functional domains: a variable amino-terminal region (A/B); a highly conserved DNA binding domain (DBD, or C) that consists of two zinc-finger motifs and, a variable hinge (linker) region (D) which connects the DBD to the ligand binding domain (LBD, or E/F) in the C-terminal (carboxyl-terminal) (Fig. 1.1A). The DBD mediates NR's specific interaction with DNA, while the LBD is essential for hormone binding and is also involved in dimerization with other transcription factors and the recruitment of transcription co-factors (Lonard and O'Malley B, 2007). Transcriptional functions of nuclear receptors are mediated by the recruitment of cofactors that are broadly defined as coactivators and corepressors, which can modify the local chromatin environment or interact with components of the core transcriptional machinery to regulate transcription. Previous studies reveal a common mechanism that controls the precisely regulated switch of nuclear receptors from gene repression to gene activation. Nuclear receptors, such as thyroid hormone receptors (TRs) and retinoid acid receptors (RARs) bind to the response elements on the promoters of their target genes in the absence of their cognate ligand and function as potent repressors by the interacting with specific corepressor proteins, including NCoR corepressor complex (Perissi et al., 2004; Zamir et al., 1996). Upon ligand binding, NRs change conformation in a way that favors corepressor

release and recruitment of coactivator complexes (Zamir et al., 1996). TBLR1, a WD-40-containing NCoR interacting factor may serve as a specific adaptor to target NCoR to proteasome complex and mediate the exchange of the NR corepressors for coactivator upon ligand binding (Perissi et al., 2004).

4.2. COUP-TF gene family — COUP-TFs (chicken ovalbumin upstream promoter transcription factors) protein family is one of the most characterized members of the steroid/thyroid nuclear receptor superfamily. Because their cognate ligand has yet to be defined, COUP-TFs are classified as orphan receptors. In mammals, three COUP-TF genes have been cloned, named COUP-TFI (also known as Nr2f1 or EAR3), COUP-TFII (*Nr2f2* or *ARP1*) and COUP-TFIII (*Nr2f6* or *EAR2*). The COUP family of proteins play unique roles in fetal development, including neurogenesis and angiogenesis, and possibly in metabolic homeostasis in adult organisms (Pereira et al., 2000). COUP-TF genes are highly conserved between species (see COUP-TFI in Fig.1.1B). COUP-TFI and COUP-TFII share a high degree of sequence homology within DNA (DBD) and ligand (LBD) binding domains, but harbor divergent amino terminal regions (Avram et al., 1999). In the mouse, the single deletion of COUP-TFI or COUP-TFII results in perinatal and embryonic lethality, respectively, possibly due to disrupted neuronal development (COUP-TFI$^{-/-}$) (Qiu et al., 1997; Studer et al., 2005; Zhou et al., 1999) and aberrant angiogenesis, skeletal muscle development and cardiac development (COUP-TFII$^{-/-}$) (Lee et al., 2004; Pereira et al., 1999). COUP-TFIII, which is more distantly related to either COUP-TFI or –TFII than the latter two are to each other (Avram et al., 1999; Robinson-Rechavi et al., 2003), also plays an important role(s) in development. COUP-TFIII$^{-/-}$ mice exhibit disruption of noradrenergic homeostasis along with enhanced nociception and

dysregulation of genes that are important for proper circadian timing (Warnecke et al., 2005). These findings highlight the absolute essentiality of the COUP-TF family of orphan nuclear receptors in developmental processes, and indicate that these proteins do not function redundantly in vivo.

Despite of the intensive studies of COUP-TF family proteins, the precise molecular mechanism underlining their important physiological roles remains elusive. COUP-TFs were generally considered as transcription repressors; however, growing evidence suggests that COUP-TFs can also function as activators of transcription. All COUP-TF family members bind to a variety of directed repeats, including DR1, DR3, DR4 and DR5 of the 5' – AGGTCA – 3' motif (Park et al., 2003), which can also be bound by many other nuclear receptors. In transient transfection assays, it has been demonstrated that COUP-TFs homodimerize and heterodimerize with other COUP-TF subfamily members (Avram et al., 1999; Cooney et al., 1992) or other nuclear receptors, such as retinoic acid (RAR), retinoid X (RXR) and vitamin D3 (VDR) receptors (Ben-shushan et al., 1995; Cooney et al., 1993; Cooney et al., 1992; Kliewer et al., 1992). Therefore, it has been suggested that COUP-TFs may inhibit transcription activation mediated by hormone nuclear receptors through direct competition for the available binding sites. Besides functioning as passive repressors, COUP-TFs also appear to possess intrinsic repression activity based on the fact that fusion of its putative ligand binding domain (LBD) to the GAL4 DNA binding domain retained its ability to repress basal transcriptional activity (Leng et al., 1996). It is likely that COUP-TFs can repress transcription through a mechanism similar to that described for other hormone nuclear receptors, which associate with corerpessor complex in the absence of stimulus (ligand binding). Moreover, COUP-TFI may recruit distinct protein

complexes, such as transcription coactivator or corepressor complex, to specific promoter under a specific cellular context, thereby mediating different effects on transcription.

5. Ctip2 — Ctip2, which is an essential protein for post-natal life in the mouse, plays crucial roles in the development, and presumably function, of several organ systems, including the central nervous (Arlotta et al., 2005), immune (Wakabayashi et al., 2003b), and cutaneous (Golonzhka et al., 2009a; Golonzhka et al., 2009b) systems. Moreover, Ctip2 may function as a tumor suppressor in T cells, primarily based on loss of homozygosity studies (Bezrookove et al., 2004; Girdwood et al., 2003; Goldberg et al., 2007; MacLeod et al., 2004; Nagel et al., 2003; Okazuka et al., 2005; Przybylski et al., 2005; Su et al., 2004; Warnecke et al., 2005), and the demonstration of dysregulated Ctip2 expression/function in 20% of all T-cell acute lymphoblastic leukemias (T-ALL) (Goldberg et al., 2003; Sun et al., 1999). T-ALL represents a particularly aggressive form of hematopoietic cell cancer that disproportionately afflicts infants and children (Goldberg et al., 2003; Sun et al., 1999). Thus, the definition of the molecular basis for the function of Ctip2 in T cells, including the elucidation of the mechanistic basis for the regulation of Ctip2 function by cell signaling pathways and identification of the Ctip2 transcriptional regulatory network, is highly significant and of great biomedical interest.

5.1. History of the Ctip proteins — Ctip1(also known as Bcl11a) and Ctip2 (also known as Bcl11b) were originally cloned by our laboratory in 2000 based on the ability of these proteins to interact with and mediate transcriptional repression of COUP-TF orphan nuclear receptors (Avram et al.,

2000). We referred to these two proteins as COUP-TF-interacting proteins 1 and 2 (CTIP1 and CTIP2, respectively) based on our knowledge of the activities of the proteins at that time. Neal Copeland's lab later reported the identification of Bcl11a/Ctip1 as a site of retroviral integration that was causally associated with murine myeloid leukemia (Nakamura et al., 2000). Several reports were subsequently published implicating Ctip1 in human B cell leukemias (Dyer and Oscier, 2002; Kuppers et al., 2002; Martin-Subero et al., 2002; Martinez-Climent et al., 2002; Nakamura et al., 2000; Satterwhite et al., 2001).

Bcl11b (Ctip2), so named by NCBI because of its close sequence identity to Bcl11a (Avram et al., 2002), has never been implicated in B cell leukemia. *Ctip2* was recloned by Kominami's group in 2003, and referred to as radiation-induced transcript-1 (Rit-1) because they found that Ctip2 was mutated in the mouse thymus in the process of gamma radiation-induced tumorigenesis (Wakabayashi et al., 2003a). This finding led Kominami's group to hypothesize that Ctip2 may function as a tumor suppressor gene in T cells (Wakabayashi et al., 2003b), and this has been supported by reports of a link between loss of homozygosity at the human *Ctip2* locus and T cell leukemia (Bezrookove et al., 2004; MacLeod et al., 2004; MacLeod et al., 2003; Nagel et al., 2003; Okazuka et al., 2005; Su et al., 2004; Wakabayashi et al., 2003b), and the recent finding that 20% of childhood T-ALL exhibit abnormalities in Ctip2 expression (Okura et al., 1996; Przybylski et al., 2005; Su et al., 2006). Kominami's group also cloned two other splice variants of Ctip2, defined herein as Ctip2-long (containing exon 3) and Ctip2-short (lacking exons 2 and 3; see Fig. 1.2A). The organization of the mouse *Ctip2* genomic locus is shown in Figs. 1.2A and 1.2B, and it is of note that the majority of the

Ctip2 open reading frame is encoded by exon 4 of this locus (Fig. 1.2B), which was the basis for our targeting this exon by homologous recombination. Ctip2 is highly conserved at the amino acid level across species (not shown).

5.2. Ctip2 is a transcriptional regulatory protein — Ctip2 represses transcription of reporter genes in transiently transfected cells, either by tethering to other promoter-bound transcription factors or by direct, sequence-specific DNA binding activity (Avram et al., 2002; Senawong et al., 2003; Topark-Ngarm et al., 2006). The regulatory element to which Ctip2 binds is reminiscent of a GC-box as was revealed by a binding-site selection technique (Avram et al., 2002). Ctip2-mediated transcriptional repression appears to be due to the recruitment of the class III histone deacetylase (HDAC) SIRT1 (Senawong et al., 2003) and/or the class I HDACs, HDAC1 and HDAC2 (Topark-Ngarm et al., 2006), to the promoter template, the latter of which occurs within the context of the **Nu**cleosome **R**emodeling and **D**eacetylation (NuRD) complex (Cismasiu et al., 2005; Topark-Ngarm et al., 2006). Ctip2 has also been reported to sequester and target transcriptional activators, such as HIV Tat, to heterochromatic loci, which may represent a third mechanism of Ctip2-mediated transcriptional silencing (Marban et al., 2005). The hypothesis that Ctip2 functions as a transcriptional repressor has been supported by transcriptome analyses in mouse T cells (Kastner et al., manuscript in preparation) and human neuroblastoma cells (Topark-Ngarm et al., 2006). However, approximately one-third of the genes that were dysregulated in the DP (double positive) cells of Ctip2-null mice were down-regulated relative to control T cells, suggesting that Ctip2 may act as a transcriptional activator in some promoter and/or cell contexts. We have also found that Ctip2 functions as a transcriptional activator in a promoter

context-dependent manner (Golonzhka et al., 2009a; Golonzhka et al., 2009b). The underlying mechanism(s) for Ctip2-mediated transcriptional activation remains unknown.

5.3. Functions of Ctip2 during development of the CNS and cutaneous systems — Ctip2 is expressed early during mouse development as well as in the adult animal, and in both cases, expression is most predominant in the CNS, thymus, and skin/epithelial structures (Golonzhka et al., 2007; Leid et al., 2004). Mice null for expression of Ctip2 exhibit perinatal lethality, which is reminiscent of the COUP-TFI$^{-/-}$ phenotype (Qiu et al., 1997; Yamaguchi et al., 2004), and severe phenotypes in all tissues that express the gene. For example, Jeff Macklis' lab described defects in axonal projection by corticospinal neurons in the CNS in Ctip2-null mice (Arlotta et al., 2005), and we have observed severe defects in axonal path-finding in this and other regions of the CNS (Golonzhka et al., unpublished observations).

We have recently found that lack of Ctip2 also affects the cutaneous system: Ctip2-null mice exhibit a compromised barrier formation, leading to dehydration, electrolyte imbalances, and disruption of keratinocyte proliferation in the epidermis (Golonzhka et al., 2009b), whereas lack of Ctip2 in the oral ectoderm leads to a differentiation block in the ameloblast lineage, cells which are responsible for enamel secretion in the developing tooth (Golonzhka et al., 2009b).

5.4. Role of Ctip2 in thymocyte Development — Thymocyte development proceeds through multiple, sequential stages that are distinguished by the differential expression of cell surface markers and

increasingly restricted differentiative potential (Fig. 1.3). Multipotent progenitors, which are pluripotent cells that retain the ability to differentiate into B cells, dendritic cells (DC) or natural killer (NK) cells, first invade the thymus to enter the T cell pathway (Rothenberg and Taghon, 2005). These cells, and the next three downstream of them, are known as "double negative" (DN) cells because they express neither CD4 nor CD8 (Rothenberg and Taghon, 2005). DN1 cells, which express CD44 and c-kit then differentiate into DN2 cells and first begin to express CD25 while maintaining expression of CD44 and c-kit (Rothenberg and Taghon, 2005). The DN1→DN2 transition is a specification step, generating cells that have a more restricted differentiation potential than their immediate precursor. DN2 cells then differentiate into DN3 cells in a process known as commitment because DN3 cells are now committed to become either the αβ or γδ T cells (Rothenberg and Taghon, 2005). The third step of this pathway involves the differentiation of DN3 cells into DN4 cells, and this is easily the most complicated and least well understood step in thymocyte maturation (Rothenberg and Taghon, 2005). The DN3→DN4 transition is known as β selection, and involves a cascade of proliferation and differentiation events that are initiated by signaling of the pre-T receptor (pre-TCR) after successful recombination at the the TCRβ locus (Rothenberg and Taghon, 2005).

DN3 cells must wait until recombination at the TCRβ locus has been successfully completed before attempting to transition to DN4. This checkpoint seems to be enforced by E2A proteins, particularly E47, together with another basic helix-loop-helix protein known as HEB (Murre, 2005). Failure to complete β selection properly and in a timely manner results in cell death (Rothenberg and Taghon, 2005). DN4 cells give rise to an immature single

positive (ISP; CD8+) T cell, and this cell type subsequently up-regulates expression of CD4 to become the "double positive" (DP) T cell, which expresses the TCR and both CD4 and CD8 coreceptors (Wolfer et al., 2001). DP cells then interact with proteins of the major histocompatibility complexes (MHC), which are expressed by thymic epithelial cells (Jameson and Bevan, 1998; Kisielow and von Boehmer, 1995; Robey and Fowlkes, 1994; Wolfer et al., 2001). The avidity of the interaction of DP cells with MHCs is crucial as interactions that are too strong or too weak both lead to cell death (negative selection and death by neglect, respectively) (Wolfer et al., 2001). DP-MHC interactions that are of intermediate avidity allow the cell to survive and differentiate into single positive (SP; $CD4^+$ or $CD8^+$) T cells via a process known as positive selection (Jameson and Bevan, 1998). DP cells that interact with MHC-class I differentiate along the $CD8^+$ lineage and become known as cytotoxic T cells, while interaction of the DP cell with MHC-class II results in differentiation into $CD4^+$ T cells (helper T cells, T_H) (Jameson and Bevan, 1998). Finally, these "mature" single-positive (CD4+) T cells maintain differentiative potential in the periphery, and this most often is utilized upon stimulation with antigen, such as differentiation of $CD4^+$ cells into Th1 and Th2 cells (Rothenberg and Taghon, 2005). DP cells can also give rise to $CD4^+CD25^+$ T regulatory cells (Treg) and NKT cells though additional differentiation pathways (Rothenberg and Taghon, 2005).

The multiple differentiation and specification steps in thymocyte development are regulated through the concerted action of a series of transcription factors, including Ctip2. Germline deletion of Ctip2 results in a blockade of the DN3→DN4 transition with the complete absence of $\alpha\beta$ T cells, and this block is phenotypically similar to that seen in NCoR- (Jepsen et al.,

2000), E2A- (Bain et al., 1997), and HEB- (Bain et al., 1997; Barndt et al., 1999; Zhuang et al., 1996) null mice, conditionally null c-myb mice when crossed with the appropriate deleter strain (Bender et al., 2004), and transgenic mice ectopically expressing Id2 (Morrow et al., 1999) or a dominant negative form of HEB (Barndt et al., 2000) in thymocytes. In contrast, deletion of Ctip2 at the DP stage by use of a CD4-cre transgene allows development of DP cells but not transition to the SP stage (Albu et al., 2007). Thus, Ctip2 plays an important role in at least two stages of T cell development: β selection (DN3 → DN4 transition) and in the DP → SP transition.

6. Research Objectives — in the studies described below, we first purified a cellular complex harboring the orphan nuclear receptor COUP-TFI using a TAP (tandem affinity purification) strategy and studied how this COUP-TFI complex regulates transcription of a gene involved in the TNFα signaling pathway in Chapter 2. In chapter 3, we addressed how the transcription regulatory activity of Ctip2 is regulated by PTMs, including phosphorylation and SUMOylation, by MAPK pathways in primary mouse thymocytes. We propose that Ctip2 is switch from a repressor to an activator of transcription through PTMs upon activation of T cell signaling pathways, contributing to positive selection of DP thymocytes.

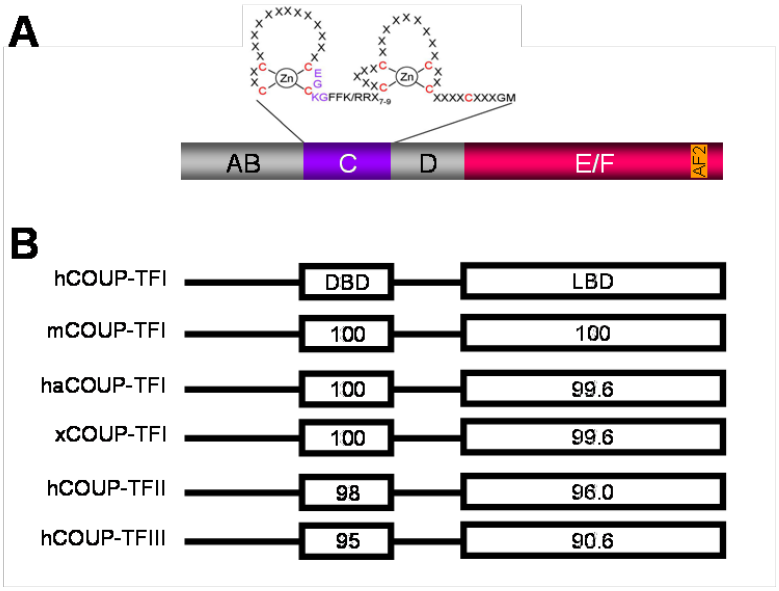

Figure 1.1. COUP-TF gene family. **(*A*),** Schematic diagram of the common modular structure of hormone nuclear receptor. DNA binding domain (C domain) comprised of two Cys2-Cys2 Zinc fingers. Conserved cysteines and a typical P-box, (CEGCKG) are highlighted. The AF2 (activation function-2) domain is located to the C-terminal of the ligand binding domain (E/F) domain. **(*B*),** Sequence homologies within the COUP-TFI gene family. Abbreviation used here: h, human; m, mouse; ha, hamster; x, Xenopus; z, zebrafish

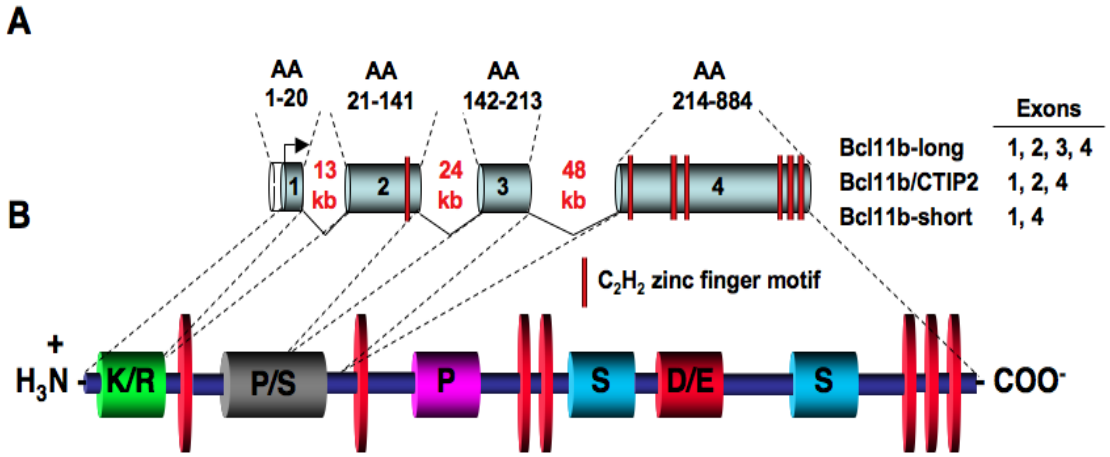

Figure 1.2. Mouse Ctip2 locus. **(*A*)**, organization of mouse Ctip2 genomic locus and splice variants. **(*B*)**, relationship of exonic structure to the protein. Single letter is the amino acid abbreviation

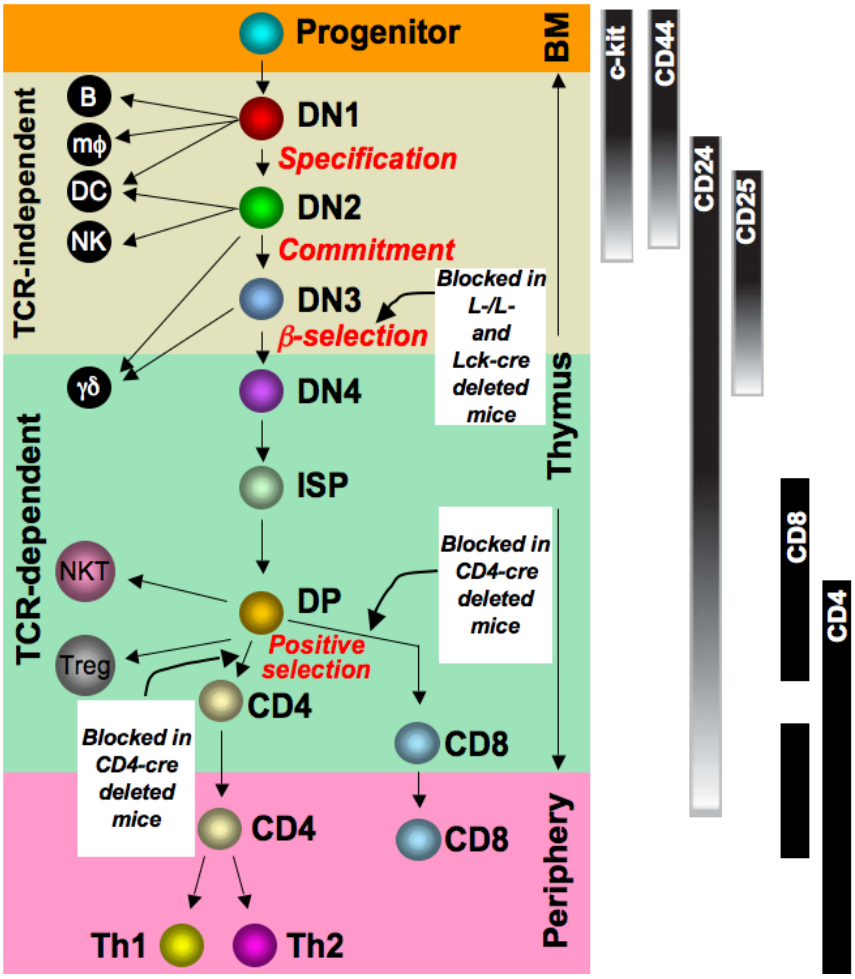

Figure 1.3. Outline of T cell development. This figure was adapted from Ref (Rothenberg and Taghon, 2005) and depicts the bone marrow-derived progenitor, which invades the thymus and undergoes at least four $CD4^{-}CD8^{-}$ (DN1-4) stages of differentiation prior to the onset of CD8 expression (immature single positive [ISP] cell), and subsequently CD4 expression to yield double positive (DP, CD4+CD8+) cells. See text for more details. Mice that are null for Ctip2 expression from the two-cell stage onwards (L-/L-) or from the DN2 cell onwards (by Lck-cre-mediated deletion) arrest at the DN3 stage, possibly due to a failure to transition from DN3 to DN4 cells (β selection). In contrast, mice with a targeted deletion of Ctip2 in DP cells (CD4-cre-excised) arrest there and only inefficiently give rise to either CD4+ or CD8+ SPs. The vertical bars to the right approximate cell-specific expression of each of the cell surface markers indicated, which is used as a means to define all cells in the T cell lineage.

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CHAPTER 2

A CHICKEN OVALBUMIN UPSTREAM PROMOTER-TRANSCRIPTION FACTOR I (COUP-TFI) COMPLEX REPRESSES EXPRESSION OF THE GENE ENCODING *TUMOR NECROSIS FACTOR ALPHA-INDUCED PROTEIN 8*

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ABSTRACT

The orphan nuclear receptor COUP-TFI plays key roles in development and homeostasis. A tandem affinity purification (TAP) procedure revealed that COUP-TFI associated with a number of transcriptional regulatory proteins in HeLa S3 cells, including NCoR, TIF1β/KAP-1, HDAC1, and the SWI/SNF family member Brahma. The pro-apoptotic protein DBC1 was also identified in COUP-TFI complexes. In vitro experiments revealed that COUP-TFI interacted directly with NCoR, but in a manner different from that of other nuclear receptors. DBC1 stabilized the interaction between COUP-TFI and NCoR by interacting directly with both proteins. The gene encoding the anti-apoptotic protein, tumor necrosis factor α induced protein 8, was identified as being repressed by COUP-TFI in a manner that required several of the component proteins of the COUP-TFI complex. Finally, our studies highlighted a central role for COUP-TFI in the induction of the TNFAIP8 promoter by TNFα. Together, these studies identify a novel COUP-TFI complex that functions as a repressor of transcription and may play a role in the TNFα signaling pathways.

INTRODUCTION

The eukaryotic genome is highly compacted into chromatin, an organized mélange of nucleic acid and histone proteins (Narlikar et al., 2002). Chromatin serves several important cellular functions. First, it solves a packing problem by extensively compacting the DNA into a bundle small enough to fit into the eukaryotic nucleus. Second, chromatin modifications underlie the basis of epigenetic regulation of gene expression, providing scalable control of gene expression that is critical for the maintenance of cellular homeostasis,

differentiation, and proliferation (Goldberg et al., 2007; Kadonaga, 1998).

Nuclear receptors (NRs) belonging to steroid/thyroid hormone receptor superfamily are ligand-dependent transcription factors that regulate critical processes in growth, development, and homeostasis (Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003). Some nuclear receptors, such as thyroid hormone receptors (TRs) and retinoic acid receptors (RARs), bind to regulatory elements in the target gene promoters in the absence of their cognate ligands. These apo-receptors function as repressors of transcription by interactively recruiting NCoR- or SMRT-containing corepressor complexes, which harbor multiple HDACs, to the promoter template (Perissi et al., 2004; Zamir et al., 1996). Agonist binding to NRs appears to stabilize a receptor conformation that is not permissive for corepressor binding, but instead favors the cyclical recruitment of a series of multi-protein, coactivator complexes, many of which contain multiple histone modifying enzymes, to the responsive promoter (Perissi et al., 2004). The dynamic, agonist-driven exchange of transcriptional co-regulatory proteins on nuclear receptor-bound, promoter templates likely underlies the molecular basis of regulation of gene expression by this family of transcription factors (Glass and Rosenfeld, 2000).

Three mammalian genes encoding COUP-TF proteins, which are orphan members of the steroid/thyroid hormone receptor superfamily, have been cloned (Avram et al., 1999; Toulouse et al., 2003): *COUP-TFI* (also known as *Nr2f1* or *EAR3*), *COUP-TFII* (*Nr2f2* or *ARP1*), and *COUP-TFIII* (*Nr2f6* or *EAR2*). COUP proteins play unique roles in fetal development, including neurogenesis and angiogenesis, and possibly in metabolic homeostasis in adult organisms (Pereira et al., 2000). In the mouse, deletion of COUP-TFI or COUP-TFII

results in perinatal and embryonic lethality, respectively, possibly due to disrupted neuronal development (Qiu et al., 1997; Studer et al., 2005; Zhou et al., 1999) and aberrant angiogenesis, skeletal muscle development and cardiac development (Lee et al., 2004; Pereira et al., 1999). COUP-TFIII$^{-/-}$ mice exhibit disruption of noradrenergic homeostasis in the locus coeruleus and rostral cerebral cortex, along with enhanced nociception and dysregulation of period 1 and period 2, clock genes that are important for proper circadian timing (Warnecke et al., 2005). These findings highlight the absolute essentiality of the COUP-TF family of orphan nuclear receptors in developmental processes, and indicate that these proteins do not function redundantly in vivo.

A number of COUP-interacting proteins have been described, and the majority of these were identified and/or characterized by yeast two-hybrid systems: TFIIB (Zhang and Dufau, 2003), a p56^{lck} ligand (Marcus et al., 1996), NCoR and SMRT (Shibata et al., 1997), Sp1 (Rohr et al., 1997; Zhang and Dufau, 2003), Alien (Dressel et al., 1999), CTIP1 and CTIP2 (Avram et al., 2000), Tat (Rohr et al., 2000), FOG-2 (Huggins et al., 2001), CIP-1 (Kobayashi et al., 2002) and CIP-2 (Kurihara et al., 2002), and Ubc9, an E2 conjugating enzyme of the small ubiquitin-related modifier (SUMO)-1 family (Kobayashi et al., 2004; Kurihara et al., 2005). To our knowledge, none of these COUP-interacting proteins have been demonstrated to be recruited to the promoter of target genes in a COUP-dependent manner, and a systematic study of COUP-TFI complexes in mammalian cells has not been conducted.

In the present studies, we identified a number of COUP-TFI-interacting proteins using a TAP strategy, and we found that the orphan receptor and

several of these proteins co-occupied the promoter of a COUP-TFI target gene that was first identified herein, *TNFAIP8*. Our data strongly suggest that the induction of *TNFAIP8* by TNFα involves relief of COUP-TFI-mediated repression of the corresponding promoter. It is believed that TNFAIP8, via direct inhibition of caspase activity, serves to dampen the apoptotic response of cells that are stimulated by TNFα (Kumar et al., 2004). Thus, our findings implicate COUP-TFI in the TNFα signaling pathways in mammalian cells.

MATERIALS AND METHODS

Cell culture — HeLa S3 and 293T cells were grown at 37°C in DMEM (Gibco) media with 10% FBS (Atlas Biologicals) and 1% penicillin/streptomycin (Invitrogen). SK-N-MC cells were grown under the same conditions except that 1% sodium pyruvate (Invitrogen) was added to the media.

Constructs and generation of stable cell lines — The plasmid pOZ-COUP was prepared by PCR amplification of human COUP-TFI with primers containing appropriate restriction sites for insertion into the pOZ-N vector, (a bicistronic retroviral expression vector, which was a kind gift from Pat Nakatani of the Dana-Farber Cancer Institute (Nakatani and Ogryzko, 2003; Ogawa et al., 2002; Shi et al., 2003). The pOZ-N vector contains a tandem epitope tag (HA and Flag) upstream of the multiple cloning site, and an internal ribosomal entry site (IRES) downstream of the cloning site. The IRES are followed by the coding sequence of the extracellular domain of CD25 (IL2 receptor α). Thus, the pOZ-COUP vector, which was used to prepare pOZ-COUP cells (see below), encodes Flag-HA-COUP-TFI and CD25, the latter of which facilitated cell sorting of infected cells (see below). The NCoR expression vector and associated deletion mutants were previously described (Picard et al., 2004). Plasmids encoding GST fusions of COUP-TFI and NCoR fragments were prepared by inserting amplicons into pGEX2T

(Pharmacia) using standard methodology. All DBC1 constructs were kind gifts from Eileen White (Rutgers University, Ref. (Sundararajan et al., 2005). The expression vector for Flag-HA tagged full-length COUP-TFI in pcDNA3.1(+) was constructed by PCR amplification and insertion into pcDNA3.1(+). The COUP-TFI-responsive reporter construct harboring a tetramerized, COUP-TFI binding site, $(DR1)_4$-tk-CAT, was constructed in pBL2-CAT. All constructs were sequenced to confirm authenticity.

Recombinant retroviruses were produced using the BD Retro-X Universal Packaging System (BD Biosciences, Palo Alto, CA) and used to infect HeLa S3 cells growing in suspension. Following retroviral infection, the proportion of cells expressing CD25 varied from 0.5 to 15% as determined by flow cytometry on an EPICS XL flow cytometer (Beckman-Coulter) using a phycoerythrin-conjugated, anti-CD25 antibody (Miltenyi Biotec). The transduced HeLa S3 cultures were enriched for $CD25^+$ cells by magnetic sorting using either an AutoMACS system or manually using magnetic beads (Miltenyi). The enriched cells were cultured for several days to increase cell number and again subjected to the magnetic enrichment procedure. After 3 to 5 rounds of enrichment, cultures reached 96 to 98% purity as determined by flow cytometry, and were then transferred to a suspension culture for large-scale production. Purified cell populations have maintained 96-98% purity after more than four months in continuous culture as determined by weekly flow cytometric analyses. HeLa S3 cells, infected with recombinant retrovirus derived from the empty vector were (pOZ-N cells) similarly purified and analyzed, and used as a control in the tandem affinity purification procedure.

Transfections —293T cells (2×10^6 cells) were plated onto a 10 cm plate and transfected 24 hrs later using the calcium phosphate method. Each transfection consisted of varying amounts of the $(DR1)_4$-tk-CAT reporter gene, FH-COUP, and an empty expression vector to standardize the total amount of

DNA transfected. The media was changed and the cells were harvested 24 and 48 hrs after transfection, respectively.

Antibodies — The anti-FLAG antibody was purchased from Sigma (St. Louis, MO). The anti-HA antibody was purchased from Roche (Indianapolis, IN). Antibodies against TIF1β, DBC1, HDAC1 and TBLR1 were obtained from Bethyl (Montgomery, TX). Anti-COUP-TFI (T19), Anti-BAF170 and -SIN3A antibodies as well as control IgGs (mouse, rabbit or goat) were purchased from Santa Cruz Biotech (Santa Cruz, CA), and anti-HDAC2 was from Abcam. The anti-HSP70 antibody was purchased from Stressgen, and anti-NCoR was a generous gift from Dr. Geoff Rosenfeld (University of California San Diego).

TAP — Approximately 3 x 10^9 purified HeLa S3 cells stably expressing FH-COUP or the control pOZ-N cells were harvested during log-phase (approximately 10^6 cells/ml). Nuclear extracts were prepared essentially as described by Shapiro and co-workers with minor modifications (Shapiro et al., 1988). Briefly, nuclear extract (approximately 150 mg) was incubated with 400 µL of anti-Flag-agarose (Sigma) for 5 hrs with constant rotation and at 4°C. This resin was then washed extensively with ice-cold PBS containing 0.05% NP40 and protein complexes were eluted with a peptide corresponding to a dimerized FLAG epitope (Sigma). The eluted material was then loaded directly onto 200 µL of anti-HA-agarose resin (Roche), which was then incubated, washed and eluted with the HA peptide (Sigma) as described above.

Protein identification by MS/MS tandem mass spectrometry — Twice-purified material was electrophoretically separated on a 4-12% SDS-PAGE gradient gel, individual proteins were visualized by a mass spectrometry (MS)-friendly coomassie stain (Biorad), excised, and subjected to an in-gel tryptic digest as previously described (Shevchenko et al., 1996).

Following digestion, samples were desalted using a microC18 Tip (Millipore) and dried. Samples were then resuspended in 5 µL of 0.1% formic and analyzed by LC/ESI MS/MS with a nano2D LC (Eksigent) coupled to an LTQ-FT mass spectrometer (ThermoElectron) using an instrument configuration as described (Yi et al., 2003). Data were collected in a data-dependent mode in which a high mass resolution/high mass accuracy MS scan (in the FT part of the instrument) was followed by MS/MS scans of the five most abundant ions from the preceding MS scan. The five selected ions for tandem MS were placed on an exclusion list and not selected for subsequent tandem mass spectrometry for 1.5 minutes, allowing less intense ions to be interrogated for tandem mass spectrometry. Proteins were identified from mass spectrometry data using a modified version of the open source X!Tandem (Beavis Informatics) automated protein database search algorithm. The score function of native X!Tandem was replaced with a dot-product based score algorithm that is compatible with Peptide Prophet (Keller et al., 2005). Search results were considered correct if at least two peptides had raw scores greater than 200 for +1 ions, 300 for +2 ions, and 300 for +3 ions, % Ions of greater than 15%, Peptide Prophet scores greater than 0.9, and if the identification did not appear in a blank portion of the gel.

Co-immunoprecipitation and immunoblotting — Co-immunoprecipitations and immunoblotting were conducted as previously described (Topark-Ngarm et al., 2006).

Size-exclusion chromatography — Size-exclusion chromatography was conducted using 50 mg of nuclear extract from stably transduced HeLa S3 or control cells, and a Superose 6 column as previously described (Senawong et al., 2003; Topark-Ngarm et al., 2006).

GST pull-downs — GST pull-downs were conducted as previously described using bacterially expressed GST fusion proteins as baits and

[^{35}S]methionine-labeled proteins as prey (Dowell et al., 1997).

Reporter gene assays — Chloramphenicol acetyltransferase (CAT) reporter gene assays were conducted and quantified as previously described (Senawong et al., 2003).

ChIP studies — Both ChIP and re-ChIP studies were conducted essentially as previously described (Topark-Ngarm et al., 2006). The following primers were used for amplification reactions: TNFAIP8: forward primer: 5´-TCCTCCTTCCCTGCACGCT-3´; reverse primer: 5´-CCAGGAGCCACTTACTCGGA-3´; amplification product, 278bp. IGF2: forward primer: 5´-GATCATCGTCCAGGCAGTTT-3´; reverse primer: 5´-CTTCCCCTCCTTCAGAAACC-3´; amplification product, 227 bp.

siRNA transfections — HeLaS3 cells were transfected with 30 nmols of negative control siRNA or specific siRNA against COUP-TFI, NCoR or TIF1β using siPORT NeoFX transfection reagent (from Ambion). Medium was replaced with fresh growth medium after 24 hr, and cells were harvested for RNA extraction 48 hr after transfection.

Quantitative RT-PCR — RNA was prepared using the RNeasy Mini kit (Qiagen) and 1 µg of total RNA was reverse transcribed using reverse transcriptase and oligo dT (Invitrogen). The resulting cDNA (1 µL) was then used for the following amplification reactions: TNFAIP8 (forward primer: 5´-TGAGCTAGCATTGATGGAGA -3´, reverse primer: 5´-TCCAACATTTTGTTGATACCA -3´), and hypoxanthine-guanine phosphoribosyl- transferase (HPRT; forward primer: 5´-ATTGTAATGACCAGTCAACAGGG-3´, reverse primer: 5´-GCATTGTTTTGCCAGTGTCAA -3´). These primer sets generate amplicons of 300bp and 117bp, respectively. An Applied Biosystems 7500 real-time PCR instrument and SYBR green technology (Qiagen) was used for all qPCR

analyses.

Compound treatments and apoptosis assay — HeLa S3 cells were grown on coverslip to 70% confluency, followed by 8 hours of TNFα (5ug/ml) and CHX (30ug/ml) treatments. After drug treatment, cells were fixed with paraformaldehyde and the nuclei were stained with DAPI. Non-apoptotic cells with intacted nuclei were counted using fluorescence microscope, and the ratio of TNFα/CHX treatment to mock treatment were calculated.

RESULTS

Purification and identification of component proteins of COUP-TFI complexes — Stable cell lines were established in the HeLa S3 background using a recombinant retrovirus directing expression of COUP-TFI harboring a tandem epitope tag (FLAG-HA) at its amino terminus (FH-COUP; Fig. 2.1*A*), which allowed immunoprecipitation and immunodetection of COUP-TFI with high efficiency and specificity. The bicistronic retroviral vector used to create the cell line (pOZ-COUP) stably expressing FH-COUP also encoded the extracellular domain of IL-2 receptor (CD25), which facilitated cell sorting to enrich infected cells to near homogeneity (Fig 2.1*B*). As a control, HeLa S3 cells were infected with a recombinant retrovirus prepared using the empty, pOZ-N vector (pOZ-N cells). Both pOZ-COUP and pOZ-N cells were purified to greater than 95% homogeneity by multiple rounds of magnetic-based cell sorting (data not shown), and then expanded in suspension culture. Stable expression of FH-COUP was validated by immunoblotting (Fig. 2.1*C*), and subcellular distribution analyses demonstrated that the tagged protein was mostly localized in the nucleus, consistent with previous studies of COUP-TFI

(Kurihara et al., 2005; Wang et al., 1989; Wang et al., 1987).

Protein complexes containing FH-COUP were isolated from nuclear extracts of pOZ-COUP cells by tandem affinity purification (TAP, Fig. 2.1A), essentially as described by the Nakatani laboratory (Nakatani and Ogryzko, 2003; Shi et al., 2003). Purified protein complexes were resolved by SDS-PAGE gel, and visualized by coomassie staining (Fig. 2.S1*B*), which revealed that immunopurified FH-COUP copurified with ~ 25 other polypeptides, most of which were not present in twice-immunopurified material from pOZ-N cells (compare lanes 1 and 2 of Fig. 2.S1*B*).

Mass spectrometric analysis of FH-COUP complexes after sequential immunoaffinity chromatography revealed the co-purification of several components of transcriptional repressive complexes along with FH-COUP. These include NCoR, HDAC1, TBLR1, TIF1β/KAP-1 and MPC-3 (a chromodomain-containing polycomb protein (Table 2.1; Fig. 2.S1*B*). Brahma (Brm), an ATP-dependent, chromatin remodeling protein (Wang et al., 1996), and its associated factors BAF155 and BAF170 were also identified in FH-COUP complexes (Table 2.1, Fig. 2.S1*B*). The Brahma complex appears to be a bifunctional SWI/SNF family member, having been implicated in both transcription activation (Narlikar et al., 2002) and repression (Harikrishnan et al., 2005). Proteins not previously implicated in transcriptional regulation were also found to co-purify with FH-COUP, including the DNA repair protein DDB1, a pro-apoptotic protein that is deleted in breast cancer (DBC1), HSP70, HSP90 and HYD1, an ubiquitin ligase. Finally, several components of the splicesome assembly were identified (SFR1, SF3A1 and SF3B1). Collectively, our TAP strategy revealed that FH-COUP may associate with a number of

transcriptional regulatory proteins in HeLa S3 cells as well as other classes of proteins that have not been previously implicated in the regulation of gene expression.

Characterization of COUP-TFI-associated proteins — We undertook a multipartite approach to verify association of the identified proteins with FH-COUP complexes in mammalian cells. First, we conducted co-immunoprecipitation (co-IP) studies in nuclear extracts from HeLa S3 cells stably expressing FH-COUP. Endogenous TIF1β, NCoR, BAF170, BRM, HSP70, DBC1, and HDAC1 were efficiently co-immunoprecipitated with FH-COUP using the anti-FLAG antibody (Fig. 2.1*D*).

Second, we performed a series of reciprocal IP experiments in nuclear extracts prepared from pOZ-COUP cells. These studies utilized antibodies directed against both FH-COUP and representative, endogenous COUP-interacting proteins. The results of these studies confirmed that endogenous NCoR, TIF1β, and DBC1 were specifically immunoprecipitated with FH-COUP by the anti-Flag antibody (Fig. 2.1*E*). The reciprocal IPs also indicated that FH-COUP was immunoprecipitated with antibodies directed against TIF1β, and DBC1 (Fig. 2.1*E*). Importantly, TIF1β, NCoR, and DBC1 all appeared to co-immunoprecipitate with each other (Fig. 2.1*E*, *lanes* 4-6). These results strongly suggest that COUP-TFI stably associates with endogenous NCoR, TIF1β, and DBC1 within the same or a highly related protein complex in HeLa S3 cells.

Third, we conducted co-immunoprecipitation experiments using nuclear extracts prepared from untransfected SK-N-MC neuroblastoma cells, which

express COUP-TFI endogenously. The results of these co-IP experiments revealed the specific association of endogenous COUP-TFI with NCoR, TIF1β, BRM, and DBC1 in SK-N-MC cells (Fig. 2.1*F*).

Fourth, we verified that putative, component proteins of FH-COUP complexes co-chromatographed with FH-COUP on a Superose 6 size-exclusion column. We found that the majority of FH-COUP immunoreactivity present in nuclear extracts of pOZ-COUP cells eluted from the Superose 6 column with an apparent mass between 1-2 MDa (Fig. 2*A*). This peak of FH-COUP immunoreactivity was not symmetrical but rather eluted with a tailing shoulder extending nearly to the 669 KDa marker. All of the putative FH-COUP complex proteins examined co-eluted with FH-COUP in the high molecular mass fractions (fractions 16-20) to varying degrees, but the chromatographic behavior of these proteins differed dramatically in those fractions. This is perhaps best exemplified by TIF1β, the majority of which eluted independently of FH-COUP as a fairly symmetrical peak centered around 669 KDa (Fig. 2.2*A*, fractions 32-38). However, the leading shoulder of the TIF1β peak extended up into the mDa range of the profile, where it appeared to co-elute with FH-COUP, as well as with other putative proteins of the COUP-TF complex (Fig. 2.2*A*). Many of the other proteins (DBC1 HDAC1) of the putative COUP-TFI complex exhibited similar chromatographic behavior, which prompted us to examine if these anomalous elution patterns might result from over-expression of COUP-TFI. To test this possibility we examined the chromatographic properties of these proteins in control pOZ-N cells (prepared with the empty pOZ-N vector). In the absence of FH-COUP over-expression, TIF1β, DBC1, HDAC1 all appeared to elute from the Superose 6 column as fairly symmetrical peaks corresponding to the lower mass species observed in

pOZ-COUP cells (Fig. 2.2*B*; see also Fig. 2.2*A*). This finding suggests that stable over-expression of FH-COUP in HeLa S3 cells alters the chromatographic behavior of these proteins, consistent with the possibility that these proteins may be authentic components of FH-COUP complexes.

Considered together, these data validate the identified proteins as components of COUP-TFI complexes in mammalian cells.

COUP-TFI interacts directly with two different regions in NCoR — Because NCoR has been identified as a corepressor for several hormone nuclear receptors (Glass and Rosenfeld, 2000; Horlein et al., 1995), we hypothesized that NCoR may interact directly with COUP-TFI, nucleate the FH-COUP complex, and serve as a scaffold for other protein-protein interactions within the FH-COUP complex. Moreover, Shibata and colleagues previously reported a direct interaction between COUP-TFI and a large fragment of NCoR in yeast and in vitro, but neither defined the COUP-TFI interaction domain of NCoR precisely nor directly demonstrated a role for NCoR in COUP-mediated repression (Shibata et al., 1997). GST pull-down experiments were performed to investigate the former in detail, and these studies revealed that GST-COUP-TFI interacted with full-length NCoR (Fig. 2.3*A1*), as well as NCoR fragments containing repression domain 1 (amino acids 1-393, NCoR1; Fig. 2.3*A2*) and repression domain 4 (also known as the CBF1/Su[H] domain, corresponding to amino acids 1500-1900, NCoR5; Fig. 2.3*A6*). Other isolated regions of NCoR, including the canonical nuclear receptor interaction domain (NCoR6, Fig. 2.3*A7*), did not appear to interact with GST-COUP-TFI. In reciprocal pull-down experiments, we confirmed that COUP-TFI was specifically pulled down by both GST-NCoR1 and GST-NCoR5

(Fig. 2.S2).

The NCoR-interaction domains of COUP-TFI were similarly mapped in reciprocal pull-down experiments. These studies revealed that the isolated repression domains 1 and 4 interacted directly with full-length COUP-TFI (Fig. 2.3*B3*). However, the two NCoR fragments interacted differentially with isolated regions of COUP-TFI. For example, NCoR1, which contains repression domain 1 (RD1), interacted strongly with any deletion mutant containing the COUP-TFI DBD (*lane* 1 of Figs. 2.3*B5-7*). NCoR1 appeared to interact with zinc fingers I and II with equivalent efficiency (compare *lane* 1 of Figs. 2.3*B6* and 2.3*B7*), neither of which were as efficient as the two fingers together (*lane* 1 of Fig. 2.3*B5*). In contrast, NCoR5, which harbors RD4, interacted directly with the isolated COUP DBD (*lane* 2 of Fig. 2.3*B5*), and any mutant containing the LBD (*lane* 2 of Figs. 2.2*B9-11*). The interaction of NCoR5 with the COUP-TFI DBD differed from that of NCoR1, because NCoR5 appeared to interact with ZnFII, but not with ZnFI, and did so as efficiently as with the full DBD (compare *lane* 2 of Figs. 2.3*B5-7*). Previous work has identified the AF2 region near the carboxy terminus of COUP-TFI as an important region for COUP-TFI-mediated repression (Shibata et al., 1997). However, the AF2 domain of COUP-TFI was dispensable for the interaction of the orphan receptor with NCoR5 (*lane* 2 of Fig. 2.3*B10*), and neither the intact COUP LBD (*lane* 1 of Fig. 2.3*B9*) nor derivative mutants (*lane* 1 of Figs. 2.3*B8, 10, 11*) interacted with NCoR1. Taken all together, we conclude that two distinct domains of COUP-TFI interact differentially with two regions of NCoR: the COUP-TFI DBD was found to interact with fragments harboring both NCoR RD1 and CBF1/RD4, whereas the COUP-TFI LBD interacted only with NCoR fragment harboring the CBF1/RD4 region. Thus, NCoR appears to play a

crucial role in nucleating the large FH-COUP-containing complex that we observed in HeLa S3 cells but the manner by which COUP-TFI interacts with NCoR appears to be different from that of other members of the nuclear receptor superfamily.

DBC1 interacts directly with both COUP-TFI and NCoR — We found that COUP-TFI interacted directly with DBC1 in GST pull-down studies in a manner that required the N-, but not the C-terminus of DBC1 (lane 3 of Fig. 2.4*A*). While full-length FH-COUP interacted with wild-type DBC1 (lane 3 of Fig. 2.4*B*), we could not define the DBC1-interaction domain(s) of COUP-TFI more precisely by truncation mutagenesis as neither the isolated amino or carboxyl terminal regions of COUP interacted with DBC1 (Fig. 2.4*B*, *lanes* 4 and 5). This suggests that a higher degree of structural complexity of COUP-TFI is required for its interaction with DBC1.

DBC1 also interacted with GST-NCoR1 (Fig. 2.4*C*, *lane* 1), which contains RD1 (see above). However, unlike FH-COUP, DBC1 did not interact with NCoR5, the CBF1/RD4 domain of the corepressor (Fig. 2.S3, *lane* e4). DBC1 also did not interact with other NCoR fragments tested (data not shown). Both C- and N-terminal regions of DBC1 appear to be required for the interaction with NCoR (Fig. 2.4*C*, *lanes* 1-3).

The results described above suggest that DBC1 may function as an adaptor protein between COUP-TFI and NCoR, and this was tested in co-IP studies in HEK cells that had been transiently transfected with combinations of FH-COUP, DBC1, and NCoR. As illustrated in Fig. 2.4*D*, transfection of increasing amounts of DBC1 significantly increased the amount of NCoR that

co-immunoprecipitated with FH-COUP in 293T cells (Fig. 2.4*D*, *lane* 3-5). Collectively, our data revealed direct interactions between COUP-TFI, DBC1, and NCoR, suggesting that DBC1 may function as adaptor protein that stabilizes the interaction of COUP-TFI and NCoR, and possibly maintains the integrity of the FH-COUP complex.

NCoR, TIF1β, and DBC1 co-occupy the promoter of a FH-COUP-responsive promoter — To investigate the association of FH-COUP and its identified interaction proteins on a COUP-responsive promoter, we transiently co-transfected 293T cells with an expression vector encoding FH-COUP, and a reporter construct harboring a multimerized DR1 motif in the context of the thymidine kinase (tk) promoter, upstream of the CAT reporter (Fig. 2.5*A*). Transfection of increasing amounts of FH-COUP expression vector resulted in repression of this reporter, which reached a maximum of approximately 5-fold (Fig. 2.5*B*). We next determined if NCoR, TIF1β, and DBC1 co-occupied the COUP-responsive promoter of the CAT reporter together with FH-COUP. As expected, FH-COUP was found on this template in cells transfected with the FH-COUP expression vector but not in cells transfected with empty vector (Fig. 2.5*C*). NCoR, TIF1β, and DBC1 were all found to co-occupy the FH-COUP-responsive promoter with FH-COUP in transiently transfected cells (Fig. 2.5*D*, *lane* 5-8 of upper and lower panels, respectively). These data demonstrate that a COUP-TFI complex minimally containing FH-COUP, NCoR, TIF1β, and DBC1 is present on the promoter template of a COUP-TFI-responsive reporter gene.

The COUP-TFI complex regulates the promoter of TNFα-induced protein 8 — To extend the above results to natural, chromatinized promoters,

we first identified putative, COUP-TFI target genes using a genome-wide ChIP-chip approach (Zhang and Leid, unpublished data). From this screen we identified the anti-apoptotic gene *TNFα-induced protein 8* (*TNFAIP8*) as a putative target of COUP-TFI. The promoter region of *TNFAIP8* has not been experimentally characterized, but computational examination of the predicted promoter identified a degenerate DR1-like response element located approximately 30 bp downstream of the transcription start site. This DR1-like element is found within the putative downstream core promoter element (DPE; sequence: RGWYVT; see Fig. 2.6*A*), which has been characterized by Kadonaga and colleagues (Juven-Gershon et al., 2008). Further analyses also revealed that the TNFAIP8 promoter lacks a conventional TATA box but harbors multiple Sp1 binding sites within 100bp of the predicted start site (Fig. 2.6*A*). ChIP analyses conducted in pOZ-COUP cells validated the presence of FH-COUP on the TNFAIP8 promoter (Fig. 2.6*B*). Moreover, reChIP analyses demonstrated that FH-COUP, NCoR, TIF1β, and DBC1 all co-occupied the same fragment of the TNFAIP8 promoter (Figs. 2.6*C* and *D*).

Roles of NCoR, TIF1β, and DBC1 in COUP-TFI-mediated repression — To determine the transcriptional outcome of the interaction of the FH-COUP complex with the TNFAIP8 promoter, we first examined the effect of COUP-TFI overexpression on TNFAIP8 transcript levels in HeLaS3 cells. Quantitative RT-PCR analyses revealed that TNFAIP8 mRNA levels were downregulated (~50%) in pOZ-COUP cells relative to pOZ-N cells (Fig. 2.7*A*). This was confirmed by knocking down endogenous COUP-TFI expression, which resulted in a ~ 2 fold increase in *TNFAIP8* expression (Fig. 2.7*B*). Similarly, knock-down of NCoR and TIF1β resulted in derepression of the TNFAIP8 promoter to a level that was indistinguishable from that of COUP-TFI

knock-down (Fig. 2.7*B*). The siRNA-mediated knock-down of COUP-TFI, NCoR and TIF1β expression was verified by immunoblotting analyses (Fig. 2.S4*A*). Collectively, our results indicate that the COUP-TFI/NCoR complex represses expression of *TNFAIP8* expression in untransfected HeLa S3 cells.

We do not know if DBC1 plays a role in COUP-TFI-mediated repression of the TNFAIP8 promoter in HeLa S3 cells because we could not achieve appreciable knock-down of DBC1 expression in these cells (data not shown). However, DBC1 does appear to play a role in COUP-TFI-mediated repression in HEK293T cells, in which we could knock down DBC1 expression using an siRNA approach (Fig. 2.S4*B*). First, similar to HeLa S3 cells, *TNFAIP8* expression was also induced upon knock-down of endogenous COUP-TFI in 293T cells, suggesting that *TNFAIP8* is a bona fide target of the COUP-TFI transcriptional repressor complex in both HeLa and HEK293 cells. Importantly, FH-COUP-mediated repression of the endogenous TNFAIP8 promoter was reversed by knock-down of DBC1 (Fig. 2.7*C*). In addition, co-transfection of DBC1 stimulated significantly the FH-COUP-dependent repression of the $(DR1)_4$-tk-CAT reporter in 293T cells (Fig. 2.7*D* and Fig. 2.S4*C*). Thus, we conclude that NCoR, TIF1β, DBC1 all play key roles in COUP-TFI-mediated transcriptional repression.

Role of COUP-TFI during TNFα-induced apoptosis. *TNFAIP8* is upregulated by TNFα treatment (Kumar et al., 2000), and the TNFAIP8 protein functions as a caspase inhibitor, which appears to limit the extent of apoptosis induced by TNFα (You et al., 2001). The results presented above also identified *TNFAIP8* as target of COUP-TFI-mediated repression in HeLa S3 and HEK cells. Therefore, we investigated the possibility that COUP-TFI may

play a direct or indirect role in the induction of *TNFAIP8* by TNFα, as well as in TNFα-induced apoptosis in HeLa S3 cells. We confirmed that *TNFAIP8* was induced by approximately 4-fold by TNFα in HeLa S3 cells (Fig. 2.8*A*, *lane* 2). We also found that expression of *TNFAIP8* was induced by the protein synthesis inhibitor cycloheximide (CHX; *lane* 3), and the combination of TNFα and CHX acted synergistically to induce *TNFAIP8* in HeLa S3 cells (~ 15-fold induction; Fig. 2.8*A*). It is well established that both pro-apoptotic and anti-apoptotic pathways are activated by TNFα (Gaur and Aggarwal, 2003), and that CHX may inhibit the translation of anti-apoptotic mRNAs induced by TNFα, thus potentiating TNFα-induced apoptosis (Gaur and Aggarwal, 2003). In contrast, COUP-TFI expression was found to be divergently regulated by TNFα and CHX: TNFα downregulated COUP-TFI expression by approximately 65% (Fig. 2.8*B*, *lane* 2), whereas CHX induced COUP-TFI mRNA levels by nearly 3-fold (Fig. 2.8*B*, *lane* 3). The latter finding may suggest that a labile repressor dictates basal COUP-TFI expression levels in HeLa S3 cells. Strikingly, concomitant treatment with TNFα and CHX completely reversed the stimulatory effect of CHX on COUP-TFI mRNA levels (Fig. 2.8*B*, *lane* 4). TNFα, alone or in combination with CHX, also downregulated COUP-TFI protein levels (Fig. 2.8*C*). However, the stimulatory effect of CHX on COUP-TFI mRNA levels did not generalize to the protein level, which is likely due to the inhibitory effect of CHX on protein synthesis.

The above results prompted us to determine if TNFα/CHX-induced downregulation of COUP-TFI protein levels played a role in the induction of TNFAIP8 mRNA by TNFα/CHX, and we addressed this using two approaches. First, we examined the effect of COUP-TFI knock-down on induction of TNFAIP8 mRNA by TNFα/CHX. We found that knock-down of COUP-TFI

potentiated TNFα/CHX-mediated induction of the TNFAIP8 promoter by approximately 2-fold (Fig. 2.8*D*). Second, we compared induction of *TNFAIP8* by TNFα/CHX in cells overexpressing COUP-TFI (pOZ-COUP) and control cells (pOZ-N). We found that overexpression of COUP-TFI impaired the induction of *TNFAIP8* by TNFα/CHX, relative to control cells (Fig. 2.8*E*), and as a functional correlate of this, cells overexpressing COUP-TFI were also more sensitive to TNFα/CHX-induced apoptosis (Fig. 2.8*F*). Considered together, these results suggest that COUP-TFI plays a direct or indirect role in the induction of the *TNFAIP8* gene by TNFα, with or without inhibition of protein synthesis by CHX.

DISCUSSION

The orphan nuclear receptor COUP-TFI is a transcription factor that plays essential roles in the regulation of key biological processes, presumably in the context of a multi-protein complex. This is the first study, to our knowledge, to identify component proteins of cellular complexes containing COUP-TFI using a proteomic approach, which revealed COUP is associated with a large number of proteins in HeLa S3 cells.

As with many other nuclear receptor complexes, NCoR appears to serve as a scaffold protein that couples COUP-TFI to histone deacetylases, which may underlie the mechanistic basis for COUP-TFI-mediated transcriptional repression. However, the molecular basis for the interaction of COUP-TFI with NCoR appeared to differ from that of other, previously studied nuclear receptors, in that COUP-TFI did not interact with the canonical nuclear receptor-interactions domains located in the carboxyl terminus of NCoR (Glass

and Rosenfeld, 2000; Seol et al., 1996). Rather, the DNA and putative ligand binding domains of COUP-TFI interacted with RD1 and RD4 of NCoR, respectively. Repression domain 4 of NCoR also interacted with the COUP-TFI DBD but did so in a manner that differed from that of NCoR RD1. However, it remains unclear if the relatively small COUP-TFI DBD can interact with one or two domains of NCoR while simultaneously binding DNA in a sequence-specific manner. This seems unlikely without some type of facilitation, and we propose that the adaptor protein DBC1 functions to stabilize COUP-TFI-NCoR interaction on the promoter of target genes, such as *TNFAIP8*. Indeed, DBC1 was found to co-occupy the TNFAIP8 promoter with COUP-TFI, NCoR, and TIF1β, and all of these proteins were crucial for the repression of the TNFAIP8 promoter by COUP-TFI. Further studies are clearly needed to map the protein-protein interaction network within the COUP-TFI complex in greater detail, and to define the role of other complex proteins, such as BRM and TIF1β, in the transcriptional regulatory activity of COUP-TFI.

Previous studies by Torchia's group revealed the existence of at least two chromatographically distinct NCoR complexes: NCoR1, containing NCoR, HDAC3, TIF1β, BAF155, BAF170, and splicing factor 3A/3B; and NCoR2, containing NCoR, SIN3A, HDAC1 and HDAC1/2 (Underhill et al., 2000). Subsequently, Roeder's group described a SIN3A-less, NCoR complex containing NCoR, HDAC3, TBL, TBLR1, and GPS2 (Zhang et al., 2002), which may be the same as, or highly related to, that described by Wong's group (Yoon et al., 2003). These findings support the idea that NCoR can interact with a variety of proteins in an extremely flexible manner. The FH-COUP/NCoR complex described herein appears to be most similar to the SIN3A-less,

NCoR1 complex described by Torchia's group (Underhill et al., 2000), in that the complex that we identified clearly contains TIF1β, the Brm-associated factors, and splicing factors 3A/3B. However, unlike NCoR1 or the NCoR complex described by the Roeder (Zhang et al., 2002) and Wong (Yoon et al., 2003) laboratories, we found no evidence for the presence of HDAC3 in the FH-COUP/NCoR complex. Moreover, HDAC1, which is not present in NCoR1, was identified by peptide sequencing to be a component of FH-COUP complexes and this was verified by co-IP analyses. Considered together our findings suggest the existence of a novel NCoR complex that harbors FH-COUP, as well as other proteins, such as DBC1, that has no appreciated role in transcriptional regulation. Nonetheless, as previously observed in the case of other nuclear receptor complexes, NCoR appears to play the role of a scaffold protein in FH-COUP complexes that couples the orphan receptor to the transcriptional repression machinery (i.e., HDACs).

DBC1 is a pro-apoptotic protein that was originally cloned from human chromosome 8p21, a region that is homozygously deleted in some breast cancer cells (Hamaguchi et al., 2002). DBC1 is localized exclusively in the nucleus of healthy cells (Sundararajan et al., 2005), and has been reported to interact directly with and inhibit the catalytic activity of the class III HDAC, SIRT1 (Zhao et al., 2008). Upon apoptotic stimulation, DBC1 undergoes caspase-dependent processing with rapid degradation of the amino terminus, and the C-terminal fragment translocates from the nucleus to the mitochondria, promoting apoptosis by sensitizing cells to apoptotic signals (Sundararajan et al., 2005). As reported herein, DBC1 also appears to associate with the COUP-TFI complex on the promoter of COUP-TFI target genes, and functions to stabilize interaction between NCoR and COUP-TFI, thus, contributing to

COUP-TFI-mediated transcriptional repression. In present study, we found that the DBC1 N-terminus is required for the interaction with COUP-TFI. Therefore, as a result of caspase cleavage, DBC1 may no longer be able to interact with COUP-TFI during TNFα-induced apoptosis. Thus, it may be of interest to study other dynamic changes taking place within COUP-TFI complex during TNFα-induced apoptosis.

TIF1β, which is also known as KRAB-associated protein 1 (KAP-1) and tripartite motif containing 28 (TRIM28 in humans; Trim28 in mice), is a transcriptional corepressor of the KRAB domain-containing zinc finger protein family(Ivanov et al., 2007). TIF1β harbors multiple protein-protein interaction and functional domains, including an amino terminal region with homology to ring finger, B-box, and coiled-coil (RBCC) proteins, a heterchromatin protein 1 (HP1)-binding domain, a NCoR homology domain, and a carboxyl terminal region known as the PB region, which contains a plant homeodomain (PHD) finger and a bromodomain in tandem (Ryan et al., 1999). The PHD of TIF1β interacts strongly with the SUMO E2 transfer protein Ubc9, and functions as an intramolecular E3 SUMO ligase that directs Ubc9 to SUMOylate specific lysine residues in the bromodomain of TIF1β (Ryan et al., 1999). The SUMOylated bromodomain of TIF1β recruits the Nucleosome Remodeling and Deacetylation (NuRD) complex, which deacetylates template-associated histones, including Lys^9 of histone H3. In addition, the SUMOylated bromodomain of KAP-1 appears to recruit the lysine-specific methyltransferase SETDB1, which methylates this residue forming a binding site for HP1 on the template, thus promoting the silenced state of the promoter. TIF1β/KAP-1 is clearly an integral component of the COUP-TFI complex and is required for COUP-TFI-mediated transcriptional repression of the TNFAIP8 promoter.

However, it remains to be determined if the intramolecular E3 SUMO ligase activity of TIF1β/KAP-1 is required for the transcriptional repressive properties of the COUP-TFI complex on target gene promoters.

The SWI/SNF complex has been shown to disrupt nucleosome structure and increase DNA accessibility for transcription activators (Narlikar et al., 2002). While it is tempting to speculate that the Brahma-containing, COUP-TFI complex may be involved in COUP-TFI-mediated transcriptional activation, for example of ovalbumin (Sagami et al., 1986), cholesterol 7α-hydroxylase (Stroup et al., 1997), and aldosterone synthase (Kurihara et al., 2005) promoters, we can not rule out the possibility that Brahma and associated factors may be part of the COUP/NCoR complex that represses transcription. Indeed, recent studies have found that SWI/SNF remodeling activity of Brahma is required for transcription repression as well as transcription activation (Harikrishnan et al., 2005). Brahma appears to interact with the methyl-CpG binding protein MeCP2 to form a repressive complex that also contains SIN3A and HDAC1 (Harikrishnan et al., 2005). Moreover, a number of studies have demonstrated that Brahma is associated with HDAC1 and HDAC2 (Pal et al., 2003; Sif et al., 2001), as well as NCoR and TIF1β (Underhill et al., 2000). Further studies are required to define the specific role(s) of the SWI/SNF complex in the transcriptional regulatory activity of COUP-TFI.

TNFAIP8, which is also known as *SCC-S2* or *NDED*, is a novel oncogene that plays a role in tumor progression. The amino terminus of TNFAIP8 harbors a sequence that is highly homologous to death effector domain (DED) II of cell death regulatory protein FLIPs, which has been shown to block death receptor-mediated apoptosis by preventing caspase 8 activation (You et al.,

2001) TNFAIP8 expression is upregulated by TNFα stimulation and by activation of NF-κB in tumor cell lines(Kumar et al., 2000; You et al., 2001). Overexpression of TNFAIP8 leads to enhanced survival and inhibition of apoptosis through inhibition of the apoptotic proteins caspase 3 and caspase 8 (Kumar et al., 2004).

We identified a novel anti-apoptotic pathway that was initiated by TNFα, and involved downregulation of COUP-TFI expression, and derepression of the newly identified, COUP-TFI target gene *TNFAIP8*, and subsequent inhibition of apoptosis (Fig. 2.9). Thus, downregulation of COUP-TFI expression, which resulted in less COUP-TFI on the TNFAIP8 promoter (Fig. 2.S5) would appear to be an important component of the signaling pathway leading to induction of *TNFAIP8* expression by TNFα. At present, we do not know the mechanistic connection between TNFα signaling and repression of COUP-TFI expression. However, the proximal region of the COUP-TFI promoter harbors seven putative, but highly conserved, NF-κB binding sites, and it is conceivable that NF-κB may work through one or more of these binding sites to repress COUP-TFI expression (see Fig. 2.9) in a manner similar to that by which NF-κB represses the Bmp4 promoter (Zhu et al., 2007).

Table 2.1. Components of the COUP-TFI complexes

Protein Symbol	Genebank IDs	Unique Peptides	Function
COUP-TFI	NP_005645	12	Transcription Factor
TIF1β	NP_005753	7	Transcriptional corepressor
NCoR	NP_006302	8	Transcriptional corepressor
HDAC1	NP_004955	7	Transcriptional repressor
TBLR1	NP_078941	8	Transcriptional repression
MPC3	NP_065700	2	Transcriptional repression
TRIM33	NP_056990	4	Transcriptional corepressor
FIR	NP_055096	4	Transcriptional repression
BAF170	NP_620706	2	ATP-dependent chromatin remodeling protein
BAF155	NP_003065	2	ATP-dependent chromatin remodeling protein
BRM	NP_620614	4	ATP-dependent chromatin remodeling protein
THOC4	NP_005773	4	Transcriptional coactivator
TFIIIC	NP_036336	2	Transcriptional activator
TAF9	NP_003178	2	Transcriptional coactivator
DBC1	NP_066997	27	Apoptosis
BAT3	NP_542434	7	Apoptosis
DDB1	CAA05770	10	DNA Repair
HSP70	NP_005338	11	Protein chaperone
HSP90	NP_031381	5	Protein chaperone
hHyd1	U95000_1	49	Ubiquitin Ligase
EF1α	NP 001393	6	Elongation Factor
SFR1	NP_008855	6	Splicing Factor
SF3A1	NP_005868	3	Splicing Factor

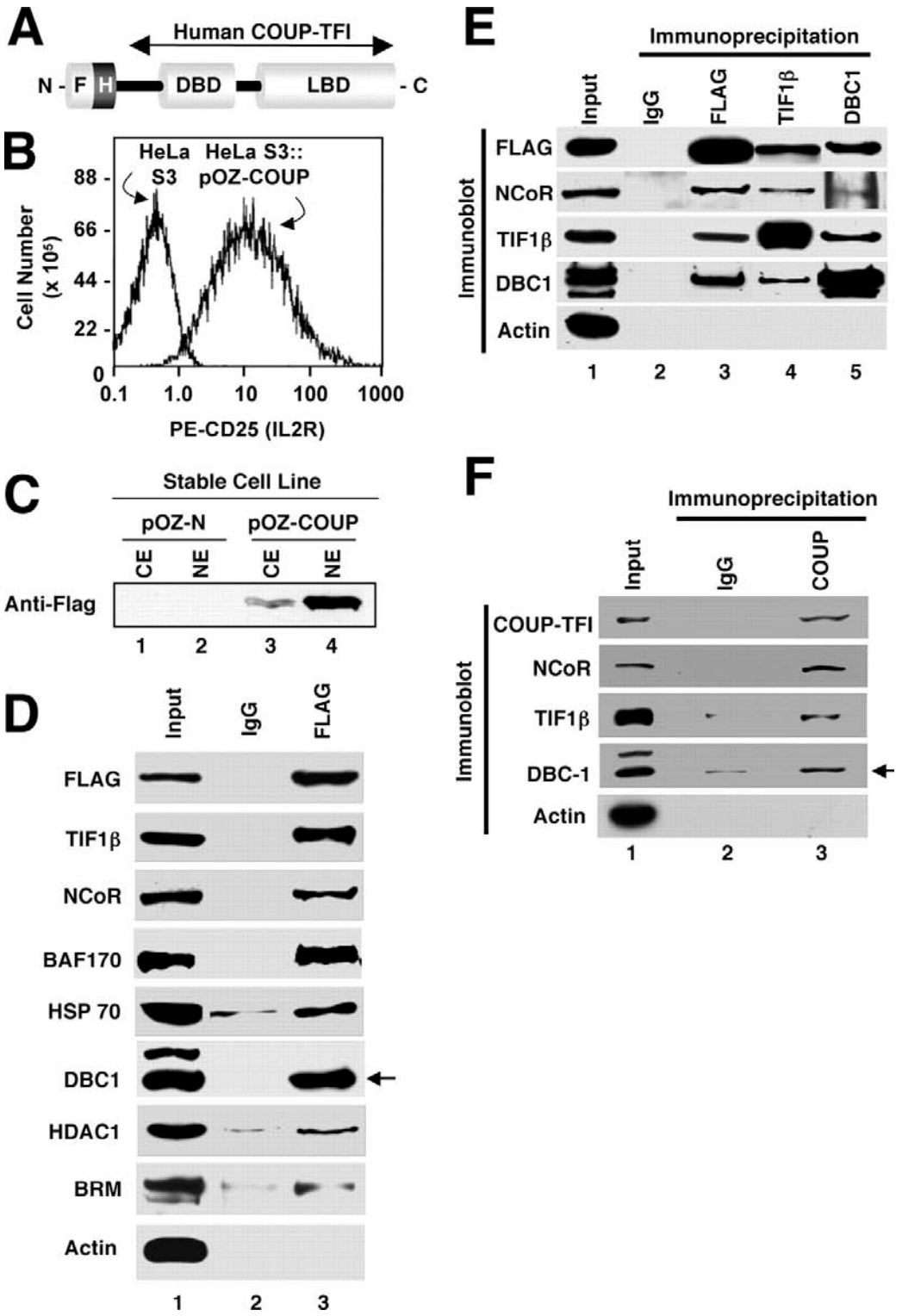

A
Human COUP-TFI
N - F H DBD LBD - C
B
HeLa S3
HeLa S3:: pOZ-COUP
Cell Number (x 10⁵)
88
66
44
22
0
0.1
1.0
10
100
1000
PE-CD25 (IL2R)
C
Stable Cell Line
pOZ-N
pOZ-COUP
CE
NE
CE
NE
Anti-Flag
1
2
3
4
D
Input
IgG
FLAG
FLAG
TIF1β
NCoR
BAF170
HSP 70
DBC1
HDAC1
BRM
Actin
1
2
3
E
Immunoprecipitation
Input
IgG
FLAG
TIF1β
DBC1
Immunoblot
FLAG
NCoR
TIF1β
DBC1
Actin
1
2
3
4
5
F
Immunoprecipitation
Input
IgG
COUP
Immunoblot
COUP-TFI
NCoR
TIF1β
DBC-1
Actin
1
2
3

Fig. 2.1. Validation of component proteins of FH-COUP complexes. (***A***) Schematic diagram of tandem epitope-tagged COUP-TFI. The sequences encoding FLAG and HA tags are located upstream of the human COUP-TFI cDNA in a retroviral vector. (***B***) Flow cytometric analysis of uninfected HeLa S3 cells and HeLa::FH-COUP cells after multiple rounds of magnetic cell purifications indicating greater than 95% purity of the latter. (***C***) Immunodetection of FH-COUP-TFI in cytoplasmic or nuclear fractions demonstrating that cells infected with FH-COUP-programmed but not control retrovirus express FH-COUP, and the majority of the fusion protein localizes in the nucleus. (***D***) Co-IP studies in pOZ-COUP cells. Nuclear lysates were immunoprecipitated with the anti-FLAG antibody or non-specific IgG, and these immune complexes were analyzed after gel electrophoresis by immunoblotting using the antibodies indicated on the left. Input represents 10% of material used for IP. (***E***). Co-immunoprecipitation analyses of FH-COUP, NCoR, TIF1β, and DBC1 using nuclear extract from pOZ-COUP cells. IPs and immunoblottings were conducted using indicated antibodies. The input lane corresponds to 5% of the nuclear extract used for IPs. (***F***) NCoR, TIF1β, and DBC1 co-IP with endogenous COUP-TFI from nuclear extracts prepared from untransfected SK-N-MC neuroblastoma cells. All studies shown in ***D-F*** are representative of 3-8 independent experiments.

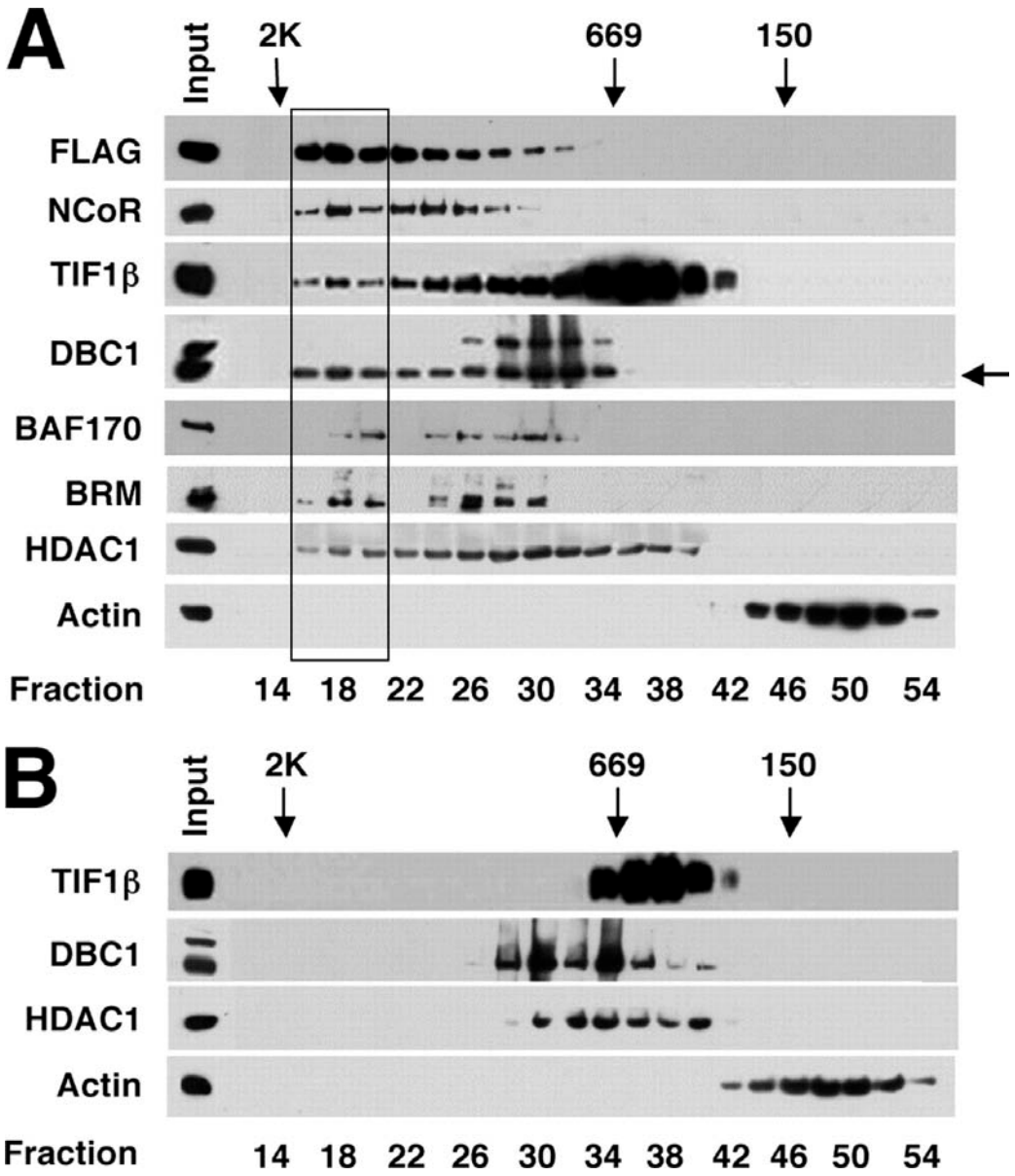

A
Input
2K
669
150
FLAG
NCoR
TIF1β
DBC1
BAF170
BRM
HDAC1
Actin
Fraction
14 18 22 26 30 34 38 42 46 50 54
B
Input
2K
669
150
TIF1β
DBC1
HDAC1
Actin
Fraction
14 18 22 26 30 34 38 42 46 50 54

Fig. 2.2. Gel filtration analysis of FH-COUP complexes. Size exclusion chromatography of nuclear extracts from pOZ-COUP (***A***) and pOZ-N (***B*)** cells using a Superose 6 column. Fractions were collected and analyzed by immunoblotting using antibodies indicated on the left. Elution peaks of the molecular weight standards are indicated at the top of the panel: blue dextran, 2000 kDa; thyroglobulin, 669 kDa; alcohol dehydrogenase, 150 kDa. Fraction numbers are indicated at the bottom of each panel.

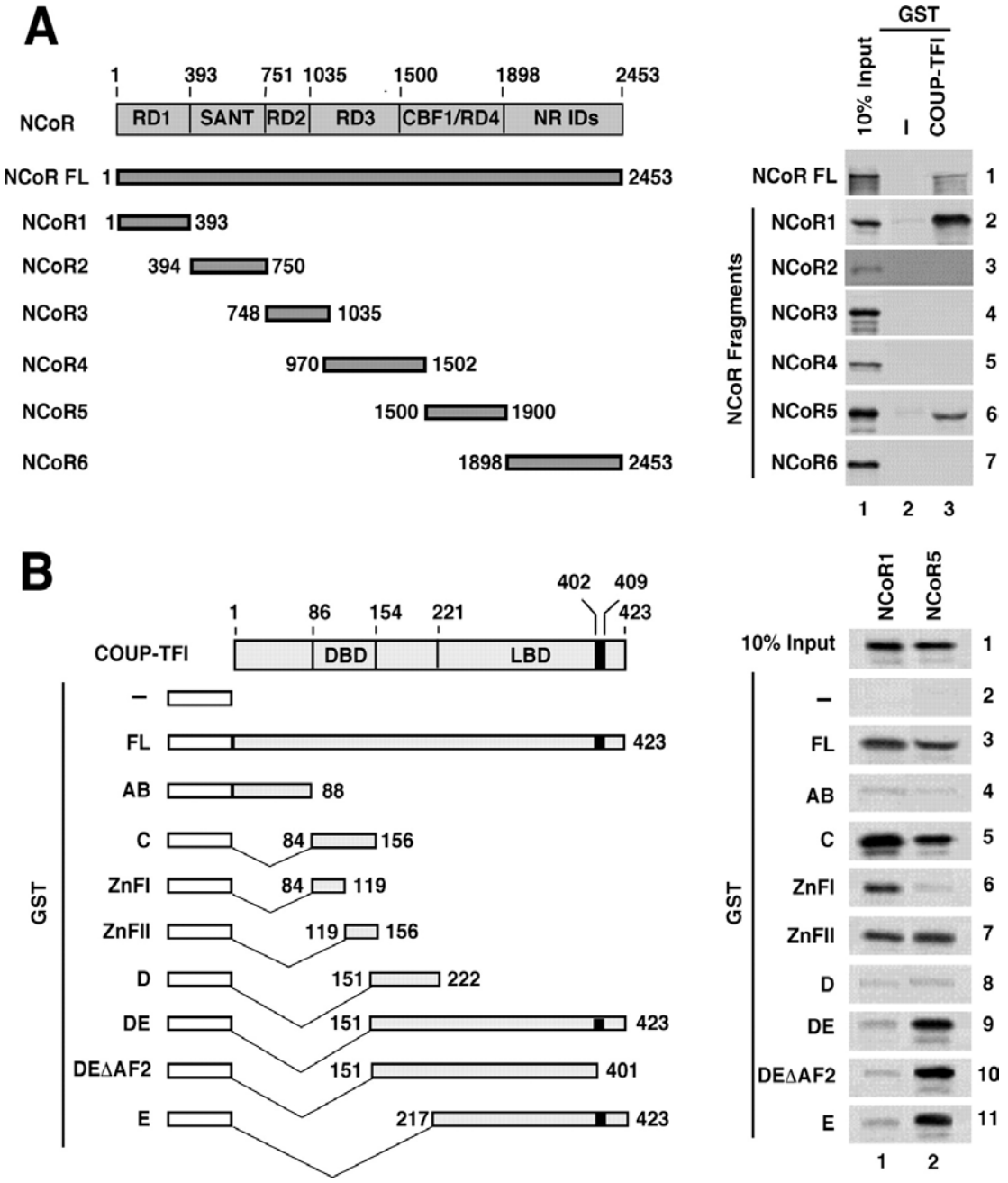
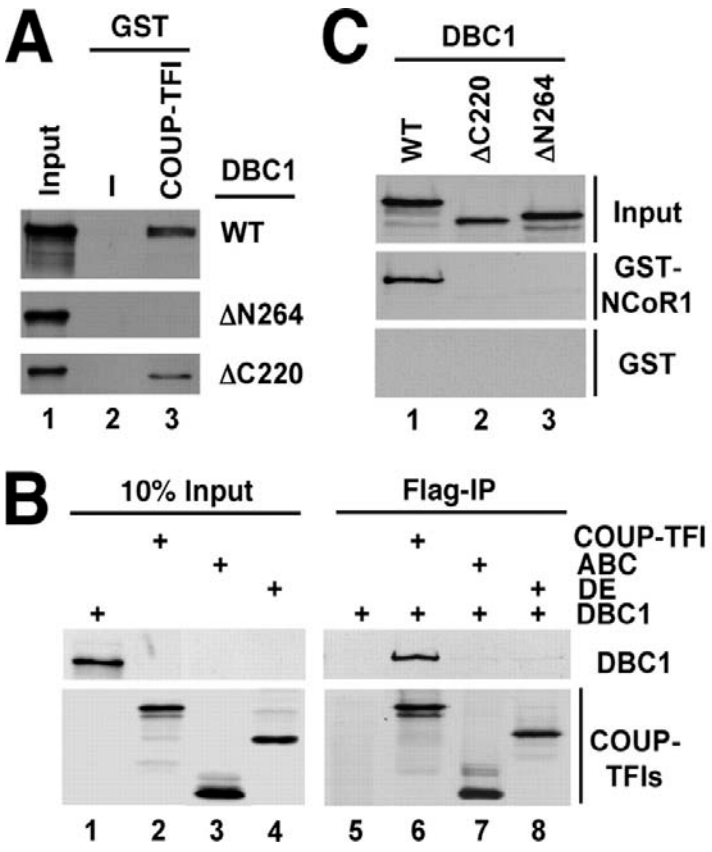

A
1
393
751
1035
1500
1898
2453
NCoR
RD1
SANT
RD2
RD3
CBF1/RD4
NR IDs
NCoR FL 1 2453
NCoR1 1 393
NCoR2 394 750
NCoR3 748 1035
NCoR4 970 1502
NCoR5 1500 1900
NCoR6 1898 2453
GST
10% Input
–
COUP-TFI
NCoR FL
NCoR1
NCoR2
NCoR3
NCoR4
NCoR5
NCoR6
NCoR Fragments
1
2
3
4
5
6
7
1 2 3
B
402
409
1
86
154
221
423
COUP-TFI
DBD
LBD
–
FL 423
AB 88
C 84 156
ZnFI 84 119
ZnFII 119 156
D 151 222
DE 151 423
DEΔAF2 151 401
E 217 423
GST
NCoR1
NCoR5
10% Input
–
FL
AB
C
ZnFI
ZnFII
D
DE
DEΔAF2
E
1
2
3
4
5
6
7
8
9
10
11
1 2

Fig. 2.3. NCoR interacts directly with COUP-TFI in an atypical manner **(*A*)** Diagram of NCoR and deletion mutants (left) used for GST pull down experiments (right). NCoR fragments were translated in vitro as [^{35}S]met-labeled proteins, and the presence of NCoR fragments in the pulldown was detected by autoradiography. *Lane* 1 in all cases was loaded with10% of input of individual NCoR fragments. **(*B*)** Diagram of FH-COUP and deletion mutants (left) used for GST pull down experiments (right) using various GST-COUP-TFI fragments and in vitro translated NCoR1 (*lane* 1) or NCoR5 (*lane* 2). The putative AF2 of COUP-TFI (amino acids 402-409) is denoted by a black box. Representative experiments are shown in


A
GST
Input
I
COUP-TFI
DBC1
WT
ΔN264
ΔC220
1 2 3
C
DBC1
WT
ΔC220
ΔN264
Input
GST-NCoR1
GST
1 2 3
B
10% Input
Flag-IP
COUP-TFI
ABC
DE
DBC1
DBC1
COUP-TFIs
1 2 3 4 5 6 7 8

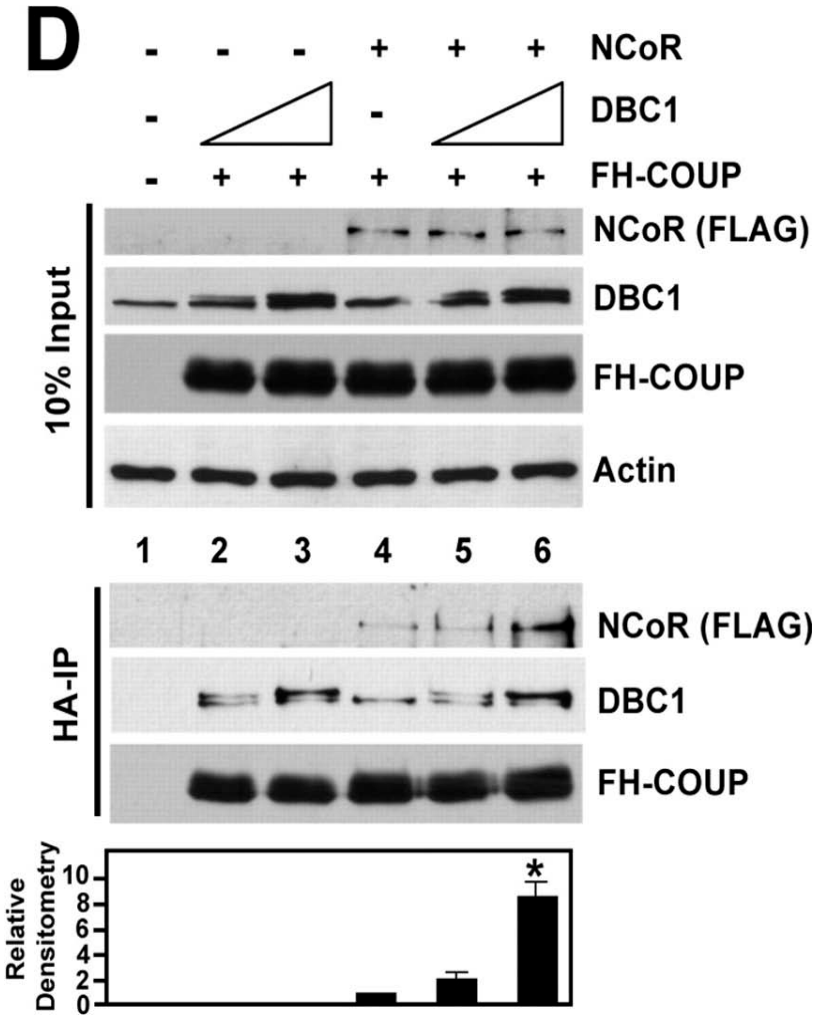

D
NCoR
DBC1
FH-COUP
10% Input
NCoR (FLAG)
DBC1
FH-COUP
Actin
1 2 3 4 5 6
HA-IP
NCoR (FLAG)
DBC1
FH-COUP
Relative Densitometry
10
8
6
4
2
0
*

Fig. 2.4. DBC1 interacts directly with both COUP-TFI and NCoR. (***A***) In vitro pull down of DBC1 by GST or GST-COUP-TFI. *Lane* 1 was loaded with 10% of the input used in pulldown reactions. DBC1 protein (full-length or truncation mutants) was translated in the presence of [^{35}S]met and detected by autoradiography. (***B***) In vitro pulldown of DBC1 by full-length FH-COUP or FH-COUP fragments by IP with the anti-Flag antibody. The upper panel corresponds the signal for full-length DBC1 ([35S]met-labeled protein), and the lower panel demonstrates that equal amount of Flag-COUP proteins were immunoprecipitated. *Lanes* 1-4 contain 10% of the input used in the IP reactions shown in *lanes* 5-8. (***C***) In vitro pull down of DBC1 and deletion mutant proteins by GST-NCoR1 and GST. DBC1 and its deletion mutants were detected as described in Fig. 4*A*. (***D***) Co-immunoprecipitation of transfected FH-COUP, DBC1, and NCoR in 293T cell extracts. Cell extracts were immunoprecipitated using anti-HA to pull down FH-COUP and immune complexes were analyzed by immunoblotting with the antibodies indicated on right. Note that lower band in the DBC1 blot (middle panel) corresponds to endogenous protein, whereas the signal for the upper band (asterisk) is derived from transfected DBC1-V5His. The NCoR expression vector used in this experiment harbors a C-terminal FLAG epitope, and transfected NCoR protein was detected using the anti-FLAG antibody. The lower panel depicts a densitometric analysis of the NCoR (FLAG) blot, indicating the amount of NCoR (FLAG) pulled down in each IP. This experiment is representative of two additional studies.

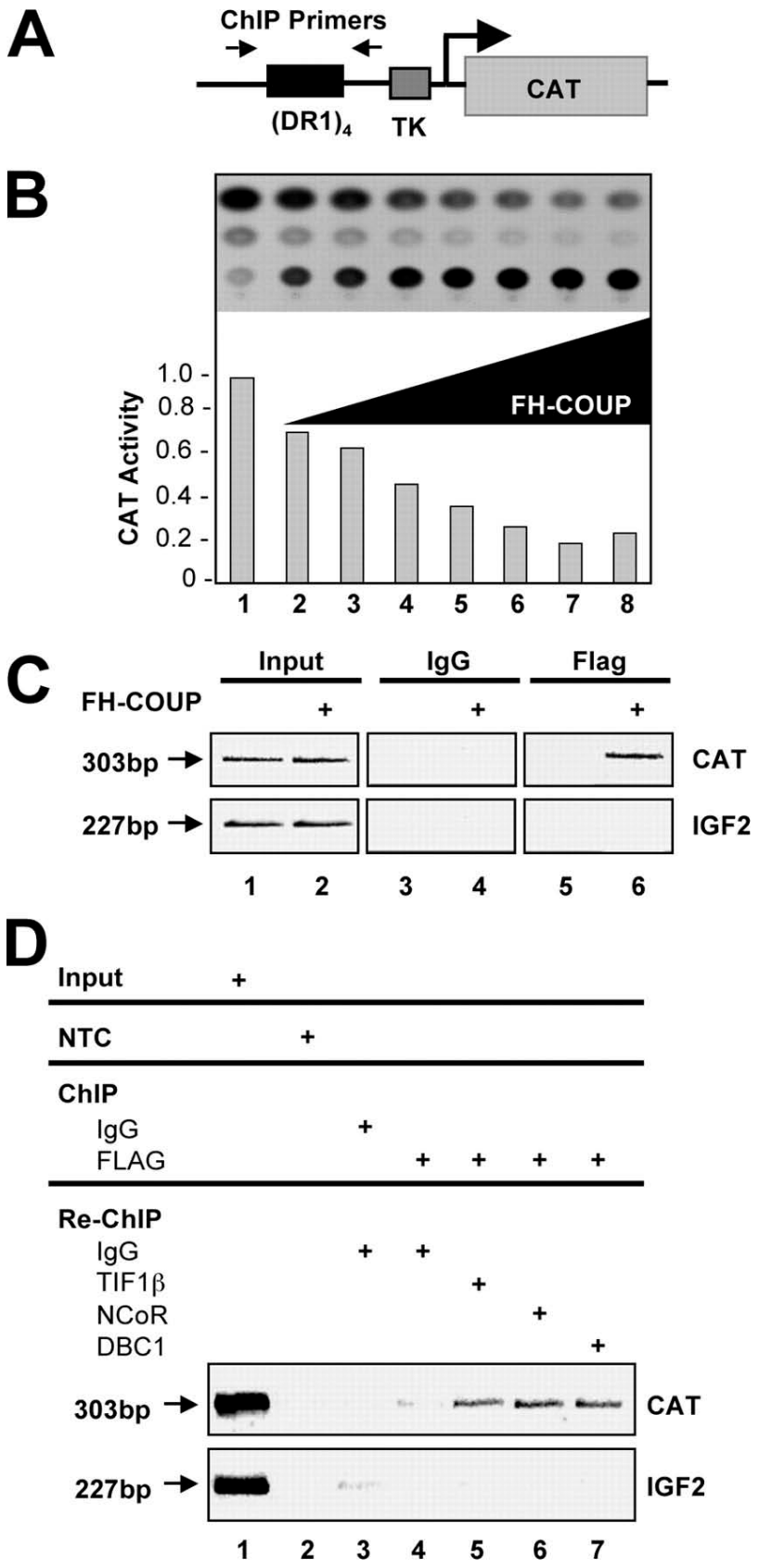

A
ChIP Primers
(DR1)4
TK
CAT
B
CAT Activity
1.0
0.8
0.6
0.4
0.2
0
FH-COUP
1 2 3 4 5 6 7 8
C
Input
IgG
Flag
FH-COUP
+ + +
303bp
227bp
CAT
IGF2
1 2 3 4 5 6
D
Input +
NTC +
ChIP
IgG +
FLAG + + + +
Re-ChIP
IgG + +
TIF1β +
NCoR +
DBC1 +
303bp
227bp
CAT
IGF2
1 2 3 4 5 6 7

Fig. 2.5. Co-occupation of a COUP-responsive promoter by FH-COUP, NCoR, TIF1β, and DBC1. (*A*) Schematic diagram of the $(DR1)_4$-tk-CAT reporter construct. ChIP primers were designed to amplify the region encompassing the multimerized DR1 element as indicated by arrows. **(*B*)** Repression of the basal CAT repression by COUP-TFI in 293T cells. Relative CAT activities are showed in the lower panel. **(*C*)** ChIP analyses demonstrate that COUP-TFI associates with the promoter region of the CAT reporter in transiently transfected cells. A region of the insulin-like growth factor 2 (IGF2) promoter (-1198 to -1424 bp upstream of transcriptional start site, 227 bp amplicon) was used as a negative control to demonstrate the specificity of FH-COUP binding to $(DR1)_4$-tk-CAT promoter. **(*D*)** Re-ChIP analyses demonstrate that FH-COUP, NCoR, TIF1β, and DBC1 co-occupy the $(DR1)_4$-tk-CAT promoter. NTC, no template control.

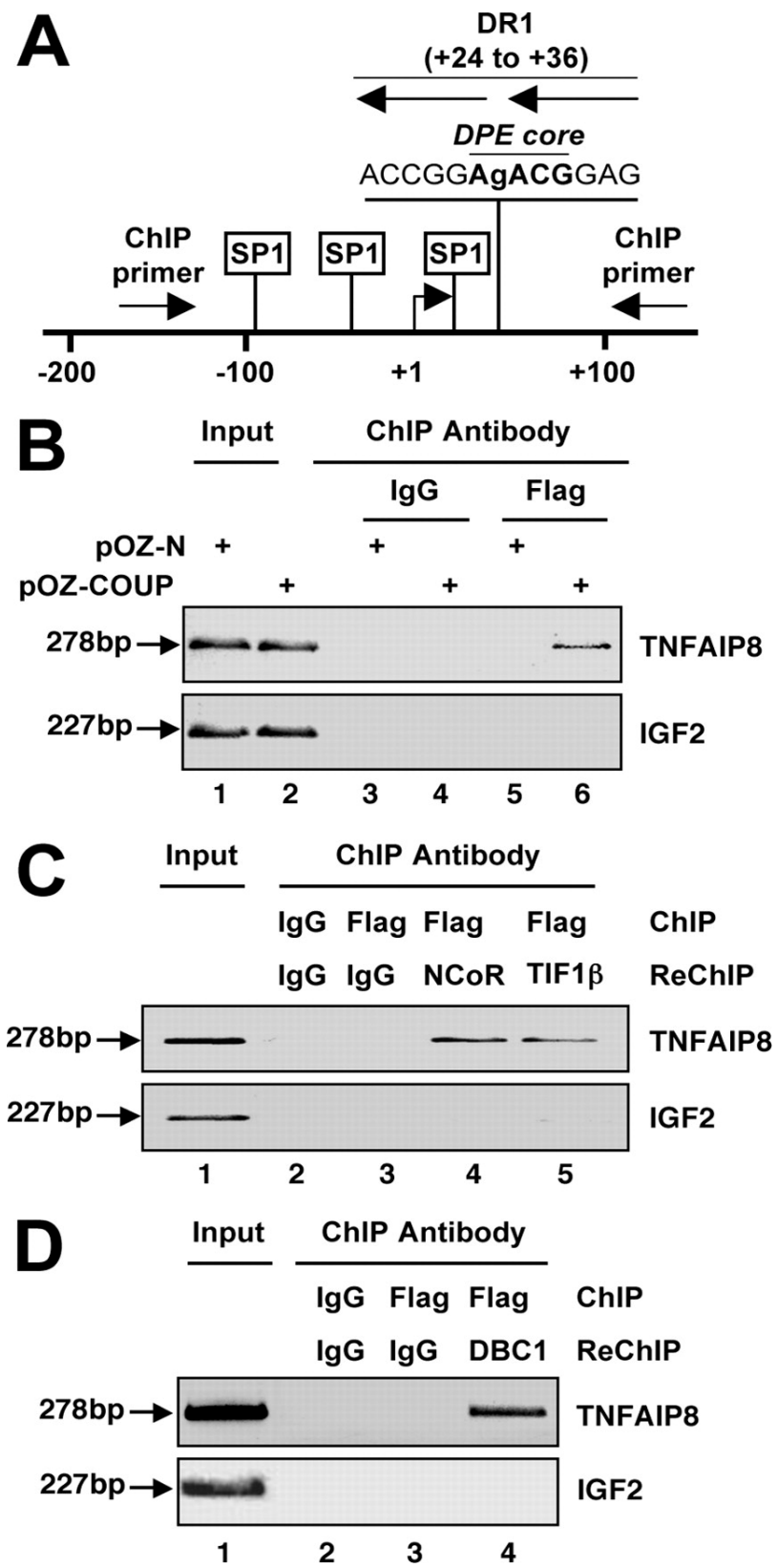

A
DR1
(+24 to +36)
DPE core
ACCGGAgACGGAG
ChIP primer
SP1
SP1
SP1
ChIP primer
-200
-100
+1
+100
B
Input
ChIP Antibody
IgG
Flag
pOZ-N
pOZ-COUP
278bp
227bp
TNFAIP8
IGF2
1 2 3 4 5 6
C
Input
ChIP Antibody
IgG Flag Flag Flag ChIP
IgG IgG NCoR TIF1β ReChIP
278bp
227bp
TNFAIP8
IGF2
1 2 3 4 5
D
Input
ChIP Antibody
IgG Flag Flag ChIP
IgG IgG DBC1 ReChIP
278bp
227bp
TNFAIP8
IGF2
1 2 3 4

Fig. 2.6. Identification of an endogenous target of COUP-TFI/NCoR complex in HeLa S3 cells (*A*) Schematic diagram of the TATA-less promoter region of the *TNFAIP8* gene highlighting the locations of GC box (SP1 binding site), DPE core element, and putative COUP-TFI response element (DR1). The location of ChIP primers, designed to amplify the region covering the putative COUP-TFI binding site, are indicated by arrows. **(*B*)** ChIP analysis demonstrating FH-COUP on the TNFAIP8 promoter in pOZ-COUP, but not pOZ-N cells. The primers used for the amplification reaction are shown schematically in ***B***. **(*C*)** and **(*D*)** Re-ChIP analyses demonstrating co-occupancy of the RAI1 promoter by FH-COUP, NCoR, TIF1β, and DBC1.

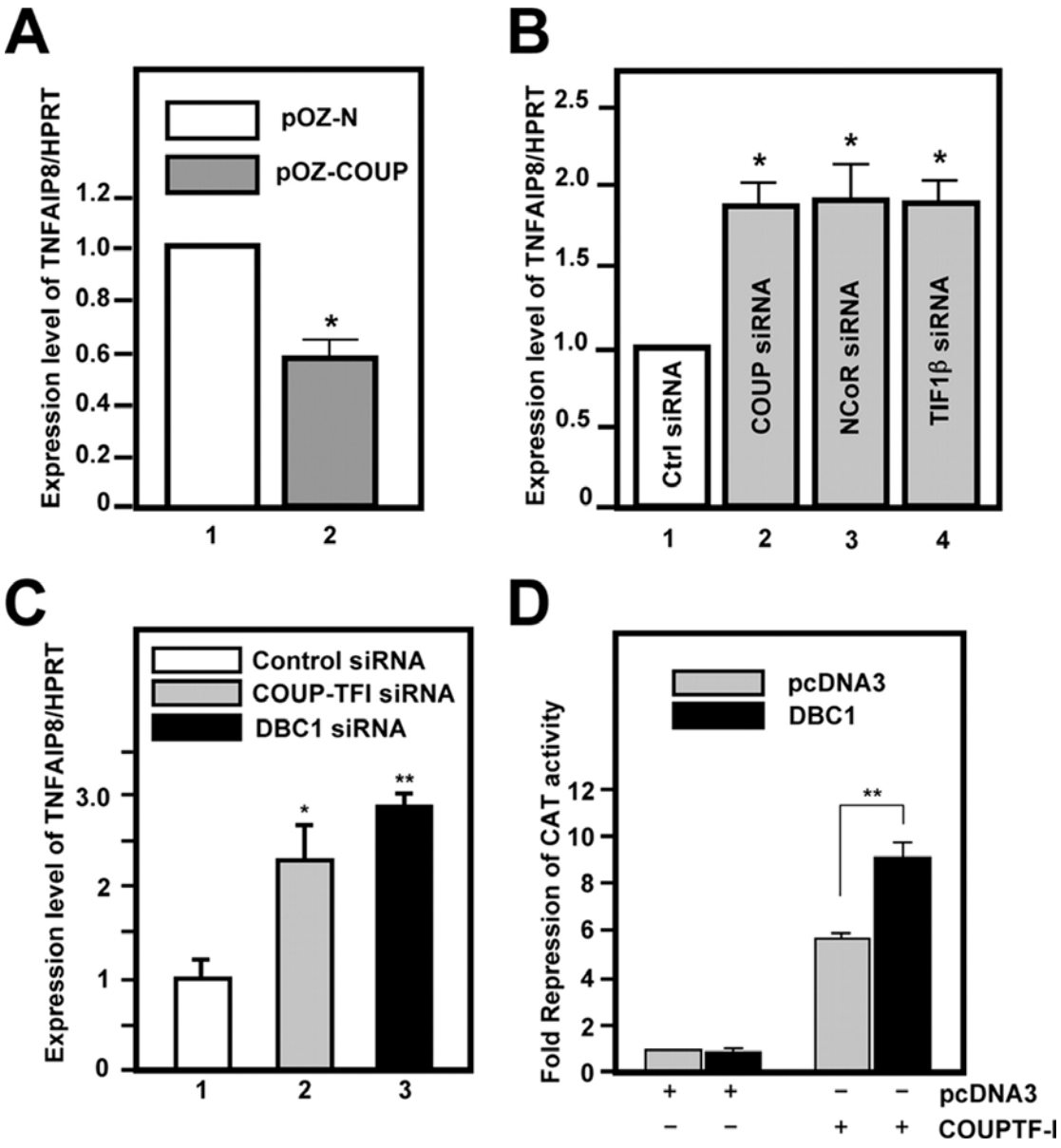

A
Expression level of TNFAIP8/HPRT
pOZ-N
pOZ-COUP
1.2
1.0
0.8
0.6
0.4
0.2
0
*
1
2
B
Expression level of TNFAIP8/HPRT
2.5
2.0
1.5
1.0
0.5
0
*
*
*
Ctrl siRNA
COUP siRNA
NCoR siRNA
TIF1β siRNA
1
2
3
4
C
Expression level of TNFAIP8/HPRT
Control siRNA
COUP-TFI siRNA
DBC1 siRNA
3.0
2
1
0
*
**
1
2
3
D
Fold Repression of CAT activity
pcDNA3
DBC1
12
10
8
6
4
2
0
**
+ + − − pcDNA3
− − + + COUPTF-I

Fig. 2.7. NCoR, TIF1β, and DBC1 are required for COUP-mediated transcriptional repression. (A) Repression of *TNFAIP8* expression by stable overexpression of FH-COUP in pOZ-COUP cells as revealed by RT-qPCR analyses. The relative fold-change of TNFAIP8 mRNA expression was calculated relative to the fold change of the expression of a house keeping gene, hypoxanthine-guanine phosphorybosyl transferase (HPRT). Bars represent mean expression levels of TNFAIP8 (± S.E.M., n = 3), relative to HPRT from independent determinations. Statistical significance was determined using a Student's t-test ($p = 0.03$, indicated by an asterisk) **(*B*)** HeLa S3 cells were transfected with negative control siRNA (Ctrl siRNA) or siRNA targeting COUP-TFI, NCoR, or TIF1β, and subjected to RT-qPCR analyses. The expression level of *TNFAIP8* was determined by RT-qPCR as described above. **(*C*)** siRNA-mediated COUP-TFI and DBC1 knockdown results in derepression of *TNFAIP8* expression in 293T cells. Transfection conditions and analyses were as described above. **(*D*)** DBC1 stimulates COUP-TFI-dependent gene repression in 293T cells, Cells were transfected with the $(DR1)_4$-tk-CAT reporter, FH-COUP-TFI, and DBC1-V5His DNA constructs as indicated. CAT activities were quantified, and the level of fold-repression was calculated relative to the basal reporter activity. Addition of DBC1 significantly stimulated fold repression of CAT activity mediated by COUP-TFI ($p < 0.01$, indicated by two asterisks).

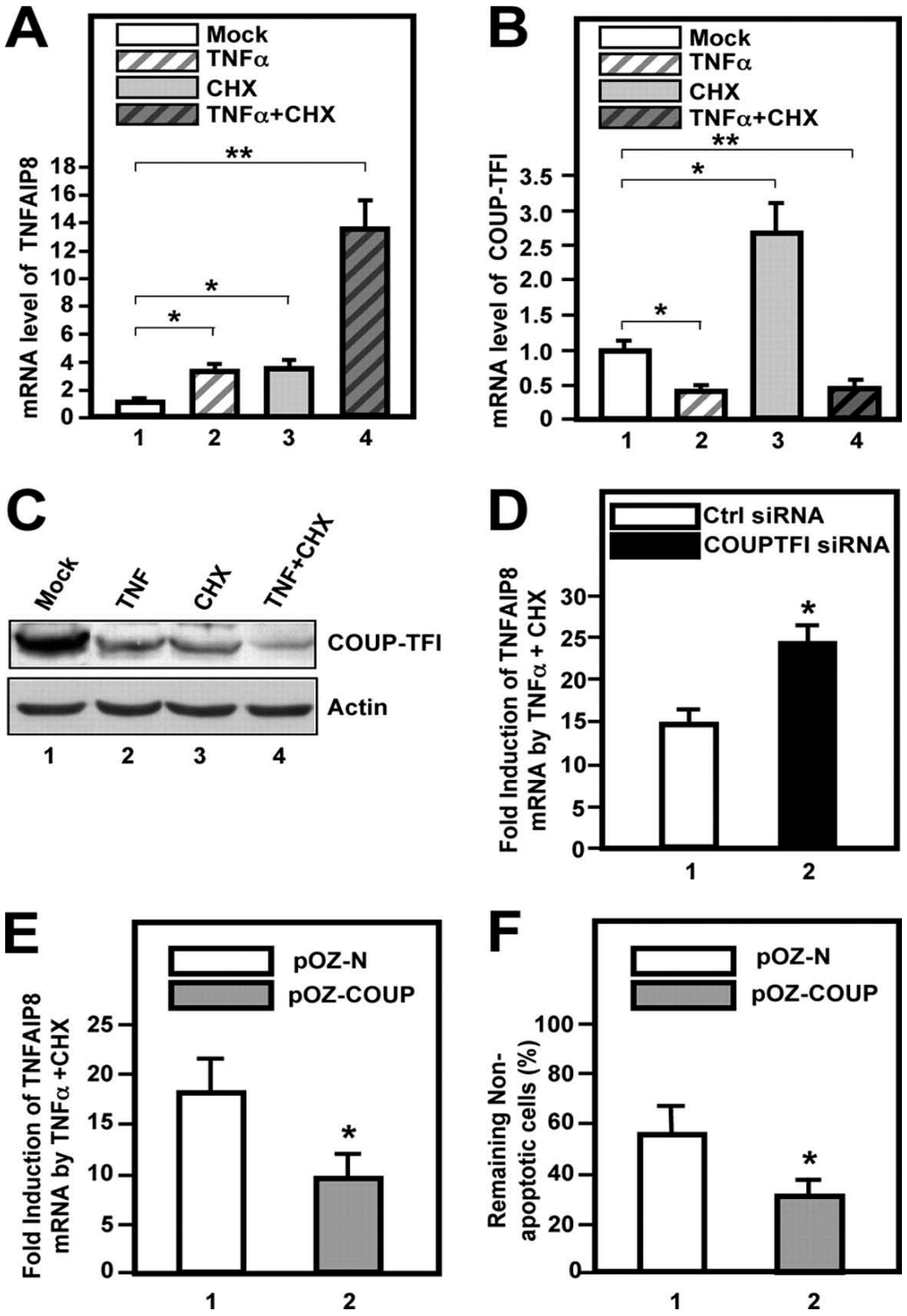

A
Mock
TNFα
CHX
TNFα+CHX
mRNA level of TNFAIP8
0
2
4
6
8
10
12
14
16
18
*
*
**
1
2
3
4
B
Mock
TNFα
CHX
TNFα+CHX
mRNA level of COUP-TFI
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
*
*
**
1
2
3
4
C
Mock
TNF
CHX
TNF+CHX
COUP-TFI
Actin
1
2
3
4
D
Ctrl siRNA
COUPTFI siRNA
Fold Induction of TNFAIP8 mRNA by TNFα + CHX
0
5
10
15
20
25
30
*
1
2
E
pOZ-N
pOZ-COUP
Fold Induction of TNFAIP8 mRNA by TNFα +CHX
0
5
10
15
20
25
*
1
2
F
pOZ-N
pOZ-COUP
Remaining Non-apoptotic cells (%)
0
20
40
60
80
100
*
1
2

Fig. 2.8. Role of COUP-TFI in TNFα/CHX–induced apoptosis. (*A*) Treatment of HeLa S2 cells with TNFα and protein synthesis inhibitor CHX synergistically induced *TNFAIP8* expression as determined by RT-qPCR. Cells treated with vehicle (DMSO, indicated as mock), TNFα (5μg/ml), CHX (30μg/ml), or TNFα/CHX for 4 hrs. TNFAIP8 transcript levels were analyzed by RT-qPCR and statistical significance was determined using a Student's t-test ($p < 0.05$, a single asterisk; $p < 0.01$, two asterisks) **(*B*)** Treatment with TNFα, but not CHX, downregulated COUP-TFI transcripts in HeLa S3 cells. COUP-TFI transcript levels were determined by RT-qPCR and expressed relative to those of HPRT as shown. **(*C*)** TNFα and CHX downregulated COUP-TFI protein levels. HeLaS3 cells were treated as indicated for 4 hrs, and cells were lysed immediately and subjected to immunoblot analyses using antibody against endogenous COUP-TFI or actin as loading control. **(*D*)** knock-down of COUP-TFI stimulated induction of *TNFAIP8* by TNFα/CHX treatment. HeLa S3 cells were transfected with COUP-TFI siRNA for 40 hrs prior to TNFα/CHX treatment. Induction of TNFAIP8 was measured by RT-qPCR as shown, . **(*E*)** *TNFAIP8* induction by TNFα/CHX was attenuated in pOZ-COUP relative to pOZ-N cells. **(*F*)** Overexpression of COUP-TFI sensitized HeLa S3 cells to apoptosis initiated by TNFα/CHX. pOZ-COUP or pOZ-N cells were grown on coverslips and treated with TNFα/CHX for 8 h. The percentage of remaining, non-apoptotic cells after this treatment was determined by DAPI staining. Single and double asterisks indicate statistical significance at the $p < 0.05$ and $p < 0.01$ levels, respectively, when comparing treatments to mock treatment (A and B), COUP-TFI-specific siRNA to control siRNA (D), and pOZ-COUP to pOZ-N cells (E, F).

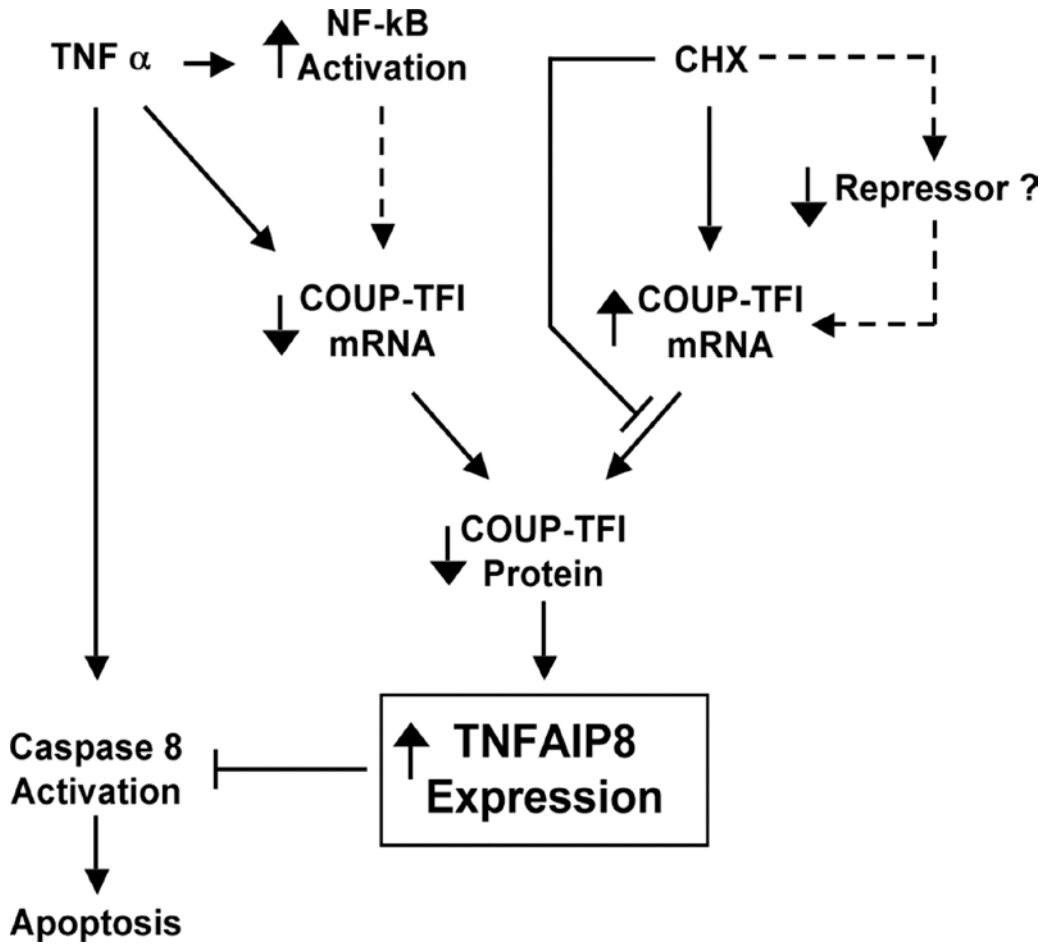


Fig. 2.9. Role of COUP-TFI during TNFα-induced apoptosis. Solid and dashed lines represent known and hypothesized events, respectively. Note that treatment with either TNFα or CHX reduces endogenous COUP-TFI protein levels, which relieves repression of the TNFAIP8 promoter, resulting in inhibition of caspase 8 activation and reduced apoptosis. Conversely, ectopically overexpressed COUP-TFI represses *TNFAIP8* expression in a manner that is insensitive TNFα, resulting in loss of TNFAIP8 protein and increased sensitivity to TNFα-induced apoptosis. Thus, COUP-TFI may play a central role in the sensitivity of a cell to TNFα-induced apoptosis.

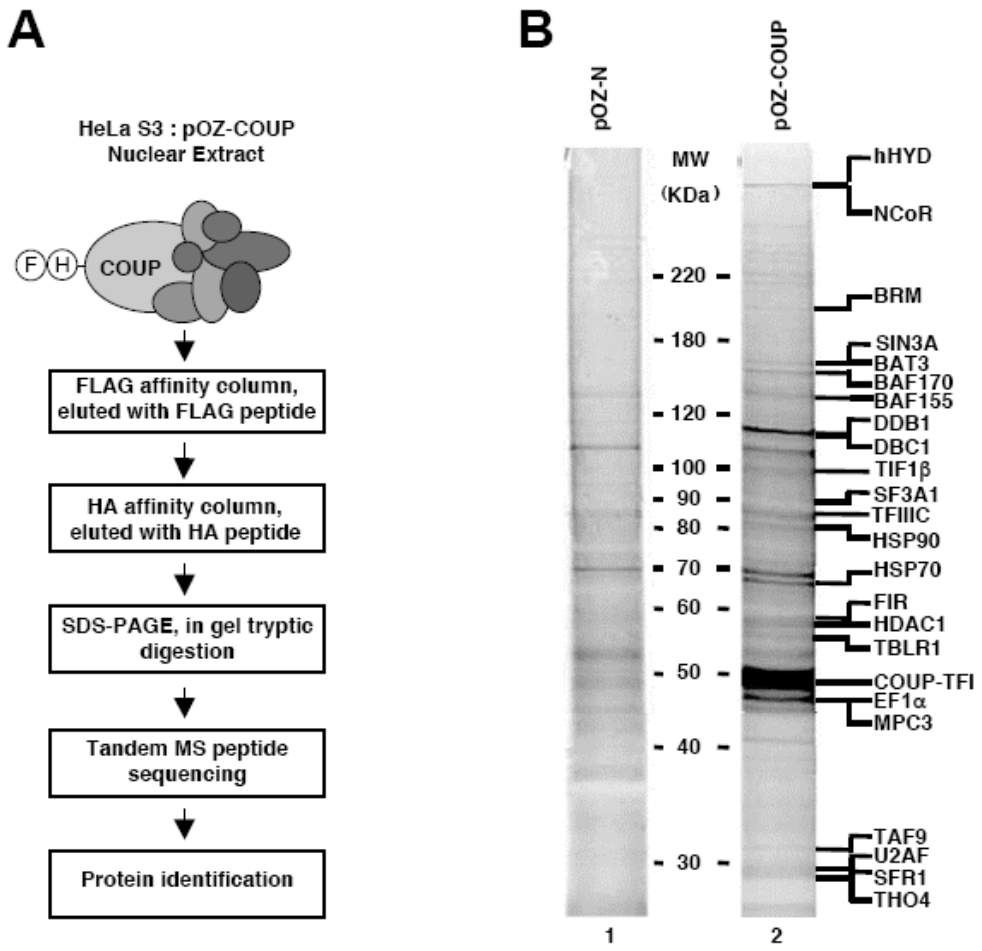


Fig. 2.S1 (A). Flow chart of the experimental design for TAP-MS/MS. **(B).** Purified COUP-TFI protein complexes were resolved on a 4-12% gradient SDS-PAGE gel and visualized by coomassie staining (lane 2). Proteins present in lane 2 (pOZ-COUP cells), but not lane 1 (pOZ-N cells) were identified by LC/ESI MS/MS using a nano2D LC (Eksigent) coupled to an LTQ-FT mass spectrometer. The identities of these proteins that have been verified by immunoblotting analyses are shown to the right of the gel.

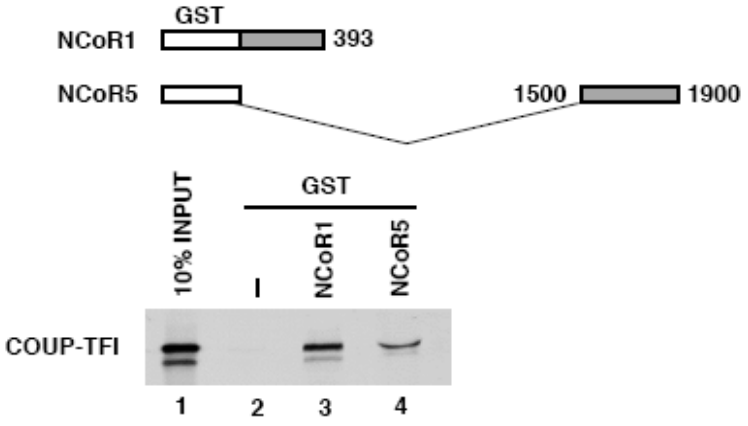


Fig. 2.S2. GST-NCoR 1 and GST-NCoR 5 interacted with in vitro translated COUP-TFI. The white box at the amino termini of GST-NCoR1 and -5 denotes GST.

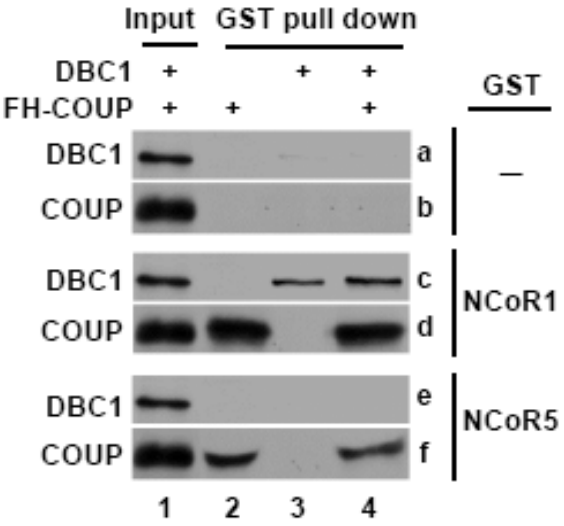


Fig. 2.S3. In vitro pull down of DBC1 and FH-COUP by GST, GST-NCoR1 and GST-NCoR5. Both DBC1 and FH-COUP used in the experiments were [^{35}S]met-labeled, as prepared by in vitro transcription/translation.

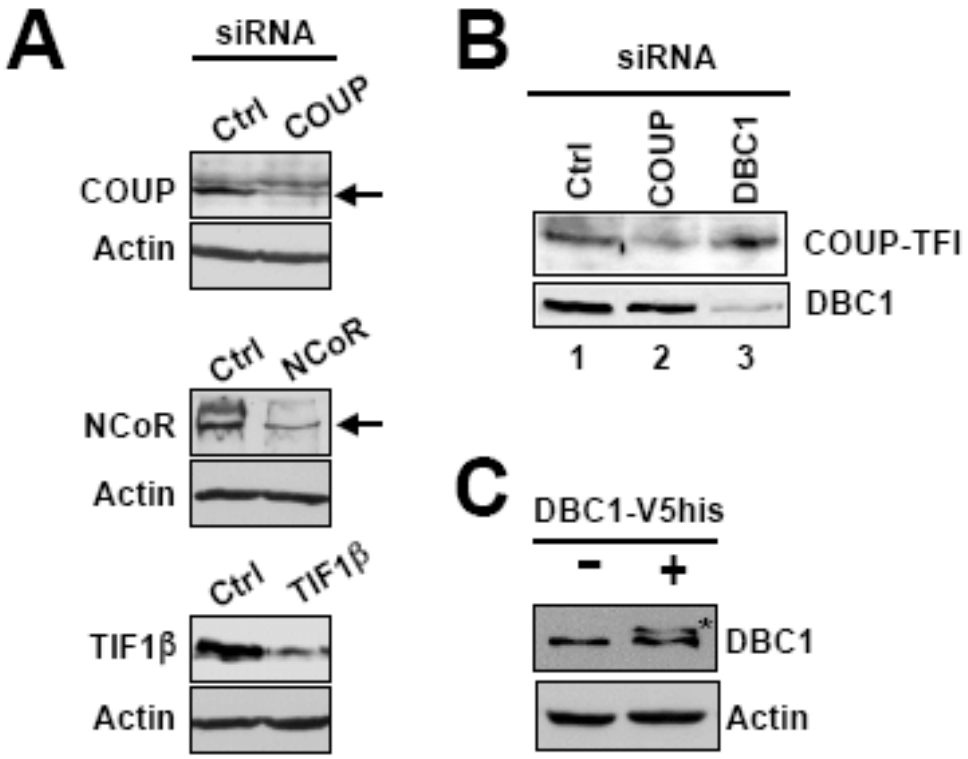


Fig. 2.S4. Immunoblotting analyses showing knock-down of (**A**) COUP-TFI, NCoR, and TIF1β by siRNA in HeLa S3 cells, and (**B**) COUP-TFI and DBC1 in HEK293T cells. **(C)** Immuno- blotting analyses showing the expression of transfected DBC1-V5his (middle panel, the upper band, indicated by an asterisk) relative to endogenous DBC1 (the lower band). Actin is used as loading control for entire figure. This figure accompanies Fig. 7B of the manuscript.

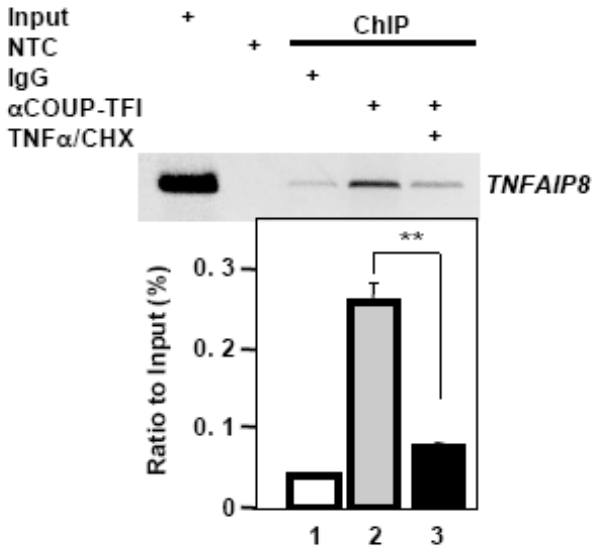


Fig. 2.S5. ChIP analysis demonstrating dismissal of endogenous COUP-TFI from the TNFAIP8 promoter upon treatment with TNFα. The gel image on top represents a single ChIP study conducting using normal PCR. The bar graph depicts the average of three independent ChIP studies conducted using qPCR to quantify amplicons from input, and IgG and anti-COUP-TFI immunoprecipitations. Values shown on the ordinate represent the ratio of immunoprecipitated to input amplicons. Cells were treated with vehicle (DMSO) or TNFα (5 µg/mL) and CHX (30µg/ml) for 4 hrs prior to formaldehyde-based crosslinking and ChIP processing as described in the manuscript. Two asterisks indicate $p < 0.01$ as determined by a T test. NTC, no template control.

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CHAPTER 3

DYNAMIC REGULATION OF CTIP2 BY PHOSPHORYLATION AND SUMOYLATION DURING IN VITRO DIFFERENTIATION OF PRIMARY THYMOCYTES

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ABSTRACT

Ctip2, also known as Bcl11b, is a C2H2 zinc finger transcription factor that is highly expressed in and plays essential yet poorly understood roles during development of T lymphocytes, the central nervous system, skin and the craniofacial complex. In this study, we use an ex vivo thymocyte differentiation system, where primary mouse thymocytes are treated with PKC activator PMA and calcium ionophore A23187 (P/A) and differentiate from mostly double positive (DP) to CD8 single positive (SP) cells. We have discovered a dynamically regulated pathway involving sequential and linked post-translational modification of Ctip2 during this process. We found that Ctip2 was rapidly phosphorylated and then quickly became dephosphorylated, followed by subsequent SUMOylation at K607 and K805 residues by treating thymocytes with P/A. SUMOylation of Ctip2 is dynamic and is tightly regulated by the phosphorylation status of Ctip2. Blockage of Ctip2 dephosphorylation completely abolished Ctip2 SUMOylation as well as the interaction between Ctip2 and Ubc9, the SUMO E2 ligase. We also found that the time course with which Ctip2 is SUMOylated after stimulation with P/A not only corresponded to the induction of the expression of Id2, a newly identified Ctip2 targeted gene, but also corresponded to the localization of Ctip2, MTA2, acetyltransferase p300 and acetylated histone H3 and H4 at the Id2 transcription start site (TSS), as revealed by ChIP analyses. Further studies reveal that p300 interacted with Ctip2, and was specifically recruited to the Ctip2 complex on the Id2 TSS in thymocytes stimulated with P/A. Thus, our work demonstrates that the transcription regulatory activity of Ctip2 is dynamically regulated by phosphorylation and SUMOylation, and SUMOylation may be a molecular

switch that converts Ctip2 from transcription repressor into an activator of the mouse Id2 promoter during thymocyte development.

INTRODUCTION

Ctip2, which is an essential transcription factor for post-natal life in the mouse, plays crucial roles in the development, and presumably function, of several organ systems, including the central nervous (Arlotta et al., 2005), cutaneous (Golonzhka et al., 2007; Golonzhka et al., 2009a) and immune systems (Wakabayashi et al., 2003b).

The multiple differentiation and specification steps in thymocyte development are regulated through the concerted action of a series of transcription factors, including Ctip2. Germline deletion of Ctip2 results in a blockade of the DN3→DN4 transition with the complete absence of αβ T cells, and this block is phenotypically similar to that seen in NCoR- (Jepsen et al., 2000), E2A- (Bain et al., 1997), and HEB- (Barndt et al., 1999; Zhuang et al., 1996) null mice, conditionally null c-myb mice when crossed with the appropriate deleter strain (Bender et al., 2004), and transgenic mice ectopically expressing Id2 (Morrow et al., 1999) or a dominant negative form of HEB {Barndt, 2000 #265} in thymocytes. In contrast, deletion of Ctip2 at the DP stage by use of a CD4-cre transgene allows development of DP cells but not transition to the SP stage (Albu et al., 2007). Thus, Ctip2 plays an important role in at least two stages of T cell development: β selection (DN3 → DN4 transition) and in the DP → SP transition.

Ctip2 was originally identified as a protein that interacted directly with the orphan nuclear receptor COUP-TF2 (Avram et al., 2000) Later studies revealed

that Ctip2 is a C_2H_2 zinc finger protein, and can bind directly to target DNA to act both as a transcriptional repressor and activator in a promoter context dependent manner (Avram et al., 2002). Ctip2 represses transcription of reporter genes in transiently transfected cells, either by tethering to other promoter-bound transcription factors or by direct, sequence-specific DNA binding activity (Avram et al., 2002; Senawong et al., 2003; Topark-Ngarm et al., 2006). The regulatory element to which Ctip2 binds is reminiscent of a GC-box, as was revealed by a binding-site selection technique (Avram et al., 2002). Ctip2-mediated transcriptional repression appears to be due to the recruitment of the class III histone deacetylase (HDAC) SIRT1 (Senawong et al., 2003) and/or the class I HDACs, HDAC1 and HDAC2 (Topark-Ngarm et al., 2006), to the promoter template, the latter of which occurs within the context of the **Nu**cleosome **R**emodeling and **D**eacetylation (NuRD) complex (Cismasiu et al., 2005; Topark-Ngarm et al., 2006). Ctip2 has also been reported to sequester and target transcriptional activators, such as HIV Tat, to heterochromatic loci, which may represent a third mechanism of Ctip2-mediated transcriptional silencing (Marban et al., 2005). The hypothesis that Ctip2 functions as a transcriptional repressor has been supported by transcriptome analyses in mouse T cells (unpublished data) and human neuroblastoma cells (Topark-Ngarm et al., 2006). However, approximately one-third of the genes that were dysregulated in the DP cells of Ctip2-null mice were down-regulated relative to control T cells, suggesting that Ctip2 may act as a transcriptional activator in some promoter and/or cell contexts. We have also found that Ctip2 functions as a transcriptional activator in a promoter context-dependent manner (Golonzhka et al., 2009a; Golonzhka et al., 2009b).

The MAP kinase pathway is implicated in both early (DN3→DN4 transition; Refs (Bommhardt et al., 1999; Crompton et al., 1996; Sharp et al., 1997) and late (positive selection (Alberola-Ila et al., 1995; Priatel et al., 2002; Swan et al., 1995; Wan et al., 2004) events in T cell development. Mice lacking upstream proteins such as Ras, RasGRP1, Raf, and PI3 kinase, as well as MEK and ERK1/2, exhibit a severe block in positive selection (Fischer et al., 2005), but disruption of genes downstream of ERK1/2, such as Sap1/Elk4 (Costello et al., 2004), Egr1 (Bettini et al., 2002), and Id3 (Rivera et al., 2000) results in less severe phenotypes, perhaps owing to functional redundancy among downstream target family members (Fischer et al., 2005) The MAP kinase pathway, like Ctip2, plays an important role in at least two stages of T cell development: β selection and positive selection.

To study how Ctip2 is regulated through T cell signaling pathways, we adapted an in vitro T cell differentiation system, in which primary thymocytes were stimulated with the protein kinase C (PKC) activator PMA and the calcium ionophore A23187, combination of which has been shown to bypass a T cell receptor (TCR) signal and activate signaling pathways necessary for initiation of single positive differentiation program in vitro. PMA directly activates several isoforms of PKC (Ryves et al., 1991), which then activate downstream kinases such as ERK1/2 and p38MAPK (Alberola-Ila et al., 1995). The calcium ionophore A23187, which mimics calcium-mediated signaling, activates p38MAPK in T cells (Matsuda et al., 1998). Upon activation, MAPKs translocate from the cytosol to the nucleus and phosphorylate transcription factors, leading to changes of gene expression and induction of positive selection (Rothenberg and Taghon, 2005). Our data presented herein suggest that the post-translational modification (PTM) status, including phosphorylation

and SUMOylation, of Ctip2 is dynamically regulated by the MAP kinase pathways, and these PTM may influence the transcriptional regulatory activity of Ctip2 on target promoters. These findings strongly suggest that Ctip2 may function as a node (transcription factor) in MAP kinase pathways downstream of TCR activation. This study contributes to a better understanding of the mechanistic basis for and the molecular consequences of cell signaling-dependent modification of Ctip2 by PTM.

METHODS AND MATERIALS

Cell culture – Primary thymocytes were isolated from 4-8 week-old C57BL/6 wild-type mice. Thymcoytes were cultured at 37°C in RPMI1640 (VWR) with 5% fetal bovine serum (FBS) (from Atlas Biologicals) and 1% penicillin/streptomysin (Invitrogen) prior to drug treatment. Jurkat, the human leukemia T cell line, was grown under the same conditions except that 10% FBS was used. HEK293T cells were grown in Dulbecco's modified Eagle's medium (VWR) with10% FBS and 1% penicillin/streptomysin.

Chemicals and antibodies – PMA was purchased from Sigma (St. Louis, MO). A23187, U0126, SB202190, GFX, MG132, zVAD were purchased from EMD Biosciences (Gibbstown, NJ). CalyculinA was purchased from cell signaling (Danvers, MA). Rat anti-CTIP2 antisera was raised against CTIP2 peptide corresponding to amino acids 25-44 and purified on a peptide affinity column. Antibodies against phosphor-threonine, phosph-ERK1/2 and phosph-p38 were purchased from cell signaling. Antibodies against SUMO1 and p300 were purchased from Santa Curz Biotechnology (Santa Cruz, CA). Antibodies against MTA2, HDAC1 and HDAC2 were purchased from Bethyl

Laboratories (Montgomery, TX). The anti-HA antibody was purchased from Roche Applied Science. The anti-Flag antibody was purchased from Sigma (St. Louis, MO).

Compound treatments – Primary thymocytes were cultured for 4 hours before treating with vehicle (DMSO) or a P/A cocktail consisting of PMA (100nM) and A23187 (500nM) for indicated times. In some studies, cells were pretreated with indicated kinase inhibitors for 30 mins prior to P/A treatment. Jurkat cells were serum starved overnight before stimulation with P/A.

DNA constructs – FCtip2 was made as previously described (Senawong et al., 2003). F-Ctip2 Lysine to Arginine mutant constructs including K607R, 805R and K607/805R (2R) were made using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). HASUMO1 and HAUbc9 DNA constructs were purchased from Addgene Inc (Cambridge, MA). HASU-FCtip2 was generated by PCR amplification of HASUMO1 with primers containing appropriate restriction sites for insertion into the N-terminal of F-Ctip2. All constructs were verified by complete DNA sequencing. The *tk* promoter was removed from pBLCAT2 vector using BamH I and Bgl II or BamH I and Xho I restriction enzymes. The BamH I/Bgl II fragment of pBLCAT2 was re-ligated to reconstitute a promoterless-CAT reporter construct. The BamH I/ Xho I promoterless-vector was used for subsequent cloning as described below. The -2.9 kb Id2-CAT reporter constructs were prepared by amplification of a 5' region of mouse *Id2* gene ranging from -2.9 kb to +152 bp, relative to TSS using appropriate primers and a DNA template from mouse ES cells (Expand long template PCR system, Roche). These amplified fragments were cloned into pCR 2.1 vector using a TA cloning kit (Invitrogen). An insert in the pCR 2.1

vector was verified by sequencing before being removed and cloned into BamH I/ Xho I sites of the promoterless-CAT reporter construct.

Transfections – 293T cells (2 X 10^6 cells) were plated onto a 10 cm plate and transfected 24 h later using the calcium phosphate method. Cells were transfected with indicated amount of DNA and an empty expression vector to standardize the total amount of DNA transfected. The medium was changed, and the cells were harvested 24 and 48 h after transfection, respectively. Jurkat cells (2 X 10^6 cells) were plated onto a 35mm dish and transfected with wild-type F-Ctip2 or F-Ctip2 mutants using TransIT-Jurkat reagent (Mirus Bio LLC). 24 h later, cells were serum starved overnight followed by P/A treatment for 60 mins prior to harvest.

Immunoprecipitation, co-immunoprecipitation and immunoblotting – To preserve phosphorylation and SUMOylation of Ctip2, cells treated with indicated compounds were harvested, lysed in a denaturing buffer containing 250mM NaCl, 2mM EDTA, 50mM NaF, 5mM sodium pyrophosphate, 5mM NEM, 0.1mM H.C. (hemin chloride) and 1% SDS, boiled for 5mins, sonicated and centrifuged to remove DNA. The resulting whole cell extracts were then diluted to 0.1% SDS and 1% Triton X200 followed by IP analyses. Immunoprecipitation assays and immunoblotting were conducted as described previously (Zhang et al., 2009). To study protein-protein interaction, whole cell extract were prepared using a similar buffer as described above, but in the absence of denaturing conditions.

Reporter gene assays – HEK293T cells were transfected and harvested 48 h after transfection. A β-galactosidase expression vector (pCMV-Sport-βGal,

Life Technologies) was co-transfected as an internal control, and β-galactosidase activity and total protein concentration were used for normalization across all samples. The relative chloramphenicol acetyltransferase was determined as described previously (Dowell et al., 1997).

Flow cytometry analysis – Primary thymocytes were treated with P/A for 2 days and labeled with FITC-labeled CD4 and APC-Alexa Fluor-labeled CD8 antibodies (purchased from eBioscience (San Diego, CA). After staining, samples were fixed with 2% PFA and store in 4°C prior to analysis on an FC 500 flow cytometer with CXP Software (Beckman Coulter)

Quantitative reverse transcription-PCR – cDNA was prepared from RNA as described previously (Zhang, 2009). The resulting cDNA was then used for the following amplification reactions: Id2, 5'- G T C C T T G C A G G C A T C T G A A T -3' (forward primer) and 5'- C T C C T G G T G A A A T G G C T G A T -3' (reverse primer); cKrox, 5'- C A C A C T G G T G A G A A G C C C T T -3' (forward primer) and 5'- C A G G T G G C A C T C A T A G G G C-3' (reverse primer); RunX3, 5'-C A G G T T C A A C G A C C T T C G A T T-3' (forward primer) and 5'-G T G G T A G G T A G C C A C T T G G G-3' (reverse primer); and GAPDH, 5'-C A T C A A C G G G A A G C C C A T -3' (forward primer) and 5'- A C A C C C A T C A C A A A C A T G G G -3' (reverse primer). These primer sets generate amplifications around 50 to 150 bp. An Applied Biosystems 7500 real-time PCR instrument and SYBR Green technology (Qiagen) were used for all quantitative PCR.

Chromatin immunoprecipitation – Both chromatin immunoprecipitation

(ChIP) and re-ChIP studies were conducted similarly as described previously (Zhang et al., 2009), except that micrococcal nuclease (MNase) was added to facilitate chromatin shearing during lysis. The resulting ChIP DNA were subjected to quantitative PCR analyses using the following amplification primers covering mId2 promoter regions (relative to transcription start site): -3Kb, 5'-T G C C A A T T C A T C A C C T G T C -3' (forward primer) and 5'- C C T C G G T T T T G T T C T C A C A G -3' (reverse primer); TSS, 5'- G G C G A G T G C A C A T A A A A G A C -3' (forward primer) and 5'- C C T C T G G T T G A G C A G T C A G T -3' (reverse primer) and 3'UTR, 5'- G G C T C G G T T C A G A A T G A A G -3' (forward primer) and 5'- T C A A A A T T C A G G C T G G A G A G -3' (reverse primer).

RESULTS

Ctip2 is a dynamically regulated phosphoprotein in mouse thymocytes — Previous studies of double positive (DP) thymocytes from Ctip2 null (*Ctip2*$^{dp-/-}$) mice revealed that Ctip2 plays key roles during T cell differentiation (Albu et al., 2007). To investigate if Ctip2 is regulated by activated T cell signaling pathways during differentiation, we stimulated primary mouse thymocyte cells with the pan-PKC activator PMA, together with the calcium ionophore A23187. This treatment regimen serves as a surrogate for activation of cell signaling pathways, including T cell receptor activation (Takahama and Nakauchi, 1996). First, we found that Ctip2 was a phosphoprotein in unstimulated mouse thymocytes (Fig. 3.1A, upper blot lane 2). Treatment of mouse thymocytes with PMA and A23187 (P/A) resulted in very rapid phosphorylation of Ctip2 on threonine residues that reached a maximum at around 5 min. (lane 3 of upper blots shown in Fig. 3.1A).

However, this phosphorylation was transient as phospho-Ctip2 (pCtip2) was rapidly dephosphorylated, reaching a phosphorylation state that was *less* than basal phosphorylation on threonine residues by 30 min. after stimulation with P/A (Fig. 3.1A, lane 4). Total levels of Ctip2 remained constant over the same time course (lower blot of Fig. 3.1A). The phosphorylation status (phospho-Thr) of Ctip2 then returned to basal levels after 1–2 hours, and this level was stable even in the presence of continuous P/A (see Fig. 3.1A, lanes 5-7).

Dynamic regulation of Ctip2 at multiple post-translational levels in stimulated mouse thymcotytes — During the course of these studies we noticed the appearance of more slowly migrating protein species, which were recognized by our anti-Ctip2 monoclonal antibody (lanes 2, 5, and 6 of the lower blot shown in Fig. 3.1A). While present in cell extracts prepared from untreated cells (lane 2 of the lower blot in Fig. 3.1A), these low mobility species seemed to disappear immediately after P/A treatment (5 min.; lane 3), and then began to reappear approximately 60 min. after initiation of P/A treatment (Fig. 3.1A, lanes 5 of lower blot). The low mobility species were even more striking when the thymocyte lysates were prepared by directly lysing cells in sample buffer containing SDS (Fig. 3.1B, lanes 4-6), which revealed the reappearance of the lower mobility species beginning at 20 min. after initiation of P/A treatment. We also noticed a small gel mobility shift of Ctip2 present at 5 and 10 mins of P/A treatment (Fig. 3.1B, compare lane 1 with lanes 2 and 3), consistent with the transient hyper-phosphorylation of Ctip2 during the initial P/A treatment as shown in Fig. 3.1A. Continued exposure of thymocytes to P/A eventually resulted in loss of the low mobility species, as well as full-length Ctip2, and this occurred approximately 8 hours after initiation of P/A treatment

and concomitant with the appearance of presumably clipped forms of the protein (Fig. 3.1C lanes 4 and 6; note that these clipped forms did not appear appreciably in the absence of P/A treatment, lane 5).

These findings demonstrate that Ctip2 is rapidly phosphorylated after treatment of mouse thymocytes with P/A, and then quickly dephosphorylated. We observed the appearance of a labile, slowly migrating Ctip2 species that appeared following the dephosphorylation step. Prolonged treatment of thymocytes with P/A led to the degradation of Ctip2, presumably by proteolytic clipping.

Ctip2 is SUMOylated in mouse thymocytes in a P/A-dependent manner —We considered two possibilities, ubiquitylation and SUMOylation, to explain low mobility Ctip2 species that appeared after the phosphorylation-dephosporylation cycle in mouse thymocytes. This hypothesis was strengthened by an experiment demonstrating that omitting N-ethylmaleimide (NEM) and hemin chloride (HC), general inhibitors of ubiquitin and SUMO hydrolases, from our extraction buffer resulted in loss of the low mobility species of Ctip2 isolated from mouse thymocytes (Fig. 3.1D, lane 4). Low mobility species of Ctip2 were also generated by treatment of Jurkat cells, a human T cell line, with P/A in a manner that required the presence of NEM and HC (supplemental Fig. 3.S1A, lane 4). However, we subsequently ruled out ubiquitylation of Ctip2 because probing Ctip2 immunoprecipitates from P/A-treated mouse thymocytes for an ubiquitin signal, as well as the reciprocal experiment, both yielded negative results (data not shown). Thus, we concluded that the low mobility species of Ctip2 likely corresponds to a SUMOylated protein. P/A-induced SUMOylation of Ctip2 was

confirmed by probing SUMO1 immunoprecipitates from P/A-treated mouse thymocytes with the anti-Ctip2 antibody (Figs. 3.1E and 3.F), and this was confirmed by the reciprocal experiment (Fig. 3.1G). These reciprocal co-immunoprecipitations revealed that: (1) a pool of Ctip2 in mouse thymocytes was SUMOylated under basal conditions (Fig. 1F and 1G, lane 1; although these cells must be cultured in the presence of serum to maintain viability so "basal" is a relative term), and this SUMOylated pool was reduced 10 min. after treatment with P/A, presumably a time corresponding to increased phospho-Ctip2 levels, (2) the SUMOylated species of Ctip2 (SU-Ctip2) reappeared beginning at 30 min. after P/A treatment, presumably corresponding to the peak of Ctip2 *dephosphorylation*, and reached a peak at approximately 60 min. (Fig. 3.1F and 3.1G, lanes 3-6). These data unequivocally demonstrate that Ctip2 undergoes a cycle of phosphorylation-dephosphorylation-SUMOylation in mouse thymocytes in response to stimulation with P/A.

Phosphorylation and dephosphorylation and SUMOylation of Ctip2 are inhibited by kinase inhibitors — We next sought to identify the cell signaling pathway(s) that may be involved in phosphorylating Ctip2 in response to P/A stimulation. U0126 (U0) and SB202190 (SB), inhibitors of the ERK1/2 kinase MEK1 and p38, respectively, decreased P/A-induced phosphorylation of Ctip2, suggesting that the ERK/2 and p38 pathways both play a role in phosphorylating Ctip2 in response to PKC activation (Fig. 3.2A, compare lanes 1-4). Much to our surprise, U0, but not SB, also inhibited the subsequent, P/A-induced dephosphorylation of Ctip2 in mouse thymocytes (Fig. 3.2B, compare lanes 2 and 3 of upper blot). This dephosphorylation experiment (Fig. 3.2B) was performed using a P/A incubation time of 60 min. to allow us to

examination the SUMOylation of Ctip2. This study revealed that U0, but not SB, also blocked the SUMOylation of Ctip2 that was induced by P/A (compare lanes 2-4 of lower blot shown in Fig. 3.2B). Blockage of the P/A-induced Ctip2 SUMOylation by U0, but not SB was further confirmed by SUMO1 immunoblotting analysis of the immunoprecipitated Ctip2 (Fig. 3.2C).

Activated ERK1/2 is a component of the Ctip2 complex in mouse thymocytes — The rapidity with which the nuclear protein Ctip2 was phosphorylated in mouse thymocytes after stimulation with P/A (~ 5 min.) was surprising, and we hypothesized that the proximal kinase(s) may pre-exist in a complex with Ctip2. To test this hypothesis, we probed anti-Ctip2 immunoprecipitates from P/A-stimulated cells with antibodies directed against activated p38 and ERK1/2 (pERK1/2). While p38 did not appear to co-immunoprecipitate with Ctip2 (not shown), pERK1/2 was found to immunoprecipitate with Ctip2, but only after treatment with P/A (Fig. 3.2D, lane 4). There are at least two possible interpretations of this result: (**1**) unactivated ERK1/2 is a component of the pre-existing Ctip2 complex and becomes activated (phosphorylated) after P/A treatment and subsequently phosphorylates Ctip2, or (**2**) neither activated nor unactivated ERK1/2 is a component of the Ctip2 complex prior to stimulation with P/A, but activated ERK1/2 associates with the Ctip2 complex after P/A stimulation. In either case, activated ERK1/2 does appear to be a component of the Ctip2 complex in mouse thymocytes after stimulation with P/A.

Inhibition of Protein Phosphatases 1 and 2A blocks Ctip2 dephosphorylation and SUMOylation — To investigate the relationship between Ctip2 dephosphorylation and SUMOylation in mouse thymoctyes in

greater detail we utilized the potent inhibitor of class 1 and 2A phosphatases, calyculin A (CalA). CalA dramatically enhanced the basal level of pCtip2 (compare lanes 1 and 4 of the upper panel of Fig. 3.2E), and that occurring at 5 min. after P/A stimulation (compare lanes 2 and 5 of Fig. 2E, upper blot). CalA also blocked the dephosphorylation of pCtip2 that normally occurs 30-60 min after initiation of P/A treatment (compare lanes 3 and 6 of Fig. 3.2E). Strikingly, calyculin A completely blocked the SUMOylation reaction that occurs at 60 min. after stimulation with P/A (compare lanes 3 and 6 of the lower blot shown in Fig. 3.2E). These data demonstrate that: (1) Ctip2 is a dynamically regulated phosphoprotein that is dephosphorylated by class 1 and/or 2A phosphatases, as well as potentially other CalA-sensitive phosphatates, and (2) dephosphorylation of Ctip2 is required in order for the protein to be SUMOylated. In addition, the absence of a phospho signal(s) in the SUMOylated Ctip2, population suggests that the phosphorylation reaction may not occur efficiently in the context of a SUMOylated protein. Alternatively, if phosphorylation occurs in the context of SUMO-Ctip2, it must trigger rapid and extensive deSUMOylation of Ctip2, most likely mediated by a SENP family protein (Di Bacco et al., 2006).

The effect of CalA on basal phosphorylation of Ctip2 described above was particularly striking, and this suggested to us that Ctip2 was likely rapidly cycling between phosphorylated and dephosphorylated states, even in the absence of P/A stimulation. To test this hypothesis, we treated mouse thymocytes with CalA in the absence of P/A stimulation and found that CalA stabilized a substantial amount of pCtip2, which was not appreciably different from the level maximally induced by P/A treatment in the absence of CalA (compare lane 2 of the upper panels of Figs. 3.2E and 3.2F). Moreover,

prolonging CalA treatment from 10-60 min. dramatically increased levels of pCtip2 in the absence of P/A stimulation (lanes 3-5 of the upper blot shown in Fig. 3.2F). This result was essentially the mirror image of SUMO-Ctip2; CalA treatment reduced levels of SUMO-Ctip2 in a time-dependent manner (Fig. 3.2F, lower blot).

The above data suggested that phosphorylation and SUMOylation of Ctip2 may be mutually exclusive processes, but did not provide a potential mechanism for this mutual exclusivity. We hypothesized that phosphorylation of Ctip2 may inhibit its interaction with the SUMO-conjugating enzyme Ubc9, the only such enzyme in the mammalian genome. Indeed, we found that Ctip2 interacted with Ubc9, as expected (Fig. 3.2G, lane 2 of upper blot arrow), and in a manner that was inhibited by CalA (compare lanes 2 and 4 of Fig. 3.2G, upper blot). These data strongly suggest that the phosphorylated form of Ctip2 does not interact efficiently with Ubc9, which may contribute to the mutual exclusivity of Ctip2 phosphorylation and SUMOylation.

Considered all together, these findings suggest that: (1) modification of Ctip2 by PTMs is a highly dynamic series of linked events, (2) phosphorylation and SUMOylation of Ctip2 are likely mutually exclusive processes, (3) phosphorylation of Ctip2 may inhibit its SUMOylation by interfering the interaction between Ctip2 protein and Ubc9.

Identification of Ctip2 SUMOylation sites — Putative Ctip2 SUMOylation sites were identified using a search algorithm (SUMOplot™; http://www.abgent.com/tools/ SUMOplot_login), and two sites identified in this way (Fig. 3.3A) were verified by site-directed mutagenesis (Figs. 3.3B-D).

Mutation of these two lysine residues to arginine (K607R and K805R) resulted in loss of Ctip2 SUMOylation in both HEK (lane 8 of Fig. 3.3B, and lane 7 in Fig. 3.3C) and Jurkat cells (lane 8 of Fig. 3.3D). Interestingly, mutation of either of the primary SUMOylation sites alone resulted in hyper-SUMOylation of the remaining residue (compare lane 2 to lanes 4 and 6 of Figs. 3.3B and D; lane 4 to lanes 5 and 6 of Fig. 3.3C), suggesting that SUMOylation of these two primary sites may be linked.

Identification of Id2 as a Ctip2 target gene —Id2 has been identified as a Ctip2 target gene in DP cells by expression profiling analyses (Kastner et al., manuscript in preparation), and microarray data were confirmed by RT-qPCR (Fig. 3.4A). We found that Ctip2 yielded a very strong ChIP signal when using primers over the most distal of these highly conserved regions in both Jurkat cells and mouse thymocytes (Fig. 3.S1B and Fig. 3.4B). We cloned approximately 3.1 kb of the mouse Id2 promoter, which encompassed both conserved regions, and inserted it in front of a CAT reporter gene to determine if Ctip2 repressed this promoter in transient transfection experiments. Wild-type Ctip2 repressed expression of the Id2 promoter strongly, whereas a mutant of Ctip2 that does not bind DNA (not shown) was less effective (Fig. 3.4C). We identified a putative Ctip2 binding site (Avram et al., 2002) within this region, and demonstrated that Ctip2 bound directly to an annealed oligonucleotide spanning this site (Fig. 3.4D)..

In vitro differentiation of DP thymocytes by PMA and A23187 — Previous studies have found that treatment of primary thymocytes with PMA and A23187 bypasses TCR signaling and trigger downstream signaling pathways to induce T cell differentiation in vitro (Ohoka et al., 1996; Takahama

and Nakauchi, 1996). We found that P/A treatment for two days induced DP differentiation with a CD8 bias (Fig. 3.5A) , and this was consistent with RT-qPCR analyses of treated cells, which demonstrated P/A-dependent induction of Runx3 (Fig. 5B), a marker of $CD8^+$ cells (Woolf et al., 2003), In contrast, expression of *Zbtb7b* (also known a*s cKrox* o*r ThPok*), a marker of CD4+ SP differentiation (Dave et al., 1998), was slightly reduced by P/A treatment (Fig. 3.5B).

Induction of Id2 in stimulated T cells — Previous studies found that Id2 is essential for NK cell development (Yokota et al., 1999), but its role in DP → SP differentiation remains unknown. Analyses of Id2 expression revealed a 4-fold induction of Id2 mRNA levels in thymocytes treated with P/A (Fig. 3.5D), which was reversed by pretreating thymocytes with the ERK1/2 inhibitor U0126, but slightly enhanced by SB pretreatment (Fig. 3.5E). These results demonstrate that Id2 is induced during T cell differentiation in vitro in a MAPK-dependent manner.

Regulation of Id2 expression and Ctip2 PTM in stimulated Jurkat cells—Similar to mouse thymocytes, we found that Id2 mRNA was also induced in Jurkat cells stimulated with PMA and A23187 or PMA alone (Fig. 3.S1B). We also observed similar post-translational modification pattern of Ctip2 in Jurkat cells to that in primary mouse thymcoytes. In stimulated Jurkat cells, CTIP2 was transiently phosphorylated followed by profound dephosphorylation, SUMOylation, and proteolytic clipping eventually (data not shown). Interestingly, the time course of Ctip2 PTM appears to be slightly delayed in Jurkat cells compared to that in mouse thymocytes: the initial CTIP2 phosphorylation (data not shown) lasts up to 30 mins after stimulation (in

contrast to10 mins in thymocytes), and SUMOylation (Fig. 3.S1D) started to reappear at 60 mins (in contrast to 30mins in thymocytes) and peaked at 2h (in contrast to 1h in thymocytes). Similar to SUMOylation, induction of Id2 expression also peaked at 2h (Fig. 3.S1C). These result suggests that the dynamic post-translational modification of Ctip2 and Id2 transcription induction upon activation of T cell signaling is not limited to immature DP T cells that are immobile, but also applies to mature SP Jurkat T cells that are peripheral and proliferative.

Stimulation of mouse thymocytes with P/A alters interaction of Ctip2 and MTA2 with the Id2 promoter — Treatment of mouse thymocytes with P/A resulted in an induction of Id2 mRNA levels, which reached a maximum of ~ 4.5-fold at 60 min. after initiation of the treatment protocol (Fig. 3.6A). We noted that the time-course of P/A-induced Id2 expression also corresponded to that of P/A-induced SUMOylation of Ctip2 (Fig. 3.1F). This led us to hypothesize that the mechanism of the induction of Id2 mRNA levels by P/A treatment may involve SUMOylation and subsequent inactivation of the transcription repressive activity of Ctip2. Based on the data presented above, the Id2 promoter seemed like a useful model system to fuse our two areas of study: PTM of and regulation of gene expression by Ctip2, toward the goal of understanding the influence of cell signalling pathways on Ctip2-mediated transcriptional regulation in mouse thymocytes.

Originally, we hypothesized that loss of Ctip2 repressive activity on Id2 promoter might be due to an inability of SUMOylated Ctip2 to bind to the Id2 promoter, i.e., perhaps SUMOylation of Ctip2 inhibits its interaction with the Id2 promoter, resulting Ctip2 "falling off" the promoter and subsequent

de-repression of Id2 expression. We tested this hypothesis by conducting a kinetic ChIP assay on the Id2 promoter after treatment of thymocytes with P/A (Fig. 3.6C). We designed series of ChIP primers, covering regions including the highly conserved region at ~-3Kb, the transcriptional start site (TSS) and a region in the 3' UTR as control (see Fig. 3.6B). We found that Ctip2 interacted with both the highly conserved -3 kb region of the Id2 promoter and the TSS, but not 3' UTR region prior to treatment with P/A (Fig. 3.6C). P/A treatment actually resulted in *stimulation* of the Ctip2 ChIP signal at both the -3kb site and the TSS, both of which reached a maximum of approximately 2- and 5-fold, respectively, at 60 min. (Fig. 3.6C), corresponding to the time of maximal SUMOylation of Ctip2 (Fig. 1F). MTA2, a component of the NuRD complex with which Ctip2 interacts (Topark-Ngarm et al., 2006) displayed ChIP behaviour on the Id2 promoter that was qualitatively similar to that of Ctip2 (Fig. 3.6C). The effect of P/A treatment on the interaction of both Ctip2 and MTA2 with the human Id2 promoter in Jurkat cells was qualitatively similar to our observations in primary mouse thymocytes (see Fig. 3.S1E-F). P/A treatment did not have an effect on the interaction of Ctip2 with the Id2 3' UTR (Fig. 3.6C). Thus, we observed no diminution of Ctip2 binding to the Id2 promoter at the time of maximal Ctip2 SUMOylation, suggesting that SUMOylation does not negatively impact the ability of Ctip2 to interact with the Id2 promoter. To the contrary, it would seem that SUMOylation may increase the interaction of Ctip2 with the Id2 promoter, and may play an active role in the transcriptional induction of the Id2 promoter.

p300 is recruited to Id2 promoter in stimulated mouse thymocytes — The hypothesis that Ctip2 may be actively involved in the induction of the Id2 promoter gained additional credence with our finding that Ctip2 and

SUMOylated Ctip2 co-immunoprecipitated with the transcriptional coactivator protein p300 in a manner that was stimulated by treatment of mouse thymocytes with P/A (Fig. 3.7A; the converse was also true, see Fig. 3.7B). P/A treatment did not influence the interaction of Ctip2 with the NuRD component proteins MTA2 and HDAC1 (see bottom panels of Fig. 3.7A). Moreover, the histone acetyltransferase p300 was recruited to both the - 3 kb region and the TSS of the Id2 promoter by P/A treatment in a manner that mirrored that of Ctip2 and MTA2 (Fig. 3.7C), and similar results were obtained in Jurkat cells (Fig. 3.S1G). Robust ChIP signals were also observed for acetylated histones H3 and H4 at the TSS, consistent with the recruitment of a histone acetyltransferase and the activated state of this promoter (Fig. 3.6D and E, respectively). ReChIP assays revealed that p300 and Ctip2 co-occupied the -3 kb and TSS regions of the Id2 promoter, with the reChIP signal on the latter being stimulated approximately 60-fold after P/A treatment (Fig. 3.7C). These findings indicate that the phosphorylation → dephosphorylation → SUMOylation cycle may serve as a molecular switch that converts Ctip2 from a transcriptional repressor to an activator of the Id2 promoter in mouse thymocytes,

SUMO-Ctip2 associates with Ctip2 in cells — We created a SUMO1-Ctip2 fusion protein (see Fig. 3.8A) in order to test the hypothesis that SUMOylated Ctip2 may function as a transcriptional activator of the Id2 promoter. First, we tested the expression of the Su-Ctip2 fusion protein compared to wild-type Ctip2. Surprisingly, SUMOylated Ctip2 was barely detectable in transfected HEK cells using an anti-Ctip2 antibody (Fig. 3.8C, left blot). This lack of detection was not due to the quality of the SUMO-Ctip2 construct, because it was expressed as efficiently as Flag-Ctip2 using an in

vitro transcription/translation system (Fig. 3.8B), suggesting SUMO-Ctip2 may be preferentially degraded in HEK cells. We then determined if SUMO-Ctip2 repressed the Id2 promoter despite its extensive degradation. As expected, Flag-Ctip2 strongly repressed the Id2 promoter in a dose-dependent manner (Fig. 3.8D). However, SUMO-Ctip2 did not show any repression activity on the Id2 promoter, but rather exhibited a small but significant transcription activation activity on the Id2 promoter in a dose-dependent manner (Fig. 3.8D). This finding is consistent with the hypothesis that non-SUMOylated Ctip2 represses the Id2 promoter, whereas SUMOlated Ctip2 activates this promoter.

Next, we investigated the mechanism of SUMO-dependent degradation of Ctip2 in HEK cells. We first determined if treatment with MG132 (proteasome inhibitor) or zVAD (caspase inhibitor) treatment might block degradation of SUMO-Ctip2. We found that MG132, but not zVAD, dramatically stabilized cellular levels of SUMO-Ctip2 (Fig. 3.8E, lanes 3-4). Treatment with MG132 appeared to stablize a very high molecular weight ladder of SUMO-Ctip2 species, which resembled the characteristic ladder of a poly-ubiquitinated protein, suggesting that SUMO-Ctip2 may be modified by ubiquitination prior to proteasome-dependent degradation.

Co-expression of Flag-Ctip2 also blocked degradation of the SUMO-Ctip2 fusion protein (Fig. 3.8F). However, in this case the SUMO-Ctip2 fusion protein migrated with a substantially lower apparent mass than the MG132-stabilized protein (compare lanes 3-4 of the top blot of Fig. 3.8F with lanes 3-4 of Fig. 3.8E). Flag-Ctip2 appeared to block degradation of the SUMO-Ctip2 fusion protein in a concentration-dependent manner, and was optimal when the ratio of Flag-Ctip2:SUMO-Ctip2 was 1:1 (Fig. 3.8F),

suggesting non-SUMOylated Ctip2 may dimerize with SUMO-Ctip2 to maintain the stability of the latter. Indeed, we found that non-SUMOylated, Flag-Ctip2 co-immunoprecipitated with SUMO-Ctip2 in transfected cells (Fig. 3.8F, bottom blot), demonstrating association of the two proteins within the same complex in HEK cells. Furthermore, addition of SUMO-Ctip2 impaired the transcription repression activity mediated by non-SUMOylated Ctip2 (Fig. 3.8G).

DISCUSSION

To study how CTIP2 is regulated by T cell signaling pathways during differentiation of DP cells, we have adapted an ex vivo system in which a primary thymocyte culture was treated with phorbol ester (PMA) and calcium ionophore (A23187), the combination of which triggers signaling cascades necessary similar to those of positive selection, but bypassing the necessity of a TCR signal (Ohoka et al., 1996; Takahama and Nakauchi, 1996). We found that during the initial period of T cell stimulation by P/A, Ctip2 was dynamically regulated by phosphorylation and SUMOylation by T cell signaling pathways. These PTMs were linked to changes of the transcription regulatory activity of Ctip2 on the promoter of a newly identified endogenous Ctip2 target gene, Id2.

Ctip2 was rapidly phosphorylated during the initial 5-10 min. of stimulation, and by 30 min. Ctip2 was profoundly dephosphorylated. Subsequent studies using specific MAPK inhibitors revealed that the initial Ctip2 phosphorylation was dependent on both ERK1/2 and p38MAPK activity, whereas the following dephosphorylation was completely blocked by inhibition of ERK1/2. There are two possible explanations: First, phosphorylation by ERK1/2, but not p38, may lead to Ctip2 conformational change, which favors the recruitment of

phosphatase that dephosphorylates Ctip2. Alternatively, ERK1/2, but not p38, may phosphorylate and activate a phosphatase(s), which then dephosphorylates Ctip2.

Besides phosphorylation, we also observed a series of changes of Ctip2 SUMOylation in T cells stimulated with P/A. And interestingly, just opposite to phosphorylation kinetics, Ctip2 basal SUMOylation disappeared during the initial 5-10 min. of stimulation, reappeared at 30 mins, and reached a peak at 60 min. after initiation of of P/A treatment. Similar to Ctip2 dephosphorylation, SUMOylation of Ctip2 at 60 min. of P/A stimulation was also blocked upon ERK inhibition, suggesting that Ctip2 dephosphorylaton and SUMOylation may be linked. This hypothesis was also consistent with our finding that blocking Ctip2 dephosphorylation by calyculin A also completely inhibited SUMOylation. Overall, we interpret these finding as follows: (1). P/A stimulation results in the activation of one or more isoforms of PKC in mouse thymocytes, which then activate p38 and ERK1/2 signaling pathways, both of which contribute to the induced phosphorylation state of Ctip2. During this period, Ctip2 was also transiently deSUMOylated, suggesting that phosphorylation and SUMOylation may be mutually exclusive, and (2) within 30 min. of P/A stimulation, the Ctip2 became dephosphorylated, and this was necessary for subsequent SUMOylation, the latter of which became maximal by 60 min. after stimulation with P/A.

Our results strongly suggest that phosphorylation is one of the regulatory mechanisms that control the SUMOylation status of Ctip2. Regulation of protein SUMOylation was often found to occur at the level of the target itself, and involved other post-translational modifications, including phosphorylation.

For example, phosphorylation can enhance SUMOylation of proteins, such as HSF1 and MEF2 (Hietakangas et al., 2006). Both of these proteins are SUMOylated through the phosphorylation-dependent SUMOylated motif (PDSM), and it is also suggested that the addition of negatively charged phosphate group can enhance substrate-E2 ligase (Ubc9) interaction (Hietakangas et al., 2006). On the other hand, similar to Ctip2, phosphorylation can also negatively regulate SUMOylation, as has been reported for Elk1, p53, c-Fos and c-Jun (Bossis et al., 2005; Lin et al., 2004; Muller et al., 2000; Yang et al., 2003). In these cases, the underling mechanisms still remain largely elusive, but it is suggested that masking of the SUMO-acceptor sites and relocalization of the target are two of the possible mechanisms. For example, p53 SUMOylation is severely impaired upon its phosphorylation at the Ser-20 residue, and it is suggested that phosphorylation may reduce the affinity of the SUMOylation E2 ligase Ubc9 to p53 (Lin et al., 2004). In our study, we found that Ctip2 also interacted with Ubc9, and this interaction was lost when the cells were treated with calyculinA, which blocked dephosphorylation and induced hyper-phosphorylation of Ctip2. These data strongly suggest that the phosphorylated form of Ctip2 does not interact efficiently with Ubc9, which may contribute to the mutual exclusivity of Ctip2 phosphorylation and SUMOylation.

SUMOylation can be controlled through the action of protein kinase cascades in response to extracellular signals, which are often transduced into intracellular responses through the MAPK pathways. The ERK, p38 and JNK pathways are the three major MAP kinase cascades found in mammalian cells (Raman et al., 2007). The first evidence linking MAPK pathways with SUMOylation was demonstrated by the finding that activation of ERK-pathways caused loss of SUMOylation of the transcription factor Elk-1 (Yang et al., 2003).

SUMOylation is required for Elk-1 repression activity by recruiting HDAC2 to the target promoter (Yang and Sharrocks, 2004). And upon ERK activation, Elk-1 is phosphorylated, which resulted in loss of SUMOylation and HDAC2 association, switching Elk-1 from a transcription repressor to a transcription activator (Yang and Sharrocks, 2004). A growing number of links are now being made between protein SUMOylation and the MAPK pathways as has been reported for AIB1, ACTR, SRC-3 and p/CIP. For example, AIB1 is phosphorylated by ERK pathway, and this phosphorylation is inversely correlates with AIB1 SUMOylation (Wu et al., 2006). In our present study, we have shown that ERK1/2 pathway is also essential for dynamic interplay between Ctip2 phosphorylation and SUMOylation. Inhibition of ERK activation results in loss of Ctip2 phosphorylation as well as its subsequent dephosphorylation and SUMOylation. Furthermore, activated ERK1/2 is also found in the same protein complex with Ctip2. These findings indicate that ERK may phosphorylated Ctip2 directly and also suggest the existence of an ERK-dependent phosphatase being recruited and dephosphorylating Ctip2, which ultimately leads to Ctip2 SUMOylation.

Previous study by Iwata's group (Ohoka et al., 1996) showed that a proper combination of PMA and calcium ionophore induced differentiation of isolated DP thymocytes, and most of the cells committed into CD4 lineage, including CD4 SP and CD4+CD8^{low} T cells. However, in our study, after 2 days of culturing in the presence of PMA and A23187, most of the DP primary mouse thymocytes differentiate to CD8 single positive cells. This is consistent with our observation that Runx3 (master regulator of the CD8 SP cell differentiation (Woolf et al., 2003)) but not cKrox (master regulator of the CD4 SP cell differentiation (Dave et al., 1998)) is significantly induced upon P/A stimulation.

Id2 (inhibitor of DNA-binding 2) belongs to a member of the helix–loop–helix family of transcription factors. The basic-Helix-Loop-Helix (bHLH) transcription factors control a wide variety of developmental pathways in both invertebrate and vertebrate organisms (Murre, 2005). Recent results have shown a subset of bHLH proteins, the 'E proteins' including E12, E47, E2-2 and HEB, are particularly important for proper lymphocyte development. E protein levels are tightly controlled during thymocyte development (Murre, 2005). E47 expression is activated at the point at which thymocytes begin to commit towards the T cell lineage. E47 levels remain high and do not drop until the double positive stage, and are further downregulated upon transition to single positive stage during positive selection (Murre, 2005). The Id proteins contain only the HLH protein-protein interaction domain, but lack a basic region for DNA binding. Therefore, Id proteins can dimerize with other HLH proteins, and inhibit the function of the bHLH proteins in a dominant-negative manner by abrogating their ability to bind DNA (Benezra et al., 1990). In vertebrates, there are four members of the Id gene family: Id1- Id4 (Massari and Murre, 2000). Of the four Id members, Id2 and Id3 are the main participants that act during lymphoid development to control the activity of E proteins. Proper maturation of both CD4 and CD8 SP cells in thymocytes are impaired in Id3 null mice (Rivera et al., 2000). Additionally, ectopic expression of Id3 in a DP cell line promotes in vitro differentiation (Heemskerk et al., 1997).

Id2 is of great interest for us, as overexpression of Id2 in thymus results in a phenotype somewhat similar to that of Ctip2$^{-/-}$ mice (arrest in $\alpha\beta$ T cell development) (Morrow et al., 1999). Id2 interacts with and inhibits the DNA binding activity of the E2A proteins, E12 and E47 (Benezra et al., 1990; Sun et al., 1991; Voronova and Lee, 1994), proteins that have been demonstrated to

play crucial roles in T cell development (Bain et al., 2001; Bain et al., 1997; Bain and Murre, 1998; Bain et al., 1999; Engel et al., 2001). In addition, both ectopic overexpression of Id2 in T cells (Morrow et al., 1999) and E2A knockout (Bain et al., 1997) results in aggressive T cell lymphomas, which is also of interest for us as Ctip2 has been proposed to function as a tumor suppressor gene (Bezrookove et al., 2004; MacLeod et al., 2004; MacLeod et al., 2003; Nagel et al., 2003; Okazuka et al., 2005; Przybylski et al., 2005; Su et al., 2004; Wakabayashi et al., 2003a) and implicated in childhood T-ALL in humans (Przybylski et al., 2005; Su et al., 2006). Moreover, transgenic mice that overexpress Id2 have significant expansion of CD4-CD8+TCR- thymocyte stage, leading to a stage-specific developmental block early in thymopoiesis. However, the role of Id2 in thymocyte positive selection still remains elusive. For the first time, we found that Id2 expression is upregulated in single positive T cells compared to that in double positive T cells. Furthermore, Id2 expression is rapidly induced in P/A-stimulated thymocytes, which eventually differentiate into mostly CD8 single positive T cells. All these findings indicate a role of Id2 during thymocyte positive selection.

SUMOylation of a protein target can alter its localization, activity and stability (Geiss-Friedlander and Melchior, 2007). Numerous studies suggest that the underlying principle of SUMOylation is the alteration of intra- or intermolecular interactions of the modified substrate. Downstream consequences are mediated, at least in part, by recruiting effectors containing SUMO-interaction motif (SIM) (Perry et al., 2008). We found in our study that the time-course with which Ctip2 is SUMOylated after stimulation with P/A corresponds to the induction of the Id2 promoter by P/A, and the localization of Ctip2, MTA2, p300 and acetylated histones H3 and H4 at the Id2 transcriptional

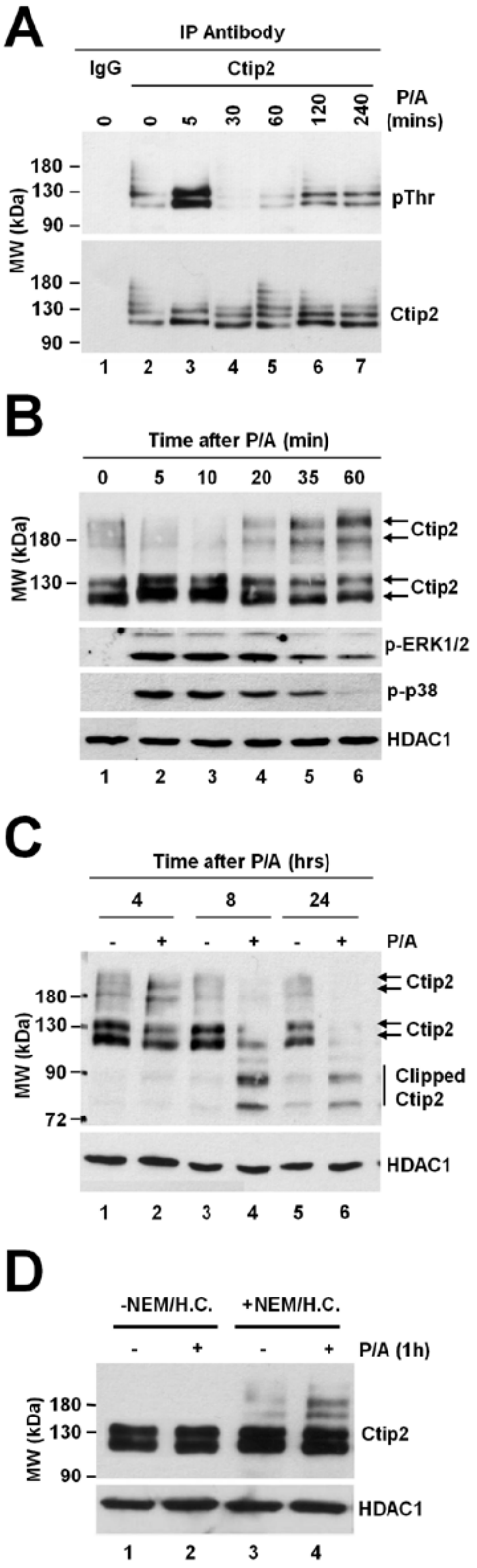
start-site. These results suggest that SUMOylation of Ctip2 may play a role in converting Ctip2 from a transcription repressor to a transcription activator. Further study of Ctip2 SUMO-fusion protein revealed that SUMO-Ctip2 has transcription activation activity even though most of the protein appears to be targeted for degradation through the Ub-proteasome pathway. Moreover, wild-type Ctip2 interacts with SU-Ctip2 and prevents SUMO-Ctip2 from being degraded. Recent studies have identified the SUMO-Targeted Ubiquitin Ligase (STUbL) family of proteins which selectively ubiquitnate SUMOylated proteins (Prudden et al., 2007). STUbLs use SIM domains to target their SUMOylated targets (Perry et al., 2008). Interestingly, through sequence homology search, we also identified a potential SIM domain located within the Ctip2 C-terminal acidic domain (*Fig S3B*). It is likely that SU-Ctip2 interacts directly with the SIM domain within another copy of non-SUMOylated Ctip2, in the absence of which STUbL will in turn be recruited to target SU-Ctip2 to the Ub-proteasome mediated degradation pathway.

We observed that after treating thymocytes with P/A for 60 min., Ctip2 was modified from mostly non-SUMOylated and phosphorylated to about half SUMOylated and mostly dephosphorylated. Based on our studies, the interaction between SUMO-Ctip2 and Ctip2 is necessary to prevent SUMO-Ctip2 from Ub-proteasome dependent degradation in order to maintain its protein stability. SUMO-Ctip2 appeared to be stable at least during the initial period of T cell stimulation. Therefore, it is likely that SUMOylation enable the formation of the Ctip2 : SUMO-Ctip2 complex, the conformation of which favors the recruitment of coactivator p300 to Id2 promoter, particularly to the TSS region. SUMOylation of Ctip2 may be the mechanistic switch to convert Ctip2 from a transcription repressor under basal condition to a transcription activator

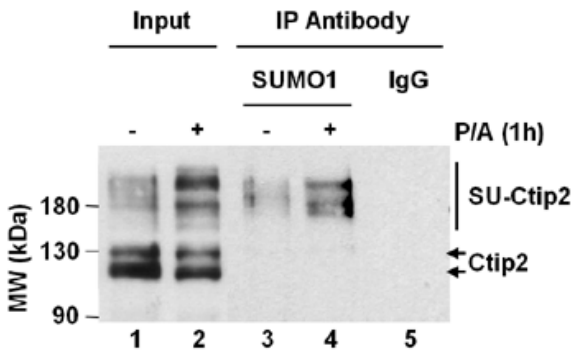
upon activation of T cell signaling pathway.

We have incorporated these data into our working model for regulation of Ctip2 by PTMs during T cell positive selection (Fig. 3.9). This model posits that (Fig. 3.9A): (1) Ctip2 exists as a basally phosphorylated protein, and functionally associates with the NuRD complex to repress transcription. (2) Ctip2 is inducibly phosphorylated in mouse thymocytes by ERK1/2 and p38, (3) the inducibly phosphorylated form of Ctip2 is a substrate for calyculin A-sensitive, class 1 and/or 2A phosphatases, which dephosphorylate phospho-Ctip2. (4) the dephosphorylated protein is a substrate for SUMO E2 ligase Ubc9, which SUMOylates Ctip2 on K^{607} and K^{805} residues. (3) SUMOylated Ctip2 associates with non-SUMOylated Ctip2 as a protein complex, which then recruits coactivator p300 to the promoter of Ctip2 target gene, leading to gene transcription activation. (5) prolonged P/A treatment eventually leads to Ctip2 degradation, possibly through targeting Ctip2 to the Ub-proteasome degradation pathway by a STUbL protein that interacts with the SUMO moiety on Ctip2. Overall, we propose that (Fig. 3.9B) during T cell differentiation, αβTCR initiates a cascade of signal pathways that activate downstream kinases. Two MAP kinases, ERK1/2 and p38 are involved in switching Ctip2 from the basally phosphorylated form to a inducibly phosphorylated form, then to a dephosphorylated, and ultimately to a SUMOylated form. We suggest that this sequence of events converts Ctip2 from a transcriptional repressor into a transcriptional activator of the Id2 promoter. The induced Id2 protein inhibits E2A activity by disrupting the heterodimer between E2A and HEB, leading to derepression of E2A-repressive targets, which promotes positive selection of DP cells to SP cells.

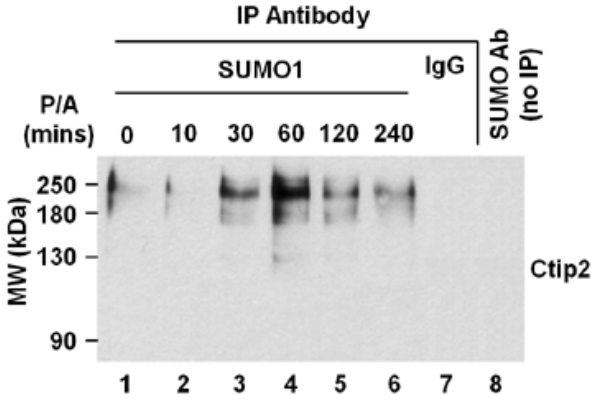
In summary, our findings in this study are the first demonstration that the transcriptional regulatory activity of Ctip2 may be influenced by post-translational modifications, including phosphorylation and SUMOylation, in the context of cell signalling pathways. These data contribute to a better understanding of the molecular mechanisms by which the transcriptional regulatory activity of Ctip2 is controlled.


A
IP Antibody
IgG
Ctip2
0
0
5
30
60
120
240
P/A (mins)
180
130
90
MW (kDa)
pThr
180
130
90
Ctip2
1 2 3 4 5 6 7
B
Time after P/A (min)
0 5 10 20 35 60
MW (kDa)
180
130
Ctip2
Ctip2
p-ERK1/2
p-p38
HDAC1
1 2 3 4 5 6
C
Time after P/A (hrs)
4 8 24
- + - + - +
P/A
180
130
90
72
MW (kDa)
Ctip2
Ctip2
Clipped Ctip2
HDAC1
1 2 3 4 5 6
D
-NEM/H.C.
+NEM/H.C.
- + - +
P/A (1h)
180
130
90
MW (kDa)
Ctip2
HDAC1
1 2 3 4

E


Input
IP Antibody
SUMO1
IgG
-
+
-
+
P/A (1h)
SU-Ctip2
Ctip2
MW (kDa)
180
130
90
1
2
3
4
5

F


IP Antibody
SUMO1
IgG
SUMO Ab (no IP)
P/A (mins)
0
10
30
60
120
240
MW (kDa)
250
180
130
90
Ctip2
1
2
3
4
5
6
7
8

G

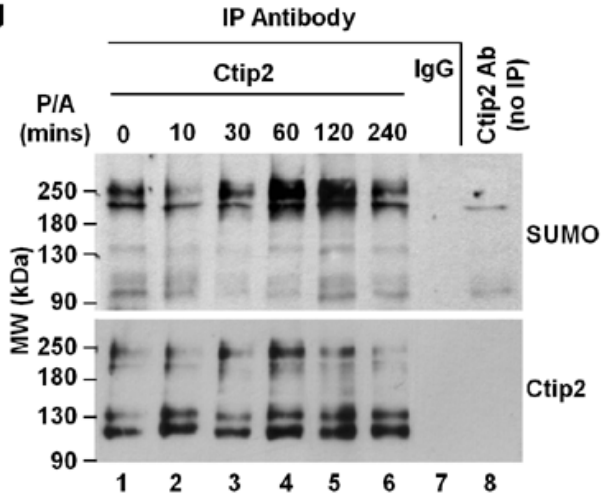
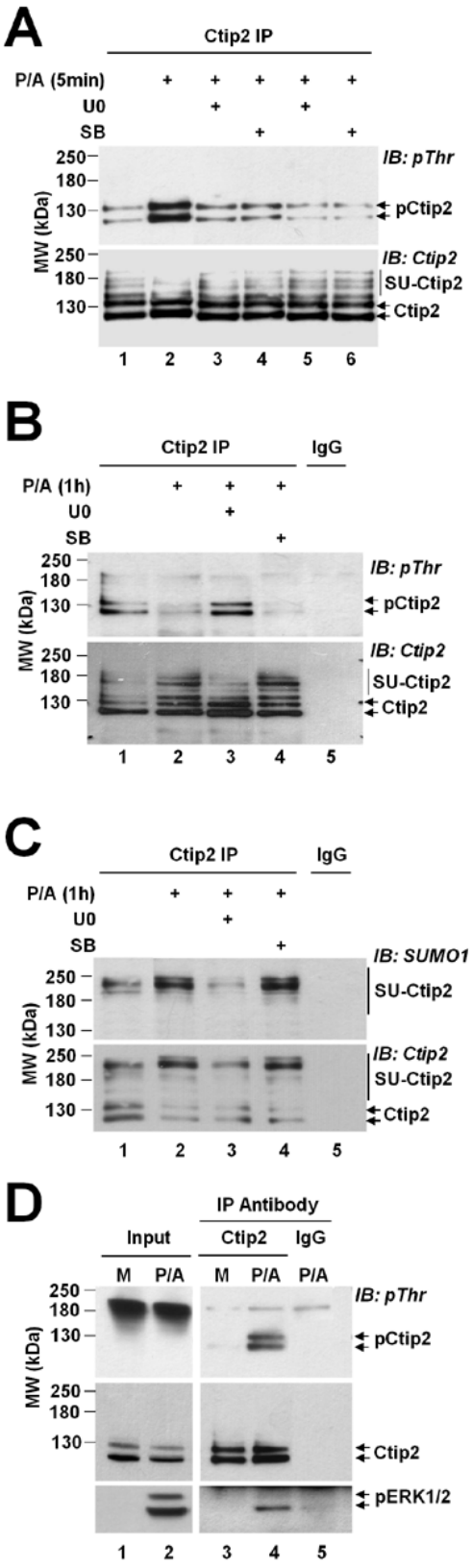
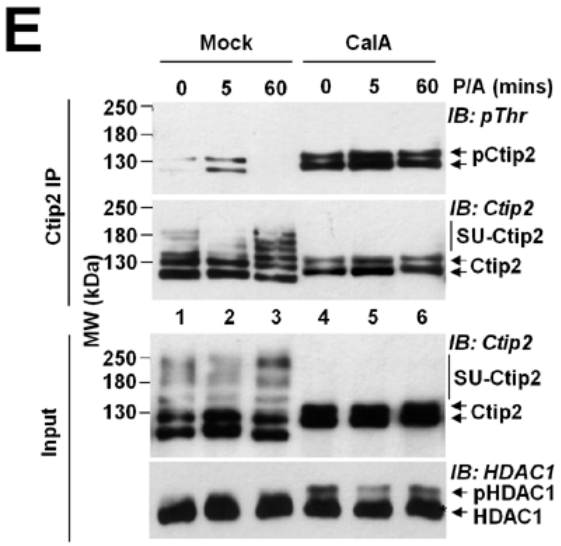
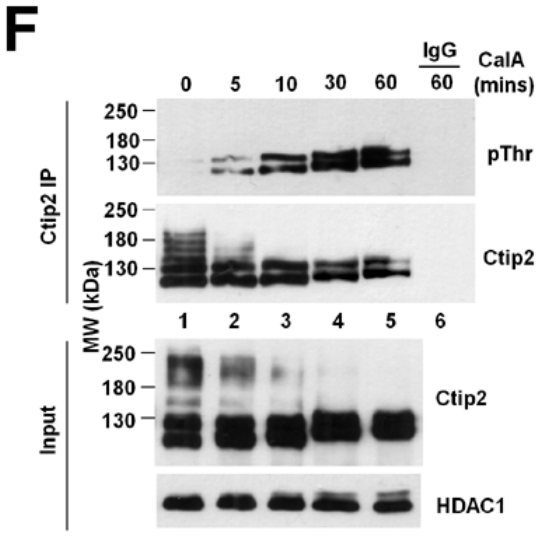

IP Antibody
Ctip2
IgG
Ctip2 Ab (no IP)
P/A (mins)
0
10
30
60
120
240
MW (kDa)
250
180
130
90
SUMO
Ctip2
1
2
3
4
5
6
7
8

Figure 3.1. Dynamic regulation of CTIP2 by phosphorylation and SUMOylation in stimulated T cells. (*A*), Time course of threonine phosphorylation of CTIP2. Immunoprecipitation (IP) experiments using CTIP2 antibody were carried out using whole cell extract (WCE) prepared from primary mouse thymocytes treated with PMA+A23187 (P/A) for indicated times. The upper blot was probed with anti-phospho-threonine antibody, and then the blot was stripped and probed with anti-CTIP2 antibody to generate the lower blot. The IP control (IgG) is shown in lane1. (***B) and (C),*** time course of the appearance of slowly migrating CTIP2 species in stimulated T cells. Primary mouse thymocytes were treated with P/A in vitro for indicated minutes, and then lysed directly in SDS-containing sample buffer prior to immunoblot analyses using indicated antibodies (the upper blot). HDAC1 blot was shown as loading control. **(*D*),** NEM and hemin chloride preserve low mobility CTIP2 species in mouse thymocytes. Mouse thymocytes were treated with P/A for 1 hour prior to extraction preparation in NEM (5mM) and hemin chloride (HC; 0.1mM). Extracts were subjected to immunoblot analysis using the anti-CTIP2 antibody, and the HDAC1 antibody as loading control. M, mock (vehicle) treatment. **(*E*),** CTIP2 is a SUMO protein and its SUMOylation is enhanced in thymocytes stimulated with P/A. Primary mouse thymocytes were treated without or with P/A for 1h, followed by lysate extraction, and immunoprecipitation using SUMO1 antibody. And the immunoprecipitates were analyzed with anti-CTIP2 antibody. The IP control (IgG) is shown in lane5. ***(F) and (G),*** Time course of CTIP2 SUMOylation. Whole cell extract (WCE) from thymocytes treated with P/A for times indicated were subjected to IP using SUMO-1 antibody, followed by CTIP2 IB analysis (as shown in *F*) or reciprocal IP using CTIP2 antibody followed by SUMO-1 IB analysis (as shown in *G*). IgG controls were shown in lane7 and antibody alone controls were shown in lane8.


A
Ctip2 IP
P/A (5min)
U0
SB
MW (kDa)
250
180
130
IB: pThr
pCtip2
IB: Ctip2
SU-Ctip2
Ctip2
1 2 3 4 5 6
B
Ctip2 IP
IgG
P/A (1h)
U0
SB
IB: pThr
pCtip2
IB: Ctip2
SU-Ctip2
Ctip2
1 2 3 4 5
C
Ctip2 IP
IgG
P/A (1h)
U0
SB
IB: SUMO1
SU-Ctip2
IB: Ctip2
SU-Ctip2
Ctip2
1 2 3 4 5
D
IP Antibody
Input
Ctip2
IgG
M P/A M P/A P/A
IB: pThr
pCtip2
Ctip2
pERK1/2
1 2 3 4 5


E
Mock
CalA
0 5 60 0 5 60 P/A (mins)
Ctip2 IP
250
180
130
IB: pThr
pCtip2
IB: Ctip2
SU-Ctip2
Ctip2
MW (kDa)
1 2 3 4 5 6
Input
IB: Ctip2
SU-Ctip2
Ctip2
IB: HDAC1
pHDAC1
HDAC1


F
IgG
CalA
0 5 10 30 60 60 (mins)
Ctip2 IP
250
180
130
pThr
Ctip2
MW (kDa)
1 2 3 4 5 6
Input
Ctip2
HDAC1

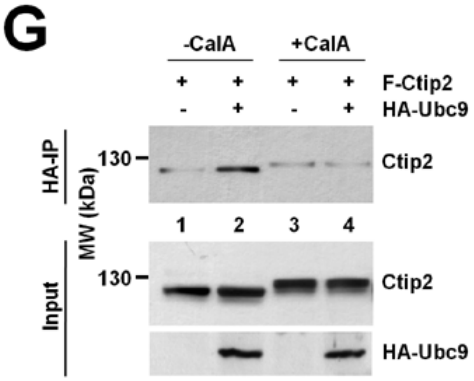

G
-CalA
+CalA
+ + + + F-Ctip2
- + - + HA-Ubc9
HA-IP
130
Ctip2
MW (kDa)
1 2 3 4
Input
Ctip2
HA-Ubc9

Figure 3.2. Effects of MAPK kinases inhibitors on P/A-induced CTIP2 phosphorylation, dephosphorylation and SUMOylation in mouse thymocytes. ***(A),*** Both ERK and p38 play roles in the initial CTIP2 phosphorylation. Five-minute treatment of mouse thymocytes with P/A in the absence or presence of U0 and SB as indicated. All samples were IP'd with anti-CTIP2 and the immunoprecipitates were subjected to immunoblotting using the indicated antibodies. **(*B*),** Inhibition of ERK but not p38 activation blocked P/A-induced CTIP2 dephosphorylation. Sixty-minute treatment with P/A and without and with inhibitors as indicated. Samples were processed as in *B*. M, mock (vehicle) treatment. **(*C*),** Effect of MAPK inhibitors on P/A-induced CTIP2 SUMOylation. Thymocytes were pretreated with MAPK inhibitors (U0 in lane3; SB in lane4) for 1 hour before P/A treatment. WCE prepared from treated thymocytes were IP's with anti-CTIP2 or non-specific IgG (lane5), followed by immunoblot analyses using SUMO and CTIP2 antibodies as indicated. **(*D*),** Activated ERK1/2 interacted with CTIP2 complex. Mouse thymocytes were treated with P/A for 5 min. and then subject to a whole cell extract procedure. The resulting lysate was then immunoprecipitated with either anti-CTIP2 Ab or non-specific IgG, and the immunoprecipitates were analyzed with anti-phospho-Thr, -Ctip2 or –pErk1/2 antibodies as indicated. **(*E*)** and ***(F),*** Effect of CalA on Ctip2 phosphorylation and SUMOylation. Mouse thymocytes were treated with 50nM CalA alone for the times indicated (*E*) or P/A for the times indicated in the absence or presence of 50nM CalA (present for 60 min. in lanes 4-6 of *F*). All samples were IP'd with the anti-CTIP2 Ab or IgG, and immunoprecipitates were subjected to immunoblotting analyses using the anti-pThr antibody. Blots were then stripped and probed for Ctip2 antibody. The lower blots indicate input CTIP2 signal, and HDAC1 blot was shown as loading control. **(*G*),** CalyculinA treatment abolished the Ctip2-Ubc9 interaction. HEK293T cells were cotransfected with Ctip2 without or with HA-Ubc9 and treated in the absence or presence of calyculinA. All samples were IP'd with the anti-HA antibody, and immunoprecipitates were subjected to immunoblotting analyses using the anti-Ctip2 antibody (the upper blot). Input signal is shown in the lower blot.

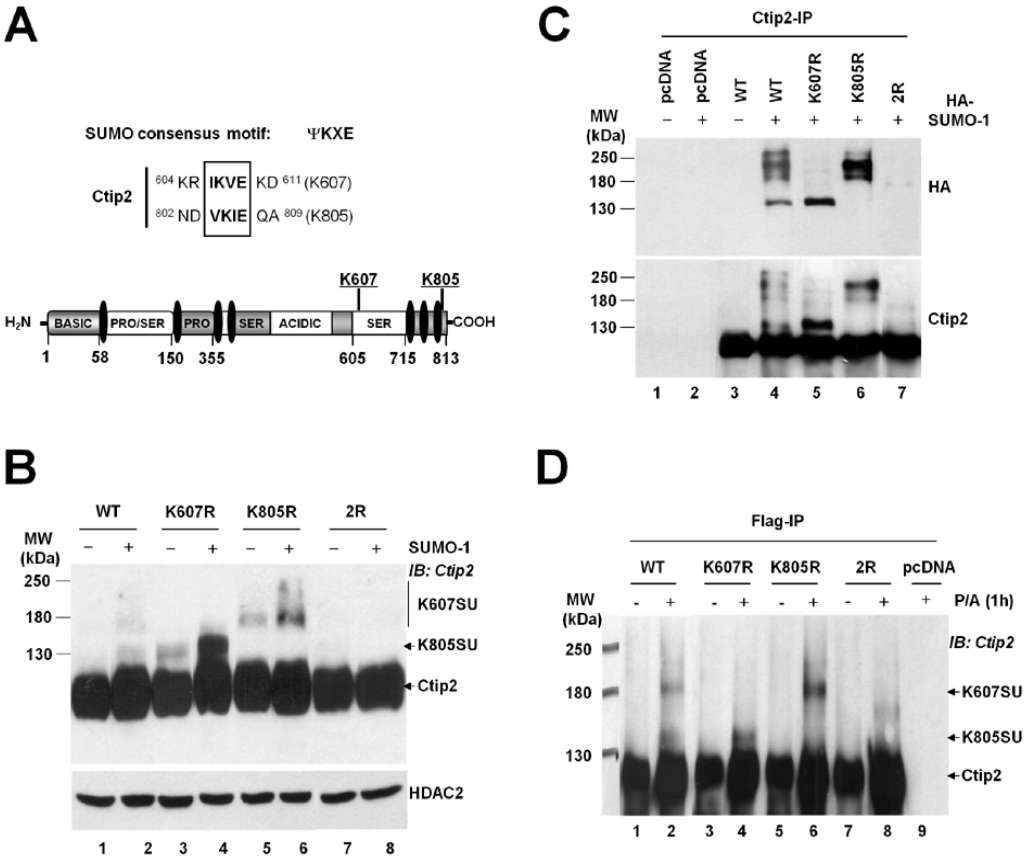

A
SUMO consensus motif: ΨKXE
Ctip2
604 KR IKVE KD 611 (K607)
802 ND VKIE QA 809 (K805)
K607
K805
H2N
BASIC
PRO/SER
PRO
SER
ACIDIC
SER
COOH
1
58
150
355
605
715
813
B
WT
K607R
K805R
2R
MW (kDa)
SUMO-1
IB: Ctip2
250
180
130
K607SU
K805SU
Ctip2
HDAC2
1 2 3 4 5 6 7 8
C
Ctip2-IP
pcDNA
pcDNA
WT
WT
K607R
K805R
2R
HA-SUMO-1
MW (kDa)
250
180
130
HA
Ctip2
1 2 3 4 5 6 7
D
Flag-IP
WT
K607R
K805R
2R
pcDNA
P/A (1h)
MW (kDa)
250
180
130
IB: Ctip2
K607SU
K805SU
Ctip2
1 2 3 4 5 6 7 8 9

Figure 3.3. Identification of CTIP2 SUMOylation sites. (*A*), Diagram of the two predicted CTIP2 SUMOylation sites. The SUMO consensus sequence is shown at the top. "ψ" refers to any large hydrophobic amino acid, and "X" can be any amino acid. **(*B*), (*C*) and (*D*),** Mapping two SUMOylation sites in Ctip2. HEK (*B* and *C*) and Jurkat (*D*) cells were transfected with expression vectors encoding Ctip2, two point mutants, and the double mutant as indicated. All constructs were epitope-tagged with the Flag peptide. Note that because wild type CTIP2 is poorly SUMOylated in HEK cells without SUMO1 overexpression (see lane1 in *B*), F-CTIP2 was co-transfected with a HA-SUMO1 expression vector. As shown in *B*, Whole cell extracts were subjected to immunoblot analysis using indicated antibodies. In *D*, WCE from transfected HEK cells were IP'd with CTIP2 antibody, and the immunoprecipitates were probed with HA antibody (the upper blot). The blot was then stripped and probed for CTIP2 antibody as shown in the lower blot. Jurkat cells (*D*), which do express high levels of Ctip2, carry out the SUMOylation reaction efficiently and without the need of over-expressed SUMO-1. Jurkat cells SUMOylate Ctip2 in a P/A-dependent manner, as was suggested in supplemental Fig.3.S2A, thus recapitulating our results using mouse thymocytes (Fig. 3.1).

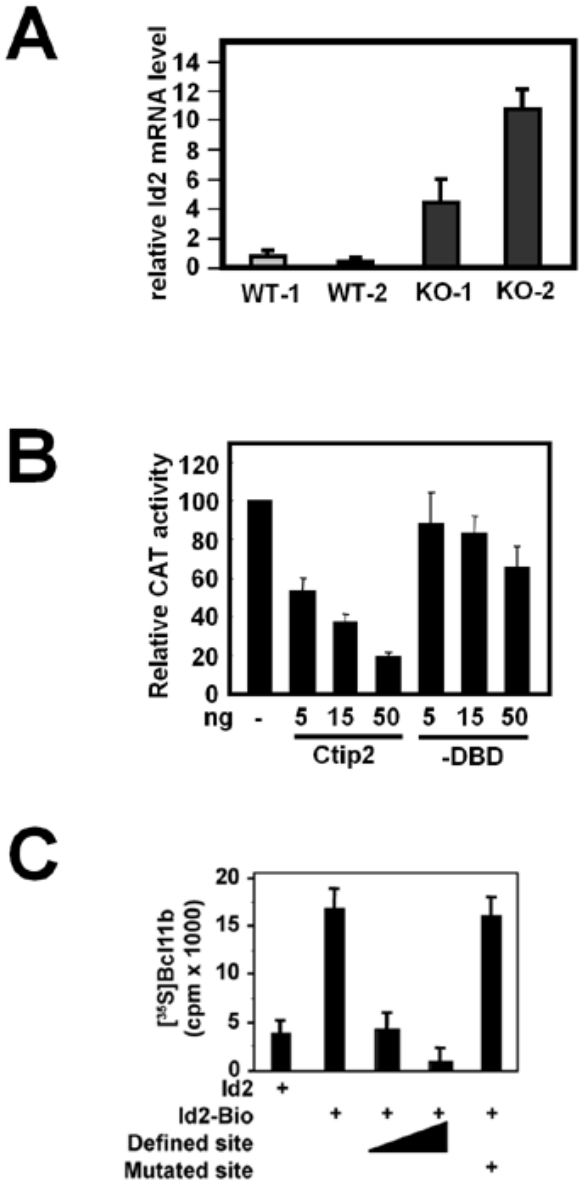

A
relative Id2 mRNA level
14
12
10
8
6
4
2
0
WT-1
WT-2
KO-1
KO-2
B
Relative CAT activity
120
100
80
60
40
20
0
ng
-
5
15
50
5
15
50
Ctip2
-DBD
C
[35S]Bcl11b
(cpm x 1000)
20
15
10
5
0
Id2
Id2-Bio
Defined site
Mutated site

Figure 3.4. Identification of Id2 as CTIP2 target gene. (*A*), Id2 was upregulated in the thymocytes of CTIP2 null mice. qPCR were performed using cDNA synthesized from mRNA extracted from thymocytes of wild type or CTIP2 null mice. GAPDH were used as control to normalize between samples. **(*B*)**, Reporter gene assays in transiently transfected 293 cells using an Id2 promoter fragment (-3.1 kb to +48, relative to the predicted start-site of transcription) CAT reporter. The amounts of wild-type Ctip2 and a DNA binding-defective mutant of Ctip2 (Avram et al., 2002) that were transfected are indicated. **(*C*)**, Avidin-Biotin complex determination assays demonstrating direct binding of Ctip2 to a biotinylated, annealed oligonucleotide derived from the distal conserved region of the Id2 promoter.

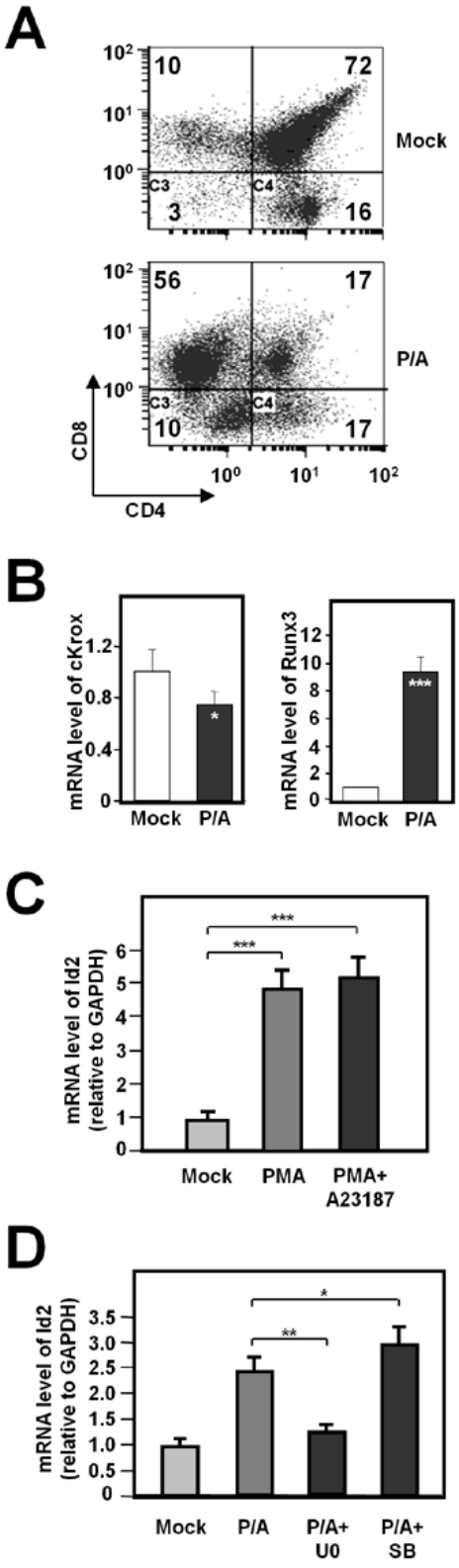
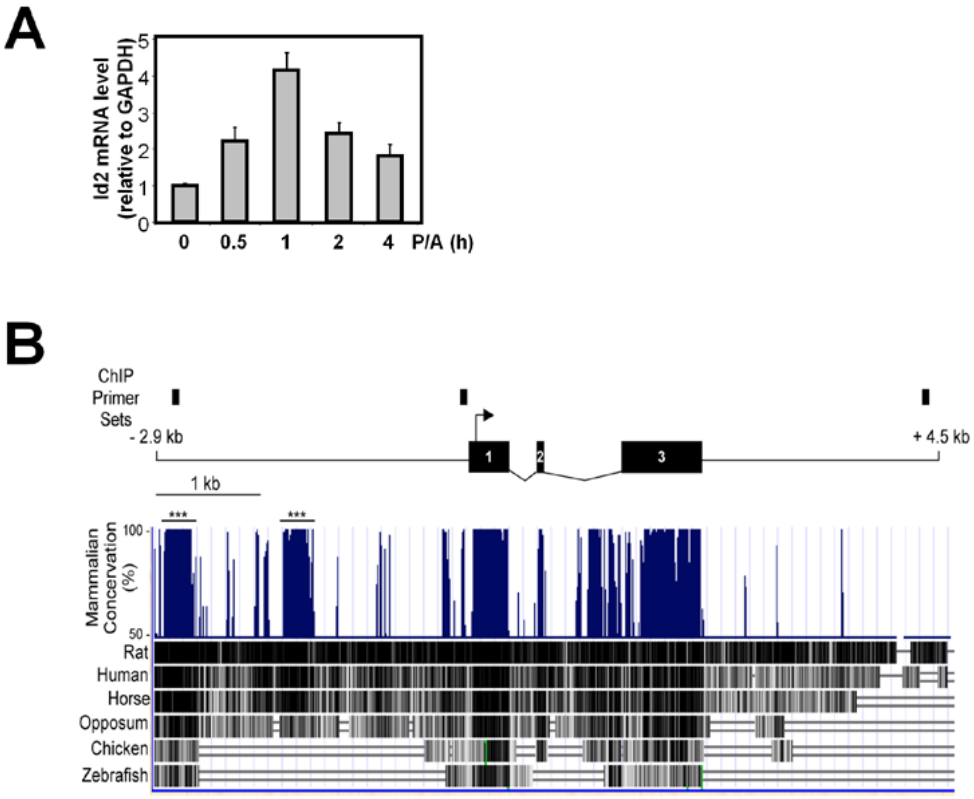
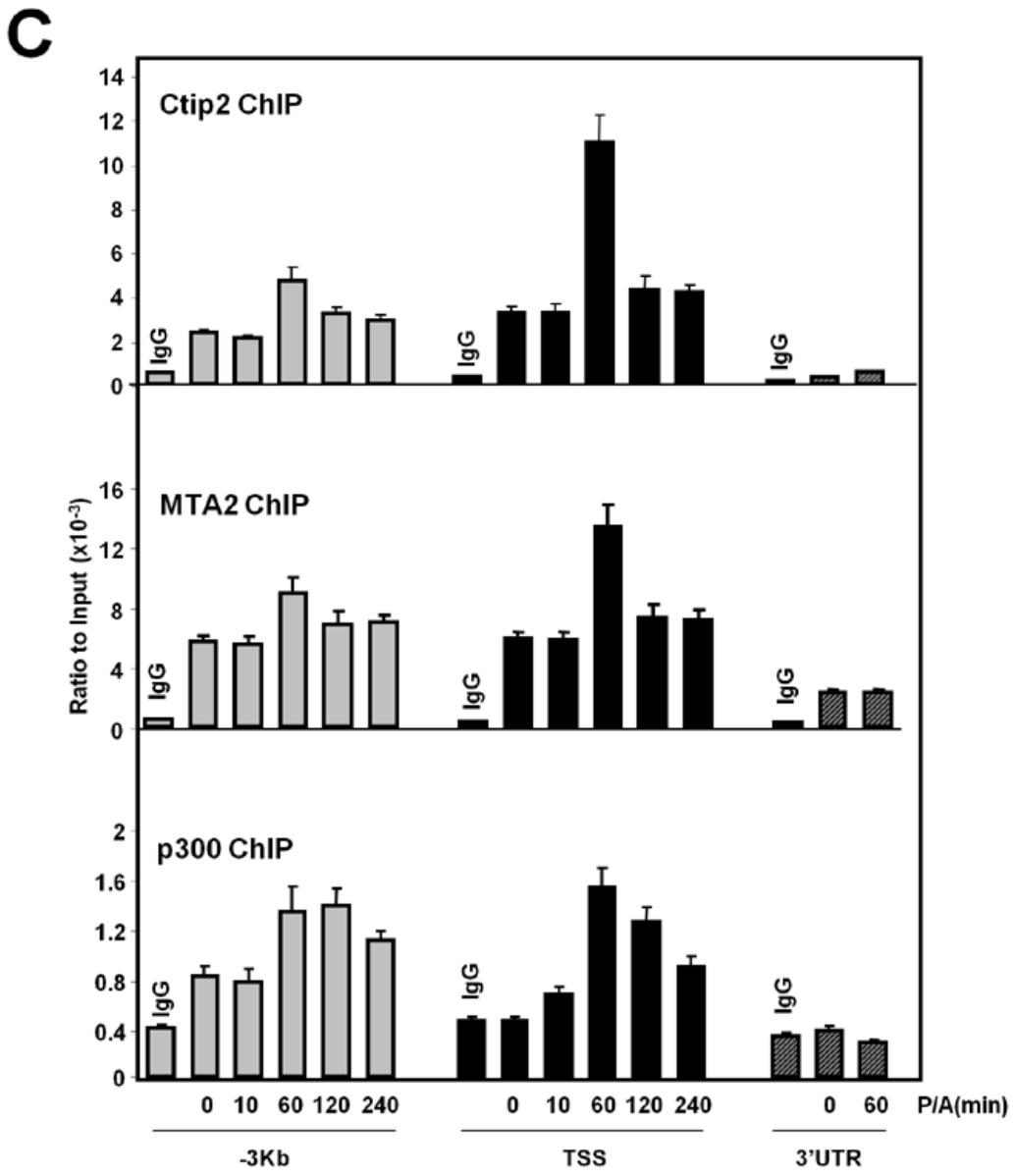
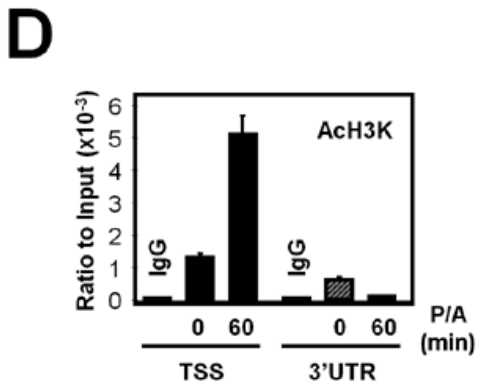

A
10
72
Mock
C3
C4
3
16
56
17
P/A
10
17
CD8
CD4
B
mRNA level of cKrox
Mock
P/A
mRNA level of Runx3
Mock
P/A
C
mRNA level of Id2 (relative to GAPDH)
Mock
PMA
PMA+ A23187
D
mRNA level of Id2 (relative to GAPDH)
Mock
P/A
P/A+ U0
P/A+ SB

Figure 3.5. Induction of Id2 upon activation of T cell signaling pathway. (*A*), Ex vivo differentiation of mouse thymocytes. Primary mouse thymocytes were cultured in vitro and treated with PMA (100nM) and A23187 (500nM) for 2 days before flow cytometry analysis of the surface expression of CD4 and CD8. **(*B*),** RT-qPCR analyses of the relative mRNA expression of cKrox and Runx3 in primary mouse thymocytes stimulated with P/A for 4 hours. GAPDH, a house keeping gene, was used as the internal control to normalize between samples. **(*C*),** RT-qPCR analyses of Id2 mRNA expression in primary mouse thymocytes treated with vehicle (Mock), PMA or PMA plus A23187 for 4 hours. qRT-PCR analyses were performed as described in *B*. **(*D*),** Thymocytes were preincubated with U0 (U0126, MEK inhibitor; 10mM; lane3) or SB (SB202190, a p38 inhibitor; 10mM; lane4) prior to P/A stimulation. RT-qPCR Analyses of Id2 mRNA expression levels were performed as described in *B*. Note that statistical significance in *B-D* was determined using Student's t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)


A
Id2 mRNA level
(relative to GAPDH)
0
1
2
3
4
5
0
0.5
1
2
4
P/A (h)
B
ChIP
Primer
Sets
- 2.9 kb
+ 4.5 kb
1
2
3
1 kb
***
***
Mammalian
Concervation
(%)
100
50
Rat
Human
Horse
Opposum
Chicken
Zebrafish


C
Ctip2 ChIP
MTA2 ChIP
p300 ChIP
Ratio to Input (x10-3)
IgG
0 10 60 120 240
0 10 60 120 240
0 60
P/A(min)
-3Kb
TSS
3'UTR


D
AcH3K
Ratio to Input (x10-3)
IgG
0 60
0 60
P/A (min)
TSS
3'UTR

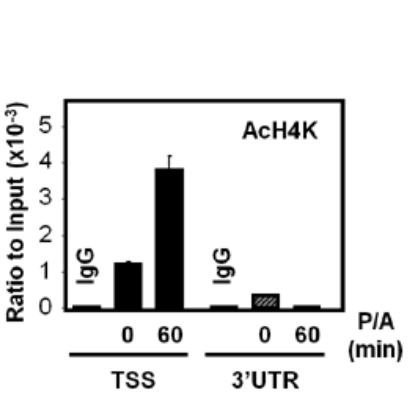
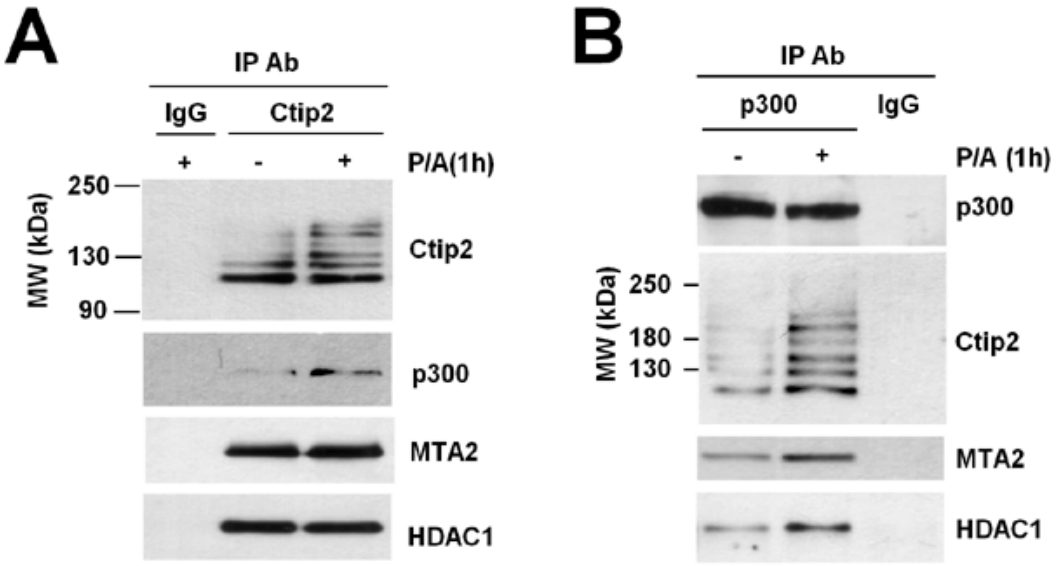

E
AcH4K
Ratio to Input (x10-3)
IgG
0 60
0 60
P/A (min)
TSS
3'UTR

Figure 3.6. Enhanced association of CTIP2, MTA2 on Id2 promoter in stimulated thymocytes. (*A*), Time course of Id2 mRNA induction in stimulated thymocytes. Primary mouse thymocytes were stimulated with P/A for times indicated prior to qRT-PCR analyses as described in Fig.3.4B. **(*B*),** organization of the mouse Id2 locus and alignment of Id2 promoter regions of numerous species. The translational start-site is indicated by a broken arrow and exons 1-3 are denoted by numbered black boxes. The location of three ChIP primer sets is also noted. Sequence identity is indicated by vertical black bars. Two highly conserved regions are indicated by asterisks. **(*C*),** Time course of CTIP2, MTA2 and p300 ChIP analyses on Id2 promoter in stimulated thymocytes. Chromatin immunopreicipitation (ChIP) experiments were performed using sheared chromatin solution from mouse thymocyte cells treated with P/A for times indicated. The purified chromatin DNA were then analyzed by qPCR using primers covering either ~-3kb of mouse Id2 promoter bearing a conserved CTIP2 binding site, the TSS, or 3'UTR region as indicated. Each bar represents the relative ratio to input of each ChIP. **(*D*) and (*E*)**, ChIP analyses of Ac-H3K (*D*) or Ac-H4K (*E*) using thymocytes treated without or with P/A for 1h. Bars represent the relative ratio to input of each ChIP through qPCR analyses using primers covering either mId2 transcription start site (TSS) or 3'UTR region as indicated.


A
IP Ab
IgG
Ctip2
+
-
+
P/A(1h)
MW (kDa)
250
130
90
Ctip2
p300
MTA2
HDAC1
B
IP Ab
p300
IgG
-
+
P/A (1h)
p300
MW (kDa)
250
180
130
Ctip2
MTA2
HDAC1

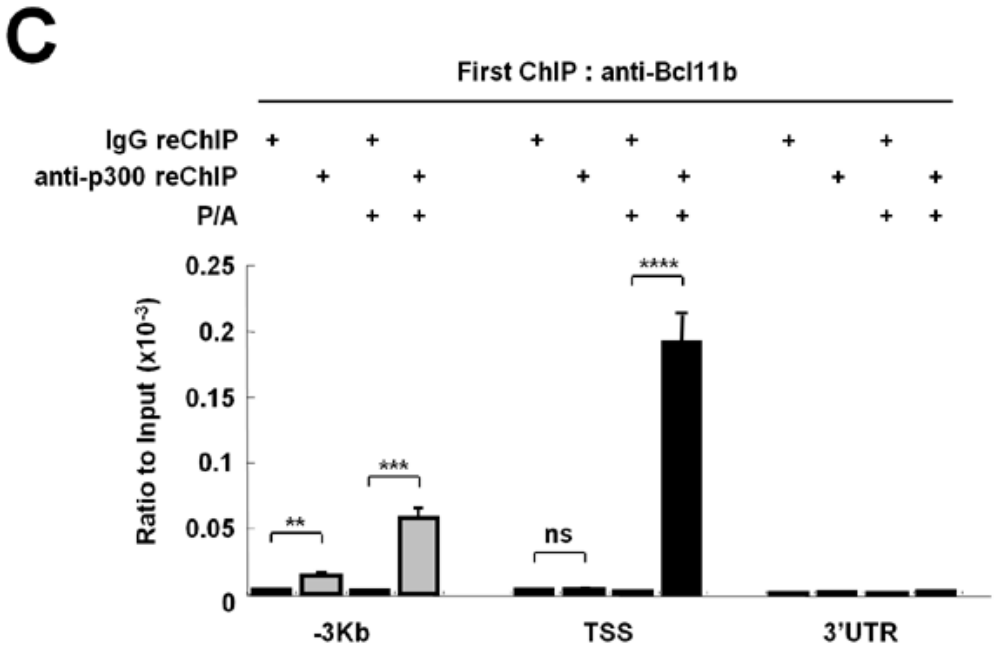

C
First ChIP : anti-Bcl11b
IgG reChIP
anti-p300 reChIP
P/A
Ratio to Input (x10-3)
0.25
0.2
0.15
0.1
0.05
0
**
***
ns
****
-3Kb
TSS
3'UTR

Figure 3.7. Recruitment of p300 to Id2 promoter by Ctip2 in stimulated mouse thymocytes. (*A*), Thymocytes were stimulated without or with P/A for 1h before lysis and IP analyses using CTIP2 antibody or IgG as control. The purified CTIP2 complex was then analyzed by immunobloting using CTIP2, p300, MTA2 and HDAC1 antibodies as indicated on the right. **(*B*)**, Lysate prepared as described above was immunoprecipated with p300 antibody or control IgG, followed by immunoblot analyses using p300, CTIP2, HDAC1 and MTA2 antibodies. **(*C*),** Thymocytes treated without or with P/A for 1h were subjected to reChIP analyses using CTIP2 as the first ChIP antibody, then p300 or control IgG as the second ChIP antibody. The purified DNA were analyzed by qPCR using primers covering either -3Kb of mId2 promoter region, transcription start site (TSS) or 3'UTR as indicated. The statistical significance was determined using Student's t test (ns, nonspecific; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$)

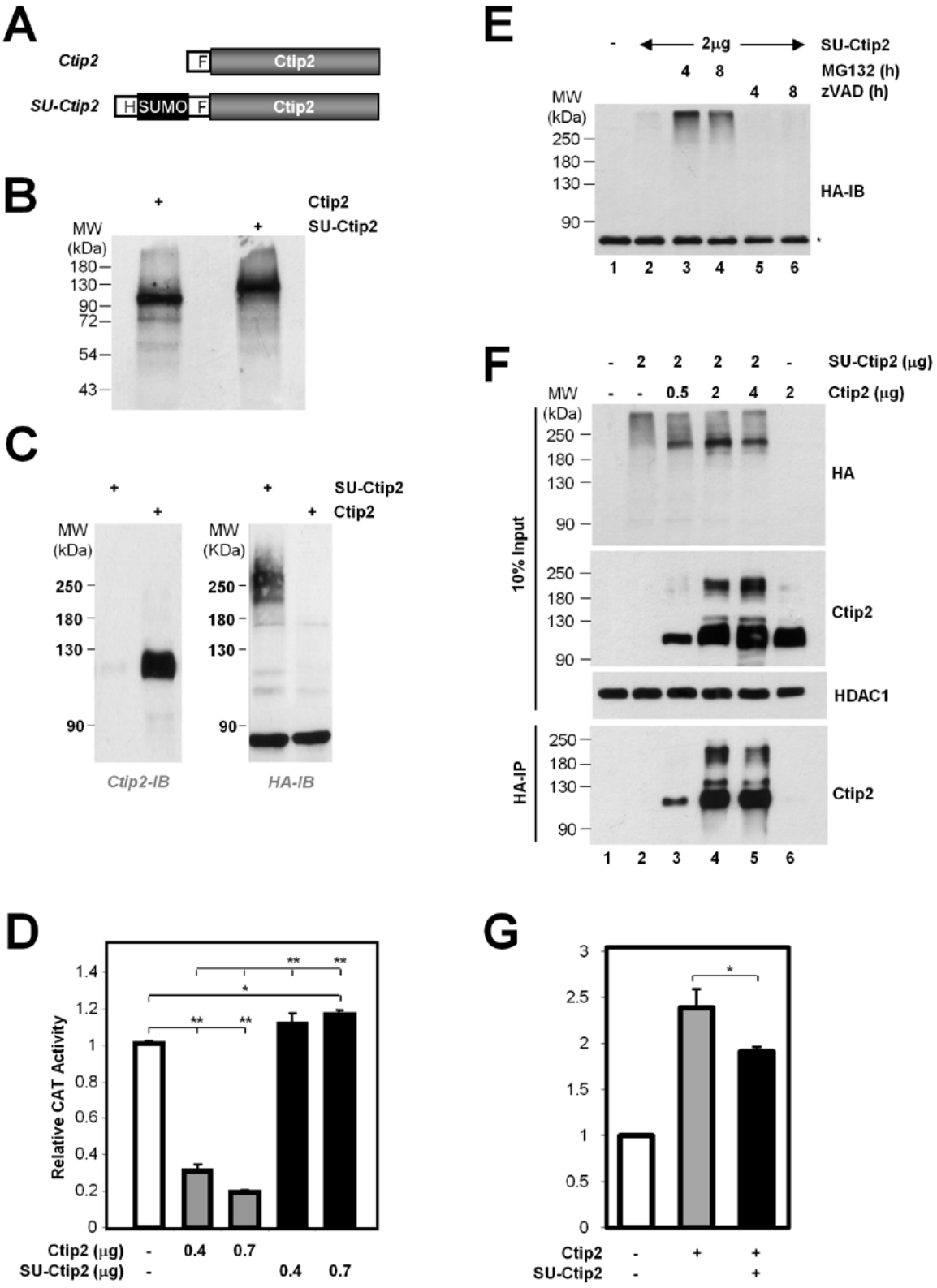
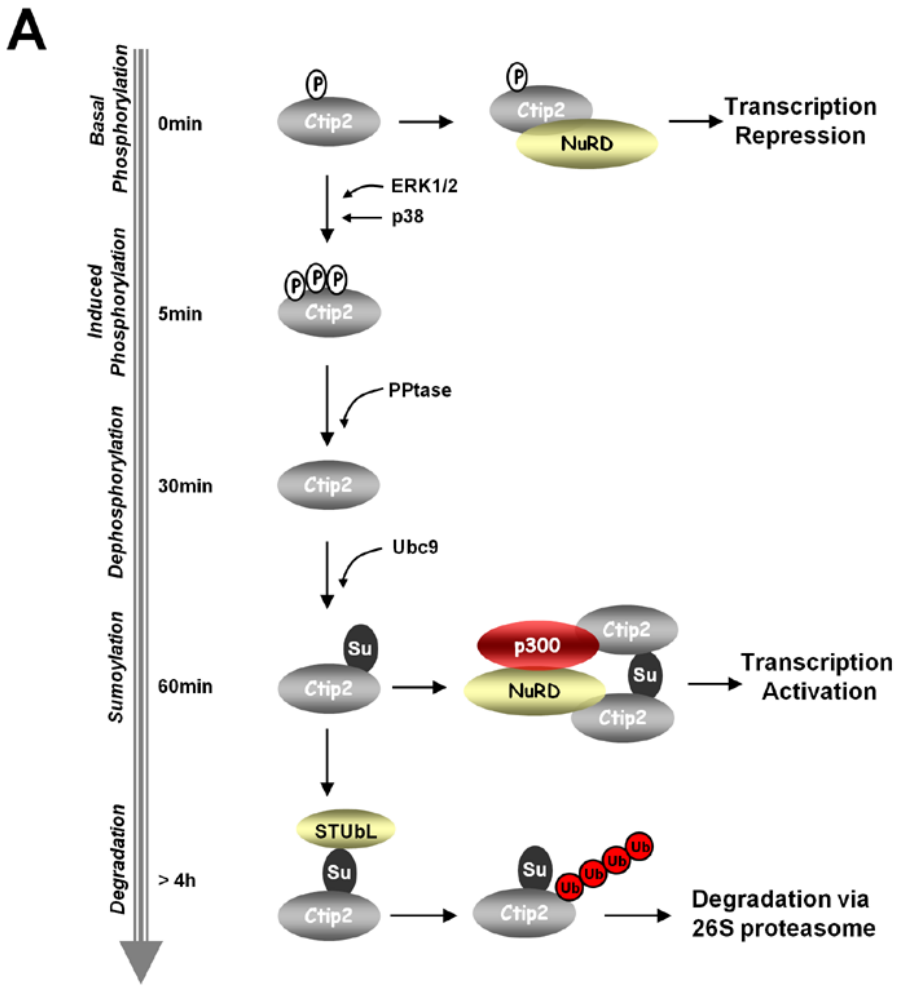

A
Ctip2
F
Ctip2
SU-Ctip2
H
SUMO
F
Ctip2
B
Ctip2
SU-Ctip2
MW
(kDa)
180
130
90
72
54
43
C
SU-Ctip2
Ctip2
MW
(kDa)
250
180
130
90
Ctip2-IB
HA-IB
D
Relative CAT Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctip2 (μg) - 0.4 0.7
SU-Ctip2 (μg) - 0.4 0.7
E
2μg
SU-Ctip2
MG132 (h)
zVAD (h)
4 8 4 8
MW
(kDa)
250
180
130
90
HA-IB
1 2 3 4 5 6
F
SU-Ctip2 (μg)
Ctip2 (μg)
- 2 2 2 2 -
- - 0.5 2 4 2
MW
(kDa)
10% Input
HA
Ctip2
HDAC1
HA-IP
Ctip2
1 2 3 4 5 6
G
3
2.5
2
1.5
1
0.5
0
Ctip2 - + +
SU-Ctip2 - +

Figure 3.8. Su-Ctip2 forms protein complex with non-SUMOylated Ctip2. (*A*), Schematic map of SUMO-Ctip2 fusion construct. **(*B*),** Expression of Su-Ctip2 in vitro. DNA constructs encoding SU-Ctip2 or wild type Ctip2 were used to express ^{35}S-labeled protein using the transcription and translation coupled reticulocyte lysate system. Protein expression was detected by autoradiography. **(*C*),** Expression of the constitutive SUMO1-Ctip2 protein in cells. HEK293T cells were transfected with the same amount of SU-Ctip2 or wild type Ctip2, and the resulting whole cell extracts were subjected to immunoblotting using Ctip2 antibody (the left blot), and the blot was then stripped and reprobed with HA antibody (the right blot). **(*D*),** Fusing SUMO1 to Ctip2 leads to a loss of the intrinsic repression activity of Ctip2. HEK293T cells were cotransfected with Id2CAT and increasing amount of SU-Ctip2 or wild type Ctip2. Relative CAT expression was measured as described in Fig. 4. **(*E*),** SU-Ctip2 is degraded through Ub-proteasome pathway. HEK293T cells transfected with SU-Ctip2 were treated with MG132 (5uM) or zVAD (5uM) for indicated hours before harvest for immunoblot analysis using HA antibody. **(*F*),** Interaction between non-SUMOylated Ctip2 and SU-Ctip2 prevents SU-Ctip2 from Ub-proteasome dependent degradation. HEK293T cells were transfected with various amount of SU-Ctip2 and wild type Ctip2 as indicated, and the resulting whole cell extracts were subjected to immunoprecipitation experiments using HA antibody. Then the purified SU-Ctip2 complex was analyzed by immunoblotting using Ctip2 antibody. 10% input signal of SU-Ctip2 (HA blot) and total Ctip2 (Ctip2 blot) were shown in the upper panels. HDAC1 blot was shown as loading control. ***(G)*,** SU-Ctip2 impairs the transcription repression mediated by non-SUMOylated Ctip2. HEK293T cells were cotransfected with Id2CAT and Ctip2 in the absence or presence with SU-Ctip2. Relative CAT expression was measured as described in Fig. 4.


A
Basal Phosphorylation
Induced Phosphorylation
Dephosphorylation
Sumoylation
Degradation
0min
5min
30min
60min
> 4h
P
Ctip2
NuRD
Transcription Repression
ERK1/2
p38
PPtase
Ubc9
Su
p300
Transcription Activation
STUbL
Ub
Degradation via 26S proteasome

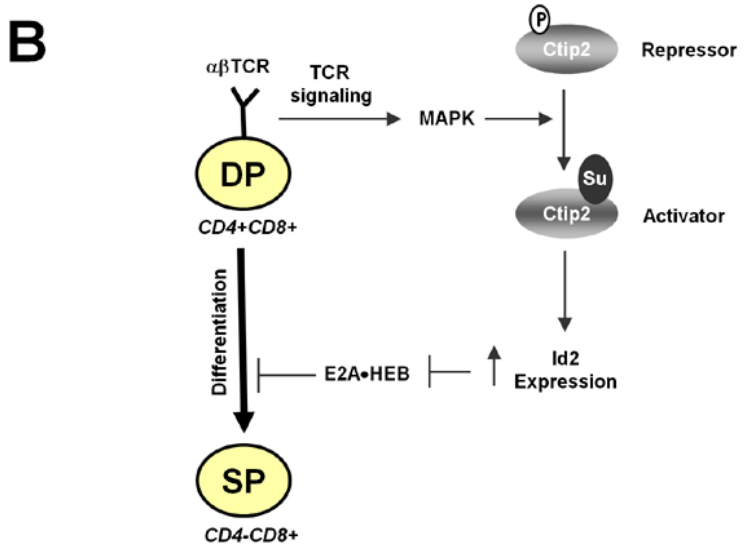

B
αβTCR
TCR signaling
MAPK
P
Ctip2
Repressor
Su
Activator
DP
CD4+CD8+
Differentiation
E2A•HEB
Id2 Expression
SP
CD4-CD8+

Figure 3.9. Model of CTIP2 regulation by T cell signaling pathways. (*A*), A model for regulation of Ctip2 by dynamic phosphorylation and SUMOylation in thymocytes stimulated with PMA and A23187. Ctip2 is basally phosphorylated and interacts with NuRD protein complex to repress target gene transcription. Ctip2 is further phosphorylated by both ERK1/2 and p38 MAPKs In thymocytes treated with P/A for 5mins. Phosphorylated Ctip2 attracts phosphatase and becomes dephosphorylated at 30mins of P/A treatment. The dephosphorylated Ctip2 interacts with SUMO E2 ligase Ubc9, leading to Ctip2 SUMOylation at 60mins of P/A treatment. SU-Ctip2 interacts with non-SUMOylated Ctip2 and recruits coactivator p300 to activate gene transcription. Prolonged treatment of P/A eventually leads to Ctip2 degradation by the STUbLs proteins recruited through the SUMO moiety on Ctip2. **(*B*),** A model for the physiological function and regulation of Ctip2 during DP T cell differentiation. MAPKs activated through TCR signaling contribute the switch of Ctip2 from a basally phosphorylated and non-SUMOylated form to the dephosphorylated and SUMOylated form, therefore converting Ctip2 from a repressor to an activator of transcription, leading to Id2 gene activation. Activated Id2 promotes DP cell differentiation by inhibiting the formation of E2A.HEB complex, resulting in derepression of E2A-repressive targets, which promotes positive selection of DP cells to SP cells.

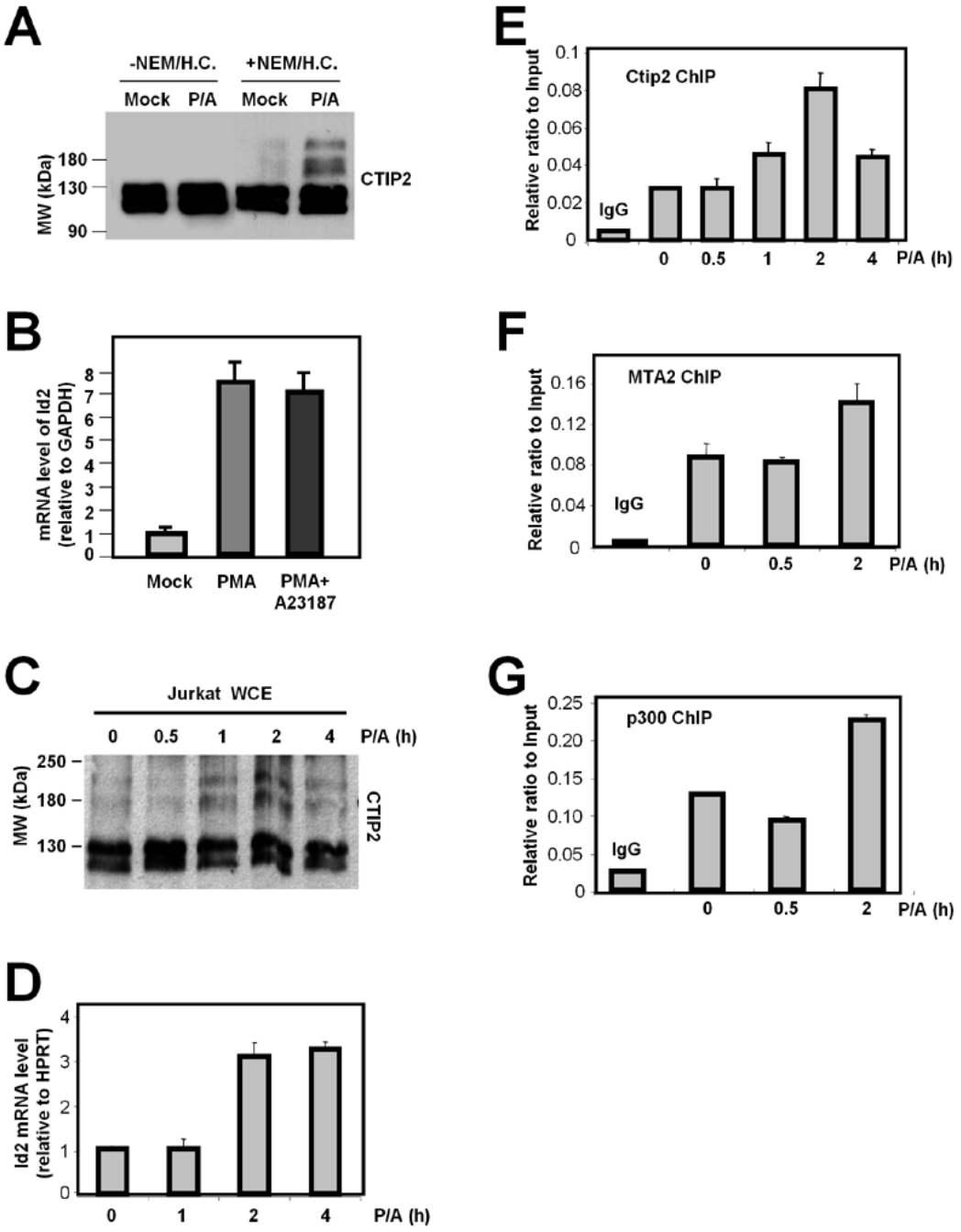

A
-NEM/H.C.
+NEM/H.C.
Mock
P/A
Mock
P/A
MW (kDa)
180
130
90
CTIP2
B
mRNA level of Id2 (relative to GAPDH)
0
1
2
3
4
5
6
7
8
Mock
PMA
PMA+ A23187
C
Jurkat WCE
0
0.5
1
2
4
P/A (h)
MW (kDa)
250
180
130
CTIP2
D
Id2 mRNA level (relative to HPRT)
0
1
2
3
4
0
1
2
4
P/A (h)
E
Ctip2 ChIP
Relative ratio to Input
0
0.02
0.04
0.06
0.08
0.1
IgG
0
0.5
1
2
4
P/A (h)
F
MTA2 ChIP
Relative ratio to Input
0
0.04
0.08
0.12
0.16
IgG
0
0.5
2
P/A (h)
G
p300 ChIP
Relative ratio to Input
0
0.05
0.10
0.15
0.20
0.25
IgG
0
0.5
2
P/A (h)

Figure 3.S1. Enhanced association of CTIP2, MTA2 and p300 on Id2 promoter in stimulated Jurkat cells. (*A*), NEM and hemin chloride preserve low mobility CTIP2 species in Jurkat cells. Jurkat cells were treated with P/A for 2 hour prior to extraction preparation in NEM (5mM) and hemin chloride (HC; 0.1mM). Extracts were subjected to immunoblot analysis using the anti-CTIP2 antibody, **(*B*),** RT-qPCR analyses of Id2 mRNA expression in stimulated Jurkat cells. HPRT, another house keeping gene, was used as the internal control to normalize between samples. **(*C*),** Time course of Id2 mRNA induction in stimulated Jurkat cells. Jurkat cells were stimulated with P/A for times indicated prior to RNA extraction and qRT-PCR analyses as described in Fig.2. **(*D*),** Time course of CTIP2 SUMOylation in Jurkat cells stimulated with P/A. Jurkat cells treated with P/A for indicated times were lysed, and subjected to immunoblotting analyses using CTIP2 antibody. **(*E*), (*F*) and (*G*)** Time course of CTIP2 (*E*), p300 (*F*) and MTA2 (*G*) occupation on Id2 promoter. Methods: Chromatin immunopreicipitation (ChIP) experiments were performed using sheared chromatin solution from Jurkat cells treated with P/A for times indicated. The purified chromatin DNA was subjected to qPCR analysis using primers covering ~-3kb of human Id2 promoter bearing a conserved CTIP2 binding site. Each bar represents the relative ratio to input of each ChIP.

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CHAPTER 4

GENERAL CONCLUSION

Transcription regulation of gene expression is one of the key processes that cells use to turn the information in genes into gene products and to control which genes are turned on, and when. Activities of transcription factors themselves have also to be tightly regulated to ensure that genes are only transcribed when appropriate signals make their way to the nucleus. In the studies described above, we have characterized in detail the molecular mechanisms of the transcription regulation mediated by COUP-TFI and Ctip2, and how these two transcription factors are regulated by TNFα signaling pathway and T cell signaling pathway, respectively.

COUP-TFI (Chicken Ovalbumin Upstream Promoter Transcription Factor-1), belonging to orphan members of the steroid/thyroid hormone receptor superfamily, plays important roles in in differentiation, patterning, axonal projection, cell migration, cortical arealization and the temporal specification of neural stem cells in the developing CNS. To study the biochemical properties underlying its important physiological functions, we used a tandem affinity purification strategy to purify COUP-TFI protein complexes from mammalian cells, and a number of COUP-TFI-interacting proteins were identified, including transcriptional regulatory proteins NCoR and KAP1 and a proapoptotic protein DBC1. In vitro pull down studies revealed that NCoR RD1 (repression domain1) and RD4 interacted directly with the DBD and LBD of COUP-TFI in an atypical manner from that of other nuclear receptors. In addition, DBC1 was found to interact with both COUP-TFI and NCoR, and may function to stabilize the interaction between COUP-TFI and NCoR in vivo.

We also demonstrated that this newly identified COUP-TFI complex

played an important role in repressing TNFAIP8 gene, which encodes an anti-apoptotic protein that is induced during TNFα-triggered apoptosis to inhibit caspase activity. We found that activation of TNFα signaling pathway led to downregulation of COUP-TFI expression, relief of COUP-TFI-mediated repression of the TNFAIP8 promoter, and subsequent inhibition of apoptosis. Thus, for the first time, our findings implicate a role of COUP-TFI in the TNFα signaling pathways in mammalian cells. The identification of oncogene TNFAIP8 as a novel COUP-TFI target gene may contribute the development of new approaches to cancer chemotherapy. For example, drugs that induce cancer cells to overproduce COUP-TF1 may improve the effectiveness of existing cancer drugs to kill cancer cells.

Ctip2 was originally identified as a COUP-TF-interacting protein, and has been shown to potentiate the transcription repression mediated by COUP-TFI in a transient transfection system. Later studies from our lab and from other groups found that Ctip2 can also bind directly and specifically to DNA and repress transcription of target promoter independently of COUP-TFI. Additionally, Ctip2 is highly expressed in a variety of cells that are devoid of COUP-TF expression, i.e. thymocytes, lymphoid derived cells and some neuronal cells, notably, the Purkinje cell layer of the cerebellum and the basal ganglia. These findings demonstrate that Ctip2 can function independently of COUP-TFs in some cell types and promoter contexts.

Previous studies discover an absolute essential role of Ctip2 during thymocyte positive selection. To study the detailed molecular mechanism of Ctip2 function during thymocyte development, we adapt an ex vivo system where combination of PMA and A23197 (P/A) triggers differentiation of primary

DP mouse thymocytes to CD8 SP thymocytes. Unlike COUP-TFI, whose function is regulated at transcription level by TNFα signaling pathway, we have discovered a dynamically regulated pathway in mouse DP cells involving sequential and linked post-translational modifications (PTMs) of Ctip2. We found that Ctip2 is rapidly phosphorylated by treatment of mouse thymocytes with P/A, and then quickly becomes dephosphorylated, leading to subsequent SUMOylation of Ctip2 at K607 and K805 residues. Prolonged treatment of thymocytes with P/A led to the degradation of Ctip2, presumably by proteolytic clipping. We demonstrated that phosphorylation is one of the regulatory mechanisms that control the SUMOylation status of Ctip2, and Ctip2 SUMOylation may be negatively regulated by its phosphorylaton status. The phosphorylated form of Ctip2 does not interact efficiently with Ubc9, which may contribute to the mutual exclusivity of Ctip2 phosphorylation and SUMOylation.

We also found in our study that the time-course with which Ctip2 is SUMOylated after stimulation with P/A corresponds to the induction of the Id2 promoter by P/A, and the localization of Ctip2, MTA2, p300 and acetylated histones H3 and H4 at the Id2 transcriptional start-site. The results of our studies indicate that these PTMs, which we have already mapped in detail using a combination of mass spectrometry and site-directed mutagenesis, may collectively serve as a molecular switch that converts Ctip2 from transcriptional repressor into an activator of the mouse Id2 promoter in DP cells.

Our studies also reveal that Su-Ctip2 interacted with Ctip2, and this interaction is crucial to protect Su-Ctip2 from degradation through the Ubiquitin-proteasome pathway. A growing body of evidence indicates that ubiquitin-mediated proteolysis is involved in regulating gene transcription and

the focus is primary on transcription activators. It is believed that the activity of transcription factors is tightly coupled to their destruction by proteasome, leading to the phenomenon of “unstable when active”. Our observation that time-course with which Ctip2 is degraded corresponds to the decline of Id2 mRNA induction after stimulation with P/A prompts us to propose a model through which Ctip2 is cleared off the Id2 promoter after appropriate amount of transcription activation is achieved: When Ctip2 is SUMOylated, it interacts transiently with the SIM (SUMO Interacting Motif) domain of another copy of non-SUMOylated Ctip2 as a complex, to positively regulate transcription of Id2 gene; when this transcription response is no longer needed, SU-Ctip2 dissociates from the protective complex structure, and its “exposed” SUMO moiety attracts STUbLs (SUMO Targeted Ubiquitin Ligases), which then recruits the Ubiquitin-proteasome system and eventually leads to the destruction of the transcription activation activity of Ctip2. Studies aimed at identifying specific STUbL protein that targets Ctip2 to degradation will further our understanding of how Ctip2 is shutting off when it is no longer needed.

These results provide the mechanistic basis for regulation of Ctip2 transcriptional activity by cell signaling pathways in T cells and contribute to a better understanding of the mechanistic basis underling its important physiological role in development from double positive to single positive thymocytes, specifically in the process of positive selection. Importantly, our work may also lead to a better understanding of the role of Ctip2 in the control of CD4+ lymphocyte function, because we have also observed a similar post-translational regulation pattern of Ctip2 in the activated Jurkat cells, the CD4-SP T cells, compared to that in DP thymocytes.

In summary, these findings described herein are significant because (1). COUP-TFI study in Chapter 2 is the first study to characterize components of COUP-TFI protein complexes on a proteomic scale and to highlight an important role of COUP-TFI during TNFα-mediated apoptosis. (2). Ctip2 study in Chapter 3 is the first demonstration that the transcriptional regulatory activity of Ctip2 may be influenced by post-translational modifications, such as phosphorylation and SUMOylation, in the context of cell signalling pathways. The work described herein brings the power of cutting-edge technology in biochemistry, pharmacology, molecular genetics, and mass spectrometry to bear on the mechanistic foundation that underlies transcriptional regulation by transcription factors COUP-TFI and Ctip2.

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