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Identification of neuronal target genes for CCAAT/Enhancer Binding Proteins

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Abstract

CCAAT/Enhancer Binding Proteins (C/EBPs) play pivotal roles in development and plasticity of the nervous system. Identification of the physiological targets of C/EBPs (C/EBP target genes) should therefore provide insight into the underlying biology of these processes. We used unbiased genome-wide mapping to identify 115 C/EBP β target genes in PC12 cells that include transcription factors, neurotransmitter receptors, ion channels, protein kinases and synaptic vesicle proteins. C/EBP β binding sites were located primarily within introns, suggesting novel regulatory functions, and were associated with binding sites for other developmentally important transcription factors. Experiments using dominant negatives showed C/EBP β to repress transcription of a subset of target genes. Target genes in rat brain were subsequently found to preferentially bind C/EBP α , β and δ . Analysis of the hippocampal transcriptome of C/EBP β knockout mice revealed dysregulation of a high percentage of transcripts identified as C/EBP target genes. These results support the hypothesis that C/EBPs play non-redundant roles in the brain.

Keywords

C/EBP β ; transcription; brain

Introduction

Since their discovery the family of CCAAT/Enhancer Binding Proteins has grown to include six members (C/EBP α , β , δ , ϵ , γ and ζ) (Johnson et al., 1986). C/EBPs are known to regulate the transcription of genes important for metabolism, differentiation and inflammation (Croniger et al., 1998; Hanson, 1998; Lekstrom-Himes and Xanthopoloulos, 1998; Poli, 1998; Ramji and Foka, 2002) and are themselves regulated at the level of transcription (Niehof et al., 1997), translation (Calkhoven et al., 1994; Lincoln et al., 1998) and post-translationally by multiple signaling pathways (Trautwein et al., 1993; Nakajima et al., 1993; Umayahara et al., 2002). C/EBPs share a highly conserved C-terminal basic amino acid-rich DNA binding and leucine zipper dimerization (bZIP) domain. With the exception of the homologous C/EBP protein C/EBP ζ (a.k.a. CHOP) (Ubeda, 1996), C/EBPs recognize the same DNA binding sequence (Falvey et al., 1996; Osada, et al. 1996). The N-terminal activation domains of C/

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EBPs are significantly less conserved than the bZIP domain, with alternative ribosomal entry sites or alternative splicing of exons generating C/EBP isoforms with additional N-terminal diversity (Descombes and Schibler, 1991; Yamanaka et al., 1997). C/EBPs dimerize not only with each other but with other bZIP proteins and dimerization outside of the immediate family modifies both DNA recognition and *trans*-activation (Vallejo et al., 1993; Hsu et al., 1994; Newman and Keating, 2003; Cai et al., 2008). The variety of DNA binding and activation domains made available through heterodimerization enables C/EBPs to interact with a broad range of DNA sequences, transcription factors, co-activators and chromatin remodeling complexes (Milos and Zaret, 1992; Falvey et al., 1996; Lee et al., 1997; Kowenz-Leutz and Leutz, 1999; Gutierrez et al., 2002; Niehof et al., 2004). These unique attributes presumably increase the number of genes that are regulated by C/EBPs and enable differential gene expression even when more than one C/EBPs family member is expressed within the same cell (Colangelo et al., 1998; Yamanaka et al., 1998; Zhu et al., 2002).

The most abundant C/EBPs in the brain are C/EBP α , β and δ , which are enriched in neurons and regulated by cAMP- and calcium-dependent signaling mechanisms (Sterneck and Johnson, 1998; Sterneck et al., 1998; Yukawa et al., 1998; Nadeau et al., 2005; Lein et al., 2007). C/EBPs have been implicated in the control of biological processes critical to neuronal development and survival, including cell fate determination (Menard et al., 2002; Paquin et al., 2005), apoptosis (Marshall et al., 2003), the synthesis of trophic factors (Symes et al., 1995; Colengalo et al., 1998; Takeuchi et al., 2002; McCauslin et al., 2006), the response to trophic factors (Sterneck and Johnson, 1998; Calella et al., 2007) and responses to brain injury and ischemia (Bernaudin et al., 2002; Cortes-Canteli et al., 2002; Soga et al., 2003; Nadeau et al., 2005; Kapadia et al., 2006). C/EBPs also have roles in transcriptional programs underlying more complex brain functions, such as learning and memory (Taubenfeld et al., 2001a; Taubenfeld et al., 2001b; Chen et al., 2003) and the effects of electroconvulsive shock (Chen et al., 2004), methamphetamine (Thomas et al., 2004) and hallucinogens (Nichols and Sanders-Bush, 2004) on brain neurochemistry.

One strategy to elucidate the underlying biology of these processes is to identify and annotate functionally related physiological targets for C/EBPs in the brain (C/EBP target genes). Classical gene promoter analysis has yielded only a limited number of neuronal C/EBP target genes, including the rate-limiting enzyme in tetrahydrobiopterin biosynthesis, GTP cyclohydrolase I (GCH1) (Kapatos et al., 2000; Kapatos et al., 2007), the structural protein α -tubulin (Gloster et al., 1994), the peptide neurotransmitter substance P (Kageyama et al., 1991; Kovacs et al., 2006), the metabotropic glutamate receptors 1 and 5 (Corti et al., 2003; Crepaldi et al., 2007), the trophic factors nerve growth factor and brain derived neurotrophic factor (Colangelo and Johnson, 1998; McCauslin et al., 2006; Callela et al., 2007), the developmentally regulated bHLH transcription factor Nex1/Math-2/NeuroD (Uittenbogaard et al., 2007) and the immediate-early genes c-fos, Egr1 and Egr2 (Metz and Ziff, 1991; Callela et al., 2007). C/EBP target gene discovery was recently accelerated, however, by a study that combined chromatin immunoprecipitation (ChIP) and DNA microarray technology (ChIP-chip) to identify new target genes regulating hepatic metabolism and cell proliferation (Friedman et al., 2004).

The overall goal of the present study was to identify genes in the brain that are regulated by C/EBPs. To accomplish this we designed a two-step process in which C/EBP β target genes were first identified in the rat PC12 pheochromocytoma cell line and then validated in the rat brain using conventional ChIP. This strategy was chosen because preliminary experiments using brain samples showed relatively low levels of enrichment by ChIP of known C/EBP β target genes. While PC12 cells are certainly not neurons, these cells express a host of neuronal genes (Angelastro et al., 2000; Impey et al., 2004) and have been used for many years to model neuronal functions (Salton et al., 1983; Rudy et al., 1987). For the identification of C/EBP

target genes we chose serial analysis of chromatin occupancy (SACO) (Impey et al., 2004), variations of which are known as GMAT (Roh et al., 2004), STAGE (Bhinge et al., 2007), SABE (Chen, 2006) and PET (Lin et al., 2007). Unlike the earlier ChIP-chip analysis of C/EBP β binding sites, which was restricted to proximal promoter regions of a limited number of genes (Friedman et al., 2004), SACO provides an unbiased genome-wide approach for the identification of all loci bound by C/EBP β . Our SACO analysis identified over one hundred C/EBP β target genes in PC12 cells and revealed a preponderance of intragenic binding sites suggesting novel transcriptional functions. A subset of these target genes were validated in the rat brain and shown to preferentially bind C/EBP α , β and δ . Subsequent analysis of the hippocampal transcriptome of C/EBP β knockout mice revealed changes in expression of many genes identified by SACO as C/EBP targets. These results support the hypothesis that C/EBPs play non-redundant roles in the brain.

Results and Discussion

C/EBP β Expression in PC12 cells

Although the PC12 cell line is known to express C/EBP β (Metz and Ziff, 1991; Sterneck and Johnson, 1998) multiple C/EBPs are often produced by the same cell (Scott et al., 1992; Doppler et al., 1995; Greenbaum et al., 1995). A crucial first step towards production of a SACO target gene library was therefore to identify which C/EBPs are found in PC12 cells. Moreover, because the pool of C/EBP target genes can be increased by activating C/EBP gene transcription (Friedman et al., 2004), it was equally important to determine whether C/EBP expression in PC12 cells can be induced by cAMP or some other second messenger.

Real-time quantitative RT-PCR analysis of growth arrested PC12 cells combined with cloning and sequencing of PCR products identified C/EBP β and δ mRNAs and showed them to be expressed at roughly equivalent amounts (Fig. 1A). Primary transcripts encoding for C/EBP α , γ and ϵ were below the limit of assay detection. Further studies focused on C/EBP β and δ and found that a 4-hour incubation with the cell-permeant cAMP analogue 8Br-cAMP increased C/EBP β mRNA levels by 6-fold, while levels of C/EBP δ mRNA were not significantly changed (Fig 1A). The different responses of C/EBP β and δ to cAMP is surprising given that the promoters of both genes contain cAMP Regulatory Elements (CRE) (Niehof et al., 1997; Cantwell et al., 1998) and are inducible by cAMP in other cell types, including neurons (Yukawa et al., 1998). Subsequent Western blot analysis of whole cell extracts showed a low basal level of the 35kDa isoform of C/EBP β which, along with the 38kDa isoform, was increased following treatment with cAMP (Fig. 1B). In contrast, C/EBP δ protein was never observed in control or 8Br-cAMP-treated PC12 cells. While this failure to detect C/EBP δ protein could be methodological the similar reactivity of the C/EBP δ and C/EBP β antibodies makes this seem unlikely (see Fig. 7A). The basis for the undetectable level of C/EBP δ protein in PC12 cells thus remains unknown but similar observations have been reported for other cell types that express a particular C/EBP transcript but no C/EBP protein (Lincoln et al., 1998). Based upon these results a SACO C/EBP β library was constructed from growth arrested PC12 cells challenged for 4 hours with 8Br-cAMP.

Characterization of the C/EBP β antibody used for SACO

The next step towards construction of the SACO library was to insure that the antibody used for ChIP specifically enriches for C/EBP β target genes. ChIP analysis of 8Br-cAMP-treated PC12 cells using a C/EBP β antibody we have used previously for this purpose (Kapatos et al., 2007) produced a 120-fold enrichment for the C/EBP β target gene GCH1 with less than a 2-fold enrichment for the house keeping gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH) (Fig. 1C). The specificity of this antibody was further assessed by ChIP of mouse liver chromatin that had been cross-linked *in situ*. This analysis showed that enrichment for

the C/EBP β target gene phosphoenolpyruvate carboxykinase (Pepck) (Croniger et al., 1998) was reduced from 45-fold in wild type animals to less than 2-fold in C/EBP β $-/-$ animals (Fig. 1D). Moreover, this decline in Pepck enrichment was specific to C/EBP β and the C/EBP β antibody since ChIP using an antibody to C/EBP α produced no difference in Pepck enrichment between genotypes (Fig. 1D). We conclude from these data that this antibody can be said to specifically precipitate C/EBP β bound to target gene DNA when ChIP enrichment is greater than 2-fold.

Identification of C/EBP β target genes in PC12 cells

Cross-linked DNA obtained from growth arrested PC12 cells treated with 8Br-cAMP for 4 hours was immunoprecipitated with anti-C/EBP β , processed through the SACO protocol and inserts containing ditags sequenced. The SACO data analysis program START was then used to identify and extract 21 bp Genomic Signature Tags (GSTs) (Marinescu et al., 2006). Based upon a previous SACO analysis of CREB binding sites (Impey et al., 2004), GSTs had to match a single gene and be located within 5000 bp upstream of a transcription start site (5P), within an exon or an intron (IN) or within 2000 bp downstream from the 3' exon (3P) (Fig. 2A). 1491 GSTs met these criteria and were aligned with high probability to genomic loci representing 115 genes. Analysis showed that 32% of these genes had one GST repeated 2 or more times. Examples of this group are synaptic vesicle protein 2b (Sv2b, 8 tags), vacuolar protein sorting 4a (Vps4a, 17 tags), protein tyrosine phosphatase 2E (Ptpn21, 2 tags) and myelin transcription factor-1 (Myt1, 2 tags). 7% of target genes, including opioid receptor like-1 (Oprl1) and metabotropic glutamate receptor subunit 7 (Grm7), were identified by multiple and repeating GSTs. The remaining 61% of target genes were marked by a single GST and include synaptoporin (Synpr) and alpha-synuclein (Snca). A complete list of all 115 C/EBP β target genes, including GST sequences, the number of times each GST was detected, gene ID, the name of the gene and gene product is available in Supplementary Table S1.

Analysis of GST locations within target genes revealed that 17% of C/EBP β binding sites are located in 5P regions, 6% in exons with 1% in exon 1, 67% in introns with 20% in intron 1 and 10% in 3P regions (Fig. 2B). The intragenic location of 73% of C/EBP β binding sites might explain why so few target genes were discovered by interrogating the proximal promoters of genes expressed in the liver (Friedman et al., 2004). Three target genes are presented in Figure 2C as examples of GST clusters and associated C/EBP β binding sites in 5P, IN and 3P locations. Sv2b is a 5P target gene with a single repeating GST located 600 bp from a C/EBP site in the proximal promoter. Oprl1 exemplifies an IN target gene and is identified by two GSTs located within exon 2, with one GST repeated 11 times, the other repeated 3 times and two C/EBP sites found 50 bp and 435 bp from these tag clusters. Vps4a is a 3P target gene identified by one GST located immediately outside of the 3' exon that repeats 17 times and has a C/EBP site within 375 bp.

In contrast to the preponderance of IN C/EBP β binding sites, a SACO dataset also generated from PC12 cells has shown CREB binding to be located primarily in 5P regions (63%), followed by IN (33%) and 3P (4%) (Impey et al., 2004). Different principal genomic locations for binding of C/EBP β and CREB may be related to differences in how these proteins couple cell signaling and gene expression. For example, CREB is not typically regulated at the level of transcription and while bound to target genes is activated by phosphorylation (Gonzalez et al., 1991; Lonze and Ginty, 2002). On the other hand, C/EBP β is regulated at the level of transcription, often by CREB (Niehof et al., 1997) and in a phosphorylation-dependent manner shuttles from the cytoplasm to the nucleus (Metz and Ziff, 1991; Chinery et al., 1997) where it is recruited to target genes (Su et al., 2003; Friedman et al., 2004; Kapatos et al., 2007). Nonetheless, the preference of C/EBP β for intragenic binding is similar to that reported for a number of other transcription factors, including Wnt/ β -catenin (Yochum et al., 2007), Foxp3

(Zheng et al., 2007), Oct4 and Nanog (Loh et al., 2006), RELA(p65) (Lim et al., 2007), Stat1 (Bhinge et al., 2007), the glucocorticoid receptor (So et al., 2007) and estrogen receptor α (Lin et al., 2007), suggesting that it is CREB and the CRE that are unique.

A phylogenetic analysis of intronic C/EBP sites in the rat genome that have been validated by ChIP (see below) revealed that seven of fifteen sites are completely conserved between rat and mouse and that two of these seven sites are also conserved in humans. The conserved mouse and rat sites are found in genes coding for dynamin 3 (Dnm3), the epidermal growth factor receptor (Egfr), thrombospondin receptor Cd47, Snca, Synpr, amyloid precursor protein (App) and voltage-dependent anion channel 1 (Vdac1). The two C/EBP sites conserved across all three species are located within intron 17 of Dnm3 and intron 1 of the Egfr and these sequences are presented in Figure 2D. The conservation of C/EBP sites in Dnm3 and Egfr suggests that these intronic elements confer critical regulatory functions. The lack of conservation in humans of the five intronic sites conserved in mouse and rat indicates that regulation of these genes by these elements has been lost in the human lineage.

While the small percentage of C/EBP binding sites located in 5P positions, such as in Sv2b, are likely to be involved in core promoter functions, the more common intragenic C/EBP β binding sites that are conserved in Dnm3 and Egfr may identify alternative promoters or distant modifiers of transcription. Cap Analysis Gene Expression (CAGE; Carninci et al., 2006) of the mouse Dnm3 and Egfr intronic binding elements indicates that there are no local transcription start sites (TSS), suggesting that when bound to these elements C/EBP β functions outside of a core promoter. In contrast, a TSS can be found within 682 bp of the C/EBP site in intron 17 of the human Dnm3 gene. Similarly, in intron 1 of the human Egfr gene two TSS are located within 331 and 1681 bp of the C/EBP site. The intronic C/EBP sites in human Dnm3 and Egfr therefore mark alternative core promoters. Interestingly, 3P C/EBP β binding sites, such as found in Vps4a, may also mark TSS but from the antisense strand, as has been reported for Wnt/ β -catenin control of antisense E2F4 transcription from the 3P region of the sense E2F4 gene (Yochum et al., 2007b).

Functional annotation of C/EBP β target genes

Functional annotation of all 115 C/EBP β target genes was performed using a combination of Gene Tools Annotation Database (Beisvag et al., 2006), Gene Ontology (Ashburner et al., 2000), DAVID Bioinformatics Database (Dennis et al., 2003), KEGG pathway, Genomatrix BiblioSphere and Ingenuity Pathways Analysis (Calvano et al., 2005). Classification of target genes according to function revealed that 31% are dedicated to cell signaling, 24% to metabolism and 19% to transport, with the remaining 26% having roles in transcription, synaptic transmission, differentiation and proliferation (Fig. 3A). The central role played by C/EBP β in one signaling network is illustrated in Figure 3B. C/EBP β and the transcription factors CREB and NF κ B are found at the center of this network, surrounded by the protein kinases PI3K, Akt, Lyn and Cdc42 as well as proteins that signal to these kinases, including calmodulin, the small GTPases Ras and Rac and the guanine nucleotide exchange factor Vav. Acting on these pathways are a number of first messengers important for neuronal function, including platelet derived growth factor (Pdgf), epidermal growth factor (Egfr), thrombospondin (Cd47), growth hormone releasing hormone (GHRHR), netrin-1 (Unc5b) and glutamate (Grm7). Based upon our SACO analysis 15 of the 23 proteins comprising this network are under direct transcriptional control by C/EBP β . The addition of these transcriptional control points to this network results in feedback loops which enable C/EBP β to regulate transcription of the same signaling proteins that modify C/EBP β function directly by phosphorylation or indirectly through CREB and NF κ B control of C/EBP β gene expression.

Given that PC12 cells are derived from the neural crest, it was not surprising to find that 54% of C/EBP β target genes are expressed in the brain. These include the neurotransmitter receptors

GABA_A receptor subunit beta and Grm7, the transcription factors Myt1 and vestigial like 4 (Vgll4) involved in neuronal and oligodendrocyte development, the clinically important Scna and App, the calcium-dependent α -latrotoxin receptor neurexin 1, the nociceptin receptor Oprl1, the signaling proteins phospholipase C beta and protein kinase C epsilon, the synaptic vesicle proteins Sv2b and Synpr and the axon guidance netrin-1 receptor gene Unc5, to name a few. It is also intriguing to find on this list multiple members of two gene families that are co-regulated by C/EBP β , the olfactory receptor genes Olf-529, -1204, -1347, -1415, and -1598, which as a class are often ectopically expressed (Feldmesser et al., 2006) and the solute carrier genes Slc-2a1, -3a1, -23a1, -36a1, -9a3, -26a3, and -16a7, which encode for membrane spanning transporter proteins. Because the depth of target gene sequencing was so shallow the known C/EBP β target genes c-fos and GCH1 (Metz and Ziff, 1991; Kapatos et al., 2007) were not identified in this screen.

Validation of PC12 cell C/EBP β target genes by ChIP

Genes selected for validation included those identified by a tag repeated at least three times, genes identified by multiple tags and genes with tags in 5P, IN and 3P regions (Fig. 4A). Individual ChIP assays on cAMP-treated PC12 cells were used as the validation tool. A locus was considered positive for C/EBP β if the average enrichment was two-fold or greater. In this analysis the C/EBP β target genes c-fos and GCH1 showed 28- and 96-fold enrichment, respectively (Fig. 4B). Overall, we validated 20 out of 22 or 91% of C/EBP β targets, some of which are presented in Fig. 4B as examples of genes with tags located in 5P, IN or 3P regions. Grm7 was one of the target genes identified by multiple GSTs that did not validate (Fig. 4B). Grm7 has numerous putative C/EBP β binding sites flanking multiple tags, making it likely that we selected the wrong binding site for validation. Although no further attempt was made to validate Grm7, the metabotropic glutamate receptors 1 and 5 are known C/EBP target genes (Corti et al., 2003; Crepaldi et al., 2007), suggesting that the Grm family of glutamate receptors are co-regulated by C/EBP β much like the olfactory receptor and solute carrier families of proteins. Based upon these observations we believe that the calculated rate of 91% for validation of targets with multiple GSTs is a conservative estimate. Because 61% of our SACO library contains genes represented by a single GST we also selected for validation a small number of genes with one tag. Eight of nine one tag genes or 89% were confirmed as target genes (Fig. 4C). Validated targets with single intronic tags included aldehyde dehydrogenase (Aldh1a4) and Scna, two genes that previously shown to have C/EBP binding sites in 5P locations (Elizondo et al., 2000; Gomez-Santos et al., 2005). Novel single tag target genes include cryptochrome 2, an essential component of the clock machinery (Van der Horst et al., 1999) and dopamine responsive-protein (Drp), which has no known function but contains a GTPase-like domain. Extrapolating from these validation rates we estimate that at least 90% of the 115 genes obtained by SACO are C/EBP β target genes. A complete list of validated target genes including sequence, number and genomic region for each GST is represented in Table 1.

Defining the C/EBP β binding site in validated target genes

The C/EBP consensus site was originally determined by *in vitro* binding assays to be either the pentameric sequence RTTGCGYAAAY (Osada et al., 1996) or RTTAYGTAAY (Falvey et al., 1996). The ambiguity of the four internal bases comprising this “consensus” illustrates the problems confronting a strictly computational approach to the discovery of C/EBP target genes. Indeed, position weight analysis (WebLogo, Crooks et al., 2004) of C/EBP binding sites from the TRANSFAC database shows the C/EBP element as the octomer TTGCGCAA, with no nucleotide preference at positions 1 and 10 (Fig. 5A). Defining the C/EBP binding site using an *in vivo* technique like SACO is even more problematic because the sequence of DNA recognized by C/EBP β is altered by heterodimerization with other bZIP proteins that are known to be expressed in PC12 cells, such as ATF-4, c-fos and c-jun (Fawcett et al., 1999; Leppa et

al., 2001). A consensual C/EBP β binding sequence derived by SACO from intact PC12 cells might therefore be expected to differ from even the “canonical” sequence shown in Figure 5A. In fact, MAPPER and TRANSFAC scores for SACO-derived C/EBP β binding sites were generally lower than expected. Accordingly, position weight analysis of binding sites from all 28 validated target genes revealed a C/EBP motif with a lower preference for T in positions 2 and 3 and no apparent base preference at positions 4, 5 and 6 (Fig. 5A).

Examples of validated target genes with non-canonical binding sites include GCH1, Drp and Sv2b with sites in 5P regions, Oprl1 with an IN site and Vps4a with a 3P site (Fig. 5B). To address whether these sites have the potential to recruit C/EBP homodimers *in vivo* we used EMSA to monitor binding of recombinant C/EBP β to double stranded oligonucleotides containing the target gene sequences shown in Figure 5B. These experiments clearly show that homodimers of C/EBP β are recruited to each of these elements but not to the negative control GCH1 CCAAT-box, which shares a CAA sequence with the C/EBP consensus but binds the heterotrimeric transcription factor NF-Y and not C/EBP β (Kapatos et al., 2000; Hirayama et al., 2001; Kapatos et al. 2007) (Fig. 5C). Identical results were obtained using C/EBP α protein (data not shown). Of particular interest was the Drp sequence which is composed of two overlapping C/EBP elements which are referred to in Fig. 5B as Sites 1 and 2. Experiments using unlabelled probes to compete binding revealed that the Drp probe binds C/EBP β with a lower affinity than a probe containing a consensual C/EBP site (Williams et al., 1995). Subsequent experiments with competing unlabelled Drp probes containing single base substitutions suggest that it is Site 1 which recruits C/EBP β (Fig. 5D). Overall, while this evidence does not preclude the binding of C/EBP β and bZIP heterodimers to these sites in intact PC12 cells it does confirm that non-canonical sites located by SACO and identified by MAPPER are *bona fide* regulatory elements capable of recruiting C/EBP β homodimers *in vivo*.

Association of C/EBP binding sites with binding sites for other transcription factors

Computational analysis predicts over 30 million C/EBP binding sites in the rat genome (Falvey et al., 1996), which is not surprising given the accepted variation in the C/EBP consensus sequence. This extravagance has spawned the hypothesis that functional C/EBP sites are defined not just by their sequence but also by adjacent binding sites for transcription factors that interact with and stabilize C/EBP binding (Friedman et al., 2004; Calella et al., 2007). In support of this concept C/EBP β binding sites in the 5P regions of liver target genes were found to be localized within 300–1000 bp of binding sites for the known C/EBP transcriptional partners IFN-response factor-1 (Hurgin et al., 2002) and PPAR γ (Tontonoz et al., 1994). Moreover, in the brain NeuroD and C/EBP β are known to functionally interact to regulate immediate early gene expression within the context of growth factor signaling (Calella et al., 2007). We therefore performed a similar comparative location analysis in search of transcription factor binding motifs within 250 bp of the C/EBP sites of 20 validated target genes functionally annotated as being expressed in the brain. This search revealed that in the rat genome 95% of these genes, including Dnm3, Egfr, App, Oprl1, Scna, Sv2b and Synpr, have binding sites for both PU.1 and Sox5 in close proximity to validated C/EBP binding sites. An analysis of whether this spatial arrangement is conserved across species indicates that both PU.1 and Sox5 elements are associated with C/EBP sites in the mouse and rat Dnm3, Egfr, Cd47, Sv2b and Snca genes. Human Dnm3 and Egfr do not have both PU.1 and Sox5 binding sites associated with conserved C/EBP sites, although in Egfr a PU.1 site is found in proximity to the C/EBP site.

PU.1 and Sox5 are known C/EBP binding partners and while PU.1 physically interacts with C/EBPs to control myeloid differentiation (Cai et al., 2008), Sox5 functions in concert with C/EBPs to regulate cartilage specific genes (Davies et al., 2007). PU.1 is a member of the E26

transformation specific (ETS) family, which has essential roles in neurogenesis (Saino-Saito et al., 2007; Tuoc and Stoykova, 2008). Sox5 is a member of the Sry-box gene family and in the developing nervous system controls the generation of corticofugal neuron subtypes (Lai et al., 2008) and the production of oligodendrocytes (Stolt et al., 2006). Although the validation of PU.1 and Sox5 as combinatorial binding partners for C/EBP awaits analysis of the developing brain, the identification of transcription factors as C/EBP target genes, including vestigial like 4 (Chen et al., 2004); transcription factor 12 (Uittenbogaard and Chiaramello, 1999; Uittenbogaard et al., 2003); Gli-Kruppel family member Hkr3 (Ruiz, 1998) and Myt1 (Nielsen et al., 2004), supports the growing consensus that C/EBPs are central to transcriptional networks that are critical for neuronal development and survival (Menard et al., 2002; Marshall et al., 2003; Paquin et al., 2005; Callela et al., 2007).

Target gene expression in PC12 cells responds to dominant negative C/EBPs

The effects of C/EBPs on transcription are known to be highly context dependent, acting as enhancers or repressors of transcription depending upon the C/EBP, the target gene and the cell type. Functional analysis of the effect of endogenous C/EBP β on endogenous C/EBP β target gene transcription was assessed here using PC12 cells stably transfected to express either Liver Inhibitory Protein (LIP) or 4H-CEBP, two dominant negative (DN) forms of C/EBP with very different modes of action. LIP is an endogenously produced N-terminal truncated form of C/EBP β that contains the bZIP domain but not the N-terminal activation domain. Homodimers of LIP thus bind DNA without activation while heterodimers of LIP with C/EBP β or some other bZIP transcription factor will bind DNA but have reduced activation potential (Descombes and Schibler, 1991). In contrast, 4H-CEBP does not bind DNA but instead prevents DNA binding by dimerizing with the basic leucine zipper and DNA binding domains of C/EBP α , β , δ , ϵ , and γ but not with unrelated bZIP proteins (Olive et al., 1997). Although the DN action of 4H-CEBP does not therefore discriminate between C/EBPs, in PC12 cells 4H-CEBP is specific to C/EBP β because these cells only express C/EBP β . Western blot analysis showed that these transgenic PC12 cell lines contain high levels of LIP or 4H-CEBP proteins (Fig 6A). Basal levels of mRNAs encoding for the target genes GCH1, App, dynamin 3 (Dnm3), Egfr and Vps4a were analyzed in these cells by real time QRT-PCR and normalized to β -actin, which was unaffected by over-expression LIP or 4H-CEBP. Figure 6B shows that each of the five target genes is transcribed in PC12 cells and that transcripts encoding for four target genes are significantly increased by expression of either one or both DN. The effect of both DN was greatest for GCH1 (5P) and Dnm3 (IN), indicating no clear relationship between DN action and the genomic location of C/EBP binding sites. App transcript levels were unaltered by expression of either DN. While the functional effects of these DN on this subset of target genes could be mediated indirectly or through C/EBP binding sites other than those reported here, they strongly suggest that in PC12 cells C/EBP β normally functions to inhibit basal transcription of some target genes. This conclusion is supported by our recent report that over-expression of C/EBP β in PC12 cells inhibits GCH1 transcription by displacing CREB from a CRE-C/EBP composite element (Kapatos et al., 2007) and by our analysis of the hippocampal transcriptome in C/EBP β knock out mice (see below).

Identification of C/EBP target genes in the rat brain

C/EBP and target gene expression is heterogeneous across brain regions and cell types (Lein et al., 2007). Moreover, the brain contains C/EBP α , β and δ as well as lesser amounts of C/EBP ϵ , γ and ζ . The first step in target gene identification was therefore to find antibodies that specifically recognize the three most abundant C/EBPs in the brain. This was accomplished by screening antibodies using Western blotting against a panel of whole cell extracts prepared from PC12 cells transiently transfected to over-express C/EBP α , β or δ . These studies showed quite clearly that the three antibodies selected by this screen, including the C/EBP β antibody used here for SACO, specifically recognize their C/EBP targets even when off-target C/EBPs

are present in excess (Fig. 7A). The second stage of the screening process involved ChIP assays performed on similarly transfected PC12 cells using GCH1 as the endogenous reporting target gene. These experiments showed that these antibodies retain their reactivity and specificity despite cross-linking of their respective antigens and the generally harsh conditions of the ChIP assay (Fig. 7B).

Based upon these encouraging results, we proceeded with ChIP experiments using antibodies to C/EBP α , β and δ and whole rat brain chromatin that had been cross-linked *in situ*. Whole brain was chosen to eliminate anatomical variation in C/EBP and target gene expression. The target genes c-fos, Drp, App, Dnm3, Egfr, Oprl1 and Scna were selected based upon prior validation in PC12 cells and their 5P and IN C/EBP binding sites. Figure 8A shows that six of these seven genes are targets for C/EBP α , with Drp the preferred target and only Oprl1 failing to reach the 2-fold cut-off for enrichment. In contrast, all seven target genes were bound by C/EBP β , with Egfr the preferred target (Fig. 8B). Of these three C/EBPs, C/EBP δ was the most discriminating towards these target genes, with c-fos, Dnm3 and Scna each falling below the 2-fold cut-off and Drp, Egfr and Oprl1 preferred equally as targets (Fig. 8C).

The results of these experiments are summarized in tabular form in Figure 8D. Of the seven target genes analyzed App, Egfr and Drp were bound by all three C/EBPs. The remaining four targets, c-fos, Dnm3, Oprl1 and Scna were all found to recruit C/EBP β and either C/EBP α or δ . These results verify this subset of genes as C/EBP targets in the brain and confirm that these three C/EBPs are recruited to 5P and IN sites originally identified by SACO in PC12 cells. While these results replicate observations that the c-fos proximal promoter is a target for C/EBP α and β (Calella et al., 2007), c-fos did not bind C/EBP δ . This selectivity and that of the other target genes most likely results from cellular heterogeneity in C/EBP and target gene expression, with positive enrichment indicating co-expression and no enrichment expression in different cells. The observation that most target genes bind multiple C/EBPs may be related to heterodimerization of C/EBP family members, a possibility that must await detailed maps of target gene and C/EBP expression in the brain. Nonetheless, the fact that most target genes identified here bind multiple C/EBPs certainly suggests differential regulation based upon the unique transcriptional or signaling properties of each C/EBP. It is possible that target genes negative for C/EBP α or δ are bound by one of the less abundant C/EBPs. Similarly, it is probable that different patterns of C/EBP and target gene association would be observed if brain regions were analyzed. It is also likely that additional C/EBP target genes will be identified in the brain using massive parallel DNA sequencing of ChIP-derived DNA (Johnson et al., 2007; Robertson et al., 2007; Barski et al., 2007; Mikkelsen et al., 2007). Finally, although no attempt was made here to modify C/EBP activity in the brain prior to ChIP analysis, based upon numerous studies we predict that activity-dependent changes in brain C/EBP expression will be found to modify C/EBP target gene selection (Taubenfeld et al., 2001a; Taubenfeld et al., 2001b; Menard et al., 2002; Marshal et al., 2003; Calella et al., 2007).

The hippocampal transcriptome in C/EBP β $-/-$ mice

As a functional test of target gene regulation by C/EBP β in the brain we next performed a genome wide comparison of gene expression in the hippocampus of wild type and C/EBP β $-/-$ mice. RNA was extracted from five same sex animals of each genotype and pooled to reduce biological variability. To minimize technical variability three separate RNA labeling reactions were produced for each genotype and hybridized to six Affymetrix 430 2.0 mouse microarrays containing over 39,000 gene transcripts (Affymetrix, Santa Clara, CA, USA). Microarray data were exhaustively analyzed by comparing genotype data files using a false discovery rate of $p \leq 0.02$ and a fold-change cut-off of 1.25 (Genomatix ChipInspector). Transcripts found to differ significantly in abundance between wild type and C/EBP β $-/-$ animals were cross-referenced to the list of 60 SACO target genes expressed in the brain. This analysis revealed

that 57% of the C/EBP β target genes discovered by SACO differ significantly between these two genotypes (Table 2). Of the 34 SACO target genes identified by microarray analysis only Aldh1a2 (the mouse homologue of rat target gene Aldh1a4) and Sv2b were decreased in C/EBP β ^{-/-} animals. In contrast, increased levels of expression were observed in C/EBP β ^{-/-} animals for the remaining 32 target genes, including Abi1, Cd47, Dnm3, Egfr, Myt1, Oprl1, Scna, Synpr and Vgl4. No difference was observed for App expression. Differences in Aldh1a2, Sv2b, Dnm3, Egfr, Oprl1 and Scna transcript abundance across genotypes were subsequently tested and validated by QRT-PCR using as template the individual RNA samples from which the RNA pools were produced for microarray analysis (data not shown). The data compiled in Table 2 show increased levels of expression for 94% of C/EBP β target genes in the hippocampus of C/EBP β ^{-/-} mice. These results are similar to the effects of DN forms of C/EBP we observed on target gene expression in PC12 cells, including unchanged App expression. The relatively small but significant increases in target gene transcripts and the results of our ChIP experiments on brain suggest that C/EBP α and δ may have assumed some of the functions of C/EBP β in C/EBP β ^{-/-} mice. Interestingly, in contrast to the results reported here an overall decline in C/EBP β target gene expression occurs in the liver of C/EBP β ^{-/-} mice (Friedman et al., 2004), suggesting tissue specific differences in the regulation of target gene transcription by C/EBP β .

In summary, this communication provides a set of novel C/EBP β target genes for further study. Functional annotation reveals that 74% of these genes are involved in cell signaling, metabolism and transport. C/EBP binding sites are overwhelmingly located within the introns of target genes and have DNA sequences somewhat different from the consensual C/EBP binding site. C/EBP binding sites in neural target genes are associated with binding sites for PU.1 and Sox5, transcription factors that interact with C/EBPs and are involved in differentiation and development of the nervous system. A subset of target genes was validated in rat brain and found to differentially recruit C/EBP α , β or δ . Analysis of the hippocampal transcriptome of C/EBP β knockout mice revealed changes in a significant percentage of transcripts previously identified as C/EBP target genes. These results support the hypothesis that C/EBP α , β and δ play non-redundant roles in transcriptional programs important to normal brain function.

Experimental Methods

Antibodies and plasmids

Rabbit polyclonal antibodies directed against C/EBP α (SC61), C/EBP β (SC150), C/EBP δ (SC636), and Green Fluorescent Protein (GFP, SC8334) were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. Antibodies to FLAG and β -actin were obtained from Sigma-Aldrich (ST. Louis, MO). Dr. Peter Johnson, NCI, NIH supplied C/EBP α , β and δ in the pMEX vector. The coding regions of these C/EBPs were cloned by PCR into the pRc/RSV vector. pRc-RSV-LIP was constructed by PCR using pRc/RSV-C/EBP β as the template. The FLAG-tagged A-ZIP dominant negative pCMV500-4H-C/EBP was obtained from Dr. C. Vinson, NCI, NIH, and cloned by PCR into pRc/RSV. The identity of all PCR products was confirmed by DNA sequencing. All PCR primer sequences used for sub-cloning are available upon request. Plasmid DNA used for transfections was purified by ion-exchange chromatography (Qiagen).

Cell culture and transfection

PC12 cells were passaged on collagen in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 5% fetal calf serum, 10% horse serum, 100 μ g/mL penicillin and 100 μ g/mL streptomycin. Cultures were maintained in a humidified atmosphere of 10% CO₂ at 37°C. For production of the SACO library and for validation of SACO target

genes 20 million PC12 cells were plated on poly-D-lysine coated 100 mm dishes in low-serum medium (0.5% fetal calf serum and 1% horse serum) and treated the next day for 4 hours with 5 mmol/L 8Br-cAMP dissolved in low-serum growth-conditioned medium. For transient transfections, cells plated on poly-D-lysine in antibiotic-free low-serum medium were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) and harvested 48 h later. For stable transfections cells plated on collagen were transfected with pRc/RSV vector, pRc/RSV-4H-C/EBP or pRc/RSV-LIP that had been linearized by digestion with *ScaI*. Transfected cells were selected in growth medium containing 1mg/ml of G418. PC12 cells that had stably incorporated transgenes were maintained under selective pressure in low-serum medium and harvested for protein and mRNA analysis.

Animal studies

All animal handling procedures were approved by the Wayne State University School of Medicine Animal Investigation Committee. Post-natal day 22 male rats were anesthetized and protein and DNA cross-linked *in situ* by cardiac perfusion at 40 ml/min with 200 ml of 1% formaldehyde dissolved in PBS followed by 200 ml of PBS containing 0.125 M glycine. Whole brains were rapidly removed, minced on ice and nuclei isolated following the protocol described below. Wild type and C/EBP β ^{-/-} mice were genotyped at birth (Screpanti, et al., 1995). Adult male wild type and C/EBP β ^{-/-} mice were perfused as described above for rats and liver was harvested for chromatin isolation. For microarray analysis five, adult male wild type and five, adult male C/EBP β ^{-/-} mice were anesthetized, brains rapidly removed and placed on ice and the hippocampus dissected under microscopic control.

Preparation of nuclei

All procedures were performed at 4°C in the presence of protease inhibitors. PC12 cells were scraped into PBS, collected by centrifugation at 200 g for 10 minutes, washed once with PBS followed by a wash with NP-40 Lysis Buffer (NB; 20 mM HEPES, 0.5% NP-40, 10mM NaCl and 3mM MgCl₂) and centrifuged at 200 g for 5 minutes. Cell pellets suspended in NB were incubated for 30 min on ice, homogenized using a Teflon pestle and the liberation of nuclei monitored by DAPI staining and fluorescence microscopy. Minced cross-linked rat brain or mouse liver was homogenized 1:10 (w:v) in NB and processed as described for PC12 cells. Nuclei from PC12 cells, mouse liver or rat brain were layered over 30% sucrose in NB and spun at 800 g for 10 minutes. Following a wash step, PC12 nuclear pellets were cross-linked with 1% fresh formaldehyde in PBS for 15 minutes at room temperature (RT). Cross-linking was stopped by addition of 100 mM Tris-HCl (pH 9.4) and 10 mM DTT at RT for 10 minutes and centrifugation at 6500 g for 15 minutes. PC12 cell, mouse liver and rat brain nuclear pellets were suspended in lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.0) containing protease inhibitors, incubated for 10 minutes, sonicated and then snap frozen at -80°C.

Chromatin immunoprecipitation

ChIP was performed as we have described previously except that isolated nuclei were used as the starting material (West et al., 2004; Kapatos, et al., 2007; Chandran et al., 2008). Unless noted all procedures were at 4°C in the presence of protease inhibitors. Sample DNA was fragmented by sonication to an average size of 400 bp, cleared of debris by centrifugation at 20,000 g and the supernatant harvested. Samples containing 25 ug of DNA were incubated overnight with 5 µg of antibody directed against C/EBP α , β , δ or GFP. After reversing cross-links and digestion of RNA and protein, immunoprecipitated DNA was isolated using the QIAquick PCR Purification system (Qiagen). Input DNA was treated identically. To determine fold enrichment each sample was analyzed by real time quantitative PCR (QPCR) (Roche LightCycler, Mannheim, Germany) using QuantiTect SYBR-Green PCR reagent (Qiagen) and

gene-specific primers (see S3 supplementary materials). Cycling parameters for all primer pairs included a hot start at 95°C for 900 s then 40 cycles of 94°C for 15 s at 20°C/s, 55°C for 25 s at 20°C/s and 72°C for 20 s at 2°C/s with a single acquisition mode. Melting curve analysis was always performed to insure amplicon homogeneity: 95°C for 5 s at 20°C/s, 65°C for 15 s at 20°C/s and 95°C for 0 s at 0.1°C/s with continuous acquisition. All samples were run in triplicate. Standard curves for each PCR product were generated by plotting *Ct* versus serial dilutions of input DNA. Sample values were calculated relative to this standard curve and fold enrichment was calculated by dividing C/EBP sample values by the average GFP sample value.

Serial Analysis of Chromatin Occupancy

SACO was performed as described by Impey (Impey et al., 2004), with minor modifications to increase ditag recovery. ChIP DNA was made blunt (DNA Terminator ER, Lucigen), adaptors (P-Cassette) were ligated and the product purified using the QIAquick PCR purification system (Qiagen). Adaptor-ligated DNA was amplified in 25 cycle PCR reactions using biotinylated primer p-Oligo. 10 µg of biotinylated product was digested with *Nla*III. *Nla*III-digested DNA was ligated to either Long-SAGE adaptor A or B and the products bound to streptavidin magnetic beads. These ligation products were cleaved with the type II restriction enzyme *Mme*I. Equal amounts of adaptor A or B containing products were ligated and amplified by PCR using ditag primers 1 and 2. PCR products were digested with proteinase K and phenol-extracted, a clean up we found to increase the efficiency of *Nla*III digestion, and then digested overnight with *Nla*III (30 U of enzyme/µg of DNA). The resulting 38 bp ditags were purified away from the *Mme*I adapters using streptavidin magnetic beads and purified further on 4% agarose gels. Ditags were isolated by electro-elution, which increased ditag recovery 3-fold over purification by polyacrylamide gel electrophoresis, and then concentrated by ethanol precipitation. Purified ditags were concatemerized in multiple reactions, each containing 1 µg of DNA. Concatemerized ditags were partially digested with 1 U of *Nla*III enzyme for 1 minute at 37°C, which increased subsequent concatemer cloning efficiency. Concatemers were gel extracted, cloned into *Sph*I cut pZero1 vector and transformed in TOP10 chemically competent *E. coli* cells. More than 400 colonies were hand picked and DNA minipreps prepared using a 96-well plate format (Invitrogen). DNA content was determined using a fluorescence-based assay (PICO Green, Molecular Probes) and 600 ng of each DNA was sequenced using the T7 universal primer (DNA Polymorphic Technology, Inc.).

SACO bioinformatics, target gene validation and functional annotation

Cloned inserts from SACO were analyzed by submitting an archive file of DNA sequences in FASTA format to the Sequence Tag Analysis and Reporting Tool program (START) available online at <http://bio.chip.org/start/> (Marinescu et al., 2006). The START program identifies and extracts 17 bp tags from insert sequences at all *Nla*III CATG sites within the rat genome and then matches the resulting tag sequences to genomic CATG sites, producing 21 bp Genomic Signature Tags (GSTs). Based upon this information, MAPPER (Multi-genome Analysis of Positions and Patterns of Elements of Regulation) (Marinescu et al., 2005) was used to identify putative C/EBP site(s) located within ± 700 bp of the tag, a distance determined by the size distribution of fragmented DNA used for ChIP. MAPPER uses binding site information obtained from TRANSFAC and JASPAR and has a lower false discovery rate than other programs designed to identify transcription factor binding sites. Individual ChIP assays were performed to confirm some of the C/EBP β target genes identified by SACO. Primers were designed by DS Gene software (Accelrys, Inc. San Diego, CA. USA) to include the C/EBP site identified by MAPPER and had the following parameters: 18–27 bases, product 100–200 bp and product Tm 66°C–78°C. In some case multiple primers were designed for loci containing more than one C/EBP site. All target gene primer sequences can be found in supplemental materials (S3). The association of validated target gene C/EBP sites with other transcription factor binding sites was investigated by first identifying target genes expressed

in the brain using Genomatix Biblisphere, the Allen Brain Atlas and then using Genomatix MatInspector to search for transcription factor binding sites located within 250 bp of the identified C/EBP binding site.

Microarray Analysis

Total RNA was isolated from the hippocampus of individual animals using the RNeasy system and treated with DNase I according to the manufacturer's instructions (Qiagen). The concentration and purity of RNA was determined spectrophotometrically (Ab260nm/Ab280nm = 2.0 – 2.2) and equal amounts were pooled for each genotype. Microarray analysis was performed by the NIH Neuroscience Microarray Consortium (UCLA DNA Microarray Core, Los Angeles, CA). The quality of the pooled RNA quality was determined using the Agilent Bioanalyzer system. Three separate labeling reactions were produced from the pooled RNA of each genotype and each labeling reaction was hybridized to a separate Affymetrix Mouse Genome 430 2.0 microarray. Six Affymetrix CEL files were imported for analysis into the Genomatix ChipInspector program (Genomatix GmbH, Munich, Germany), classified according to genotype and evaluated for legibility and average expression level. Raw data from single probes were then assigned to individual transcripts by mapping each probe sequence to the most current version of the mouse genome. Only probes mapping perfectly and uniquely were considered for further analysis. An exhaustive statistical analysis was performed in which nine comparisons were made across genotypes (SAM-Significance Analysis of Microarray with one class comparison). A False Discovery Rate (FDR) of $p \leq 0.02$, a transcript coverage of ≥ 3 probes and a cut-off log2 fold change of 0.2 (fold change of 1.25) were used to derive statistically significant differentially expressed transcripts.

Reverse Transcription and Quantitative real time PCR analysis

Total RNA was isolated using the RNeasy system and then treated with DNase I according to the manufacturer's instructions (Qiagen). 1 to 20 ng of total RNA from cultured cells or mouse brain was reverse transcribed with the Sensiscript system (Qiagen) using random primers (Promega). 10–20% of this reaction served as template for quantitative real-time RT-PCR (QRT-PCR) analysis using QuantiTect SYBR-Green PCR reagents (Qiagen). All PCR primer sequences are available in supplementary materials (S2). Control reactions minus reverse transcriptase were included for each primer pair and quantity of RNA assayed. C/EBP α , β and δ amplicons were identified by TA-cloning (pGem-T Easy, Promega) and DNA sequencing. Standard curves for each transcript were generated in distinct reverse transcription reactions using serial dilutions of pooled RNA. Target mRNA abundance was divided by β -actin mRNA abundance and expressed as relative mRNA.

Electrophoretic mobility shift assay

EMSA was performed as previously described (Kapatos et al., 2000). Briefly, 22 nt single-stranded complementary oligonucleotides containing putative target gene C/EBP *cis*-elements were annealed, end-labeled with γ -³²P ATP (3,000 Ci/mmol) and T4 polynucleotide kinase and purified using G25 spin columns. To a reaction buffer containing 12.5 mM HEPES-KOH, pH 7.9, 10% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μ g of poly(dI-dC) and 5 μ g of acetylated bovine serum albumin was added 7 ng of C/EBP β recombinant protein (GST-fusion protein containing amino acids 256–345 of the C/EBP β bZIP domain), 50,000 cpm of end-labeled probe and where noted 5X, 25X and 100X of unlabeled competitor. 20 μ l samples were incubated for 20 min at room temperature and 10 μ l was loaded on a 6% acrylamide DNA gel with 0.5 X Tris borate EDTA as the running buffer. Gels were dried, exposed to phosphorimager screens and scanned at 100 μ resolution (Typhoon, GE Healthcare, Piscataway, NJ, USA).

Western blot analysis

5–20 µg of PC12 cell protein extract was analyzed using 4%–12% Tris-Bis polyacrylamide gels (Invitrogen) and antibodies directed against C/EBP α , C/EBP β , C/EBP δ , β -actin, FLAG or GFP at a 1:1000 dilution. A chemiluminescence-based secondary antibody conjugated peroxidase reaction was performed and detected by X-ray film.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

C/EBPβ is expressed and induced by cAMP in PC12 cells. **A)** Real time QRT-PCR analysis of growth arrested PC12 cells showed C/EBPβ and δ mRNAs to be expressed at equivalent amounts. C/EBPα, γ and ε mRNAs were below the limit of assay detection defined by the no reverse transcription control. A 4-hour incubation with 8Br-cAMP increased C/EBPβ mRNA levels by 6-fold while levels of C/EBPδ mRNA were not significantly changed. **B)** Western blot analysis of whole PC12 cell extracts showed a low basal level of expression of the 35kDa isoform of C/EBPβ which, along with the less abundant 38kDa isoform, was increased 5-fold by a 4 hour treatment with 8Br- cAMP. C/EBPδ protein was not detected in control or 8Br-cAMP-treated cells. As a loading control, the blot was probed with an antibody directed against β-actin.

The C/EBPβ antibody was characterized in ChIP assays prior to use in SACO. **(C)** ChIP analysis of 8Br- cAMP-treated PC12 cells using an affinity purified rabbit polyclonal C/EBPβ antibody produced a 120-fold enrichment for the known C/EBPβ target gene GTP Cyclohydrolase I (GCH1) and less than a 2-fold enrichment for the house keeping gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH). **D)** ChIP of mouse liver chromatin cross-linked *in situ* showed enrichment for the C/EBPβ target gene phosphoenolpyruvate carboxykinase (Pepck) reduced from 45-fold in wild type animals to less than 2-fold in C/EBPβ^{-/-} animals. ChIP using an antibody to C/EBPα produced no difference between these two genotypes.

Figure 2.

Gene specific tags (GSTs) derived from SACO were used to identify C/EBP β target genes and localize C/EBP β binding sites. **A)** Schematic diagram illustrating the locations of hypothetical GSTs relative to the transcription start site, introns and exons of a hypothetical gene. 5P corresponds to regions up to 5 kb upstream of the transcription start site, IN corresponds to exons and introns and 3P corresponds to regions up to 2kb downstream from the most 3' exon. **B)** A pie chart illustrating the genomic locations of 1492 GSTs in relation to known target gene transcript start sites. 73% of GSTs are located in IN regions followed by 17% in 5P regions and 10% in 3P regions. Sixty-seven percent of GSTs were located in introns, with 20% in intron 1. **C)** Example of three target genes showing GSTs and their corresponding C/EBP β binding sites located 5P (synaptic vesicle protein 2b), IN (opioid receptor-like 1) and 3P (vacuolar protein sorting 4a). Transcriptional start sites are indicated by arrows and labeled +1. Vertical red bars represent the number of GSTs at any given location and numbers below these bars indicate position of the GST relative to +1. The sequences and locations of C/EBP binding sites falling within 700 bp of a GST were identified by MAPPER. **D)** Conservation in rat, mouse and human of the intergenic C/EBP β binding sites located in intron 17 of Dnm3 and intron 1 of Egfr.

Figure 3.

Functional annotation of 115 C/EBPβ target genes presented in pie chart format shows that 74% of target genes are dedicated to signaling, metabolism, and transport, with the remaining 26% having roles in transcription, synaptic transmission, differentiation and proliferation. Included in signaling and transport categories are multiple members of two gene families, the olfactory receptor genes and solute carrier genes. Fifty-four percent of these target genes are expressed in the brain, including GABA_A receptor subunit beta, myelin transcription factor 1 involved in oligodendrocyte development, the clinically important alpha-synuclein and amyloid precursor protein beta, the calcium-dependent α -latrotoxin receptor neurexin 1, the nociceptin receptor opioid receptor like- 1, the signaling protein phospholipase C beta, the synaptic vesicle protein 2b and the netrin- 1 receptor Unc5 involved in axon guidance. **B)** Pathway analysis of C/EBPβ and its target genes was performed using a web- based entry tool, the Ingenuity Pathway Analysis (IPA) Network Generation. The central role played by C/EBPβ in one signaling network shows the interaction of C/EBPβ with 23 proteins. C/EBPβ and the transcription factors CREB and NFκB are found at the center of this network and are surrounded by the protein kinases PI3K, Akt, Lyn and Cdc42 as well as proteins that signal to these kinases, including calmodulin, the small GTPases Ras and Rac and the guanine nucleotide exchange factor Vav. Acting on these pathways are the first messengers Pdgf, Egfr, thrombospondin (Cd47), growth hormone releasing hormone, netrin- 1 and glutamate (Grm7). Based upon SACO analysis, the direct transcriptional control of 15 of these proteins by C/EBPβ has been added to this network. The addition of these transcriptional control points results in a network where multiple signaling pathways modify C/EBPβ function, either directly by

phosphorylation or indirectly through CREB and NFkB effects on C/EBP β gene expression, while at the same time C/EBP β feeds back to regulate expression of these same signaling proteins. Abbreviations: ABI1, abl-interactor 1, Akt, thymoma viral proto- oncogene, BRAC1, breast cancer 1, CABIN1, calcineurin binding protein 1, Calmodulin, CD47, CD47 antigen (Rh- related antigen, integrin-associated signal transducer), CDC42, cell division cycle 42 homolog, C/EBPB, CCAAT/enhancer binding protein beta, CREB, cAMP responsive element binding protein, EGFR, epidermal growth factor receptor, FBNP1, formin binding protein 1, GHRHR, growth hormone releasing hormone receptor, GRM7, glutamate receptor, metabotropic 7, LYN, Yamaguchi sarcoma viral (v-yes- 1) oncogene homolog, MSH2, mutS homolog 2, NFkB, nuclear factor kappa-B, PDGF, platelet- derived growth factor beta, Ras, pleckstrin homology, PI3K, phosphoinositide- 3-kinase, PSCD3, Sec7 and coiled-coil domains 3, Rac, ras-related C3 botulinum toxin substrate, UBB, Ubiquitin B, UNC5B, unc-5 homolog B, VAV, vav 1 oncogene.

Figure 4.

Validation of C/EBPβ target genes in PC12 cells. **A)** Schematic diagram illustrating how a subset of target genes were selected for validation based upon the 5P, IN or 3P locations of C/EBPβ binding sites. **B)** Individual ChIP assays on cAMP-treated PC12 cells were used as the validation tool and genes selected for validation included those with tags repeated at least three times or with multiple tags. A locus was considered positive for C/EBPβ if enrichment was two-fold or greater (see dotted line). Ninety-one percent of 22 C/EBPβ target genes were validated, a subset of which are presented as examples of genes with GSTs located in 5P, IN or 3P regions. **C)** Eighty-nine percent of one tag genes were confirmed as target genes.

Abbreviations: GCH1, GTP cyclohydrolase I; c-fos, synaptic vesicle protein 2b; App, amyloid beta precursor protein; Dnm3, dynamin 3; Egfr, epidermal growth factor; Grm7, metabotropic glutamate receptor 7; Tmem12, transmembrane protein 12; Trim9, tripartite motif 9; Vps4a, vacuolar protein sorting 4a; Cry2, cryptochrome 2; Drp, dopamine responsive protein; Krt2-8, keratin complex 2 basic gene 8; Olf204, olfactory receptor Olf204; Aldh14a, aldehyde dehydrogenase family 1, subfamily A4; Rims4, regulating synaptic membrane exocytosis 4; Scna, alpha-synuclein; Sdfr1, stromal cell derived factor receptor 1; Kcnk10; potassium channel, subfamily K, member 10.

Figure 5. Computational and experimental analysis of C/EBPβ binding sites in validated target genes. **A)** (Top panel) WebLogo position weight matrix of the consensus C/EBPβ binding motif. (Bottom panel) WebLogo position weight matrix of the C/EBPβ binding motif derived from the C/EBPβ binding sites of 28 validated target genes. Note the differences from the consensus at nucleotide positions 2, 3 and 4. **B)** Atypical C/EBPβ binding sites (underlined) and flanking sequence of a subset of C/EBPβ target genes. Note that the Drp probe contains two overlapping C/EBP sites labeled site 1 and 2. Also shown is the C/EBPβ binding site for the known target gene GCH1 (+ GCH1) and the GCH1 CCAAT box (– GCH1), which does not bind C/EBPs. **C)** EMSA combining ³²P-labeled double stranded oligonucleotides of the sequences shown in 5B and recombinant C/EBPβ protein showed that each of these atypical C/EBP elements specifically binds homodimers of C/EBPβ, as evidenced by competition with a 100X molar excess of each respective unlabeled probe and the complete absence of C/EBPβ binding to a negative control containing the GCH1 CCAAT-box. **D)** Competition of C/EBPβ binding to ³²P-labeled Drp probe by 5X, 25X and 100X molar excess of unlabeled Drp probe, unlabeled probe containing a consensual C/EBP site (CGACTCCCTGATTGCGCCAATAGGCTCC; Williams et al., 1995) or unlabeled Drp probe containing either mutation (mt) 1 or mt 2 shown in Fig. 5B. The resulting patterns of competition indicate that C/EBPβ binds to Drp with a lower affinity than to the consensual site (note that competition by 25X Drp approximates 5X consensus). Moreover, whereas unlabeled Drp mt 1 does not compete and therefore does not bind C/EBPβ, binding is competed away by unlabeled Drp mt 2, indicating that site 1 in the labeled Drp probe binds C/EBP. Free probe is identified with an arrow on the lower left. Abbreviations: Drp, dopamine responsive protein; GCH1, GTP cyclohydrolase I; Sv2b, synaptic vesicle glycoprotein 2b; Opr1, opioid receptor like 1; Vps4a, vacuolar protein sorting 4a.

Figure 6.

Endogenous target gene expression in PC12 cells is enhanced by two dominant negatives to C/EBP β . (A) Western Blot analysis of stably transfected PC12 cells shows high levels of expression of the C/EBP β dominant negative proteins LIP and 4H-C/EBP. LIP was detected using anti-C/EBP β while FLAG-tagged 4H-C/EBP was detected using anti-FLAG. (B) Real-time QRT-PCR analysis shows that these five target genes are transcribed in PC12 cells and that mRNAs encoding for four target genes are significantly increased by LIP or 4H-CEBP. The level of App mRNA was unaffected by LIP or 4H-C/EBP. Abbreviations: GCH1, GTP cyclohydrolase I; App, amyloid beta precursor protein; Dnm3, dynamin 3; Egfr, epidermal growth factor receptor; Vps4a, vacuolar protein sorting 4a. * $p = 0.05$ compared to vector alone.

Figure 7.

Western blotting and ChIP demonstrate the specificity of antibodies to C/EBP α , β and δ . **(A)** Western blots of PC12 cells transiently transfected to express C/EBP α , β or δ along with GFP were probed with antibodies to C/EBP α , β or δ . This experiment showed that each antibody specifically recognizes the appropriate C/EBP. As a loading control, these same blots were stripped and probed with an antibody to GFP. **(B)** Real time QPCR of ChIP assays using PC12 cells transiently transfected to express C/EBP α , β or δ , antibodies to C/EBP α , β or δ , and GCH1 as the reporter target gene. These experiments confirm that antibody specificity is retained when C/EBP α , β and δ are cross-linked to genomic DNA.

Figure 8.

Real time QPCR analyses of ChIP samples from rat brain chromatin cross-linked *in situ* reveals varying degrees of overlap between target genes bound by C/EBP α , β or δ . c-fos, Drp, App, Dnm3, Egfr, Oprl1 and Scna were selected as target genes based upon their prior validation in PC12 cells, different genomic locations of C/EBP binding sites and, varying levels of expression in the whole brain ranging from high (App and Scna) to medium (Dnm3, Egfr and Oprl1) to low (c-fos and Drp). **A)** ChIP with anti-C/EBP α showed that six of these seven genes are targets for C/EBP α , with Drp the preferred target and only Oprl1 failing to reach the cut-off of 2-fold enrichment marked by the dotted line. **B)** ChIP with anti-C/EBP β showed that all seven target genes were bound by C/EBP β , with Egfr the preferred target. **C)** ChIP with anti-C/EBP δ showed that C/EBP δ was the most discriminating of the three C/EBPs, with c-fos, Dnm3 and Scna all falling below the 2-fold cut-off for enrichment and Drp, Egfr and Oprl1 preferred equally as targets. **D)** The results of these experiments are summarized in tabular form (+ and - refer to validated and non-validated target genes, respectively) and shows that only Drp, App and Egfr are bound by C/EBP α , β and δ . * $p = 0.05$ when compared with GFP. Abbreviations: Drp, dopamine responsive protein; App, amyloid precursor protein beta; Dnm3, dynamin 3; Egfr, epidermal growth factor receptor; Oprl1, opioid receptor like 1; Scna, alpha-synuclein.

Table 1
List of validated C/EBP β target genes; including GST sequence, number of GSTs and genomic location.

21 bp GSTs	# in Input	Region	GeneID	Gene	Product
GCGCACACAGCTGTGGT	4	EX2	56777	Adam1a	A disintegrin and metalloprotease domain 1
ATGCAGGAAAAAGGGGCT	1	IN8	29651	Aldh1a4	Aldehyde dehydrogenase family 1, subfamily A4
CAGTTTGGTGCTCAG	4	IN5	54226	App	Amyloid beta (A4) precursor protein
CCTCGAAGTTGTAGTGG	1	5P	171057	Cbwd1	Dopamine-responsive protein/COBW domain containing 1
ACAGGCTCCTTCACACA	2	IN2	29364	Cd47	CD47 antigen (Rh-related antigen,
GAAGCCCCATCTTACAC	1	IN4	84683	Cox4i2	Cytochrome c oxidase subunit IV isoform 2
GTATTGGCGCAAGCCATT	1	5P	170917	Cry2	Cryptochrome 2 (photolyase-like)
CTAATATAGGTCAAAGGA	2	3P	286963	Cyp2d10	Cytochrome P450, family 2, subfamily d 10
CGTGGACATCCAGAACCC	8	IN17	171574	Dmn3	testicular dynamin
TTCCCTTTCTGCCCCCT	19	IN1	24329	Egfr	Epidermal growth factor receptor
GCGTGTGCTTTGGTCAA	4	IN3	315034	Fbxo43	F-box protein 43 (predicted)
TACTTCTTGGTCCGTCC	1	3P	65272	Kenk10	Potassium channel, subfamily K, member 10
GGGCCAGTTGGGAGCCG	1	5P	25626	Krt2-8	keratin complex 2, basic, gene 8
TGGTGGAAGAAGATGCC	8	IN3	59106	Lbp2	Latent transforming growth factor beta binding
GCATTGATGAGCTGTAC	1	5P	300529	Olr1204	Olfactory receptor Olr1204
TATGATCTCTACAGGAA	24	5P	406024	Olr1347	Olfactory receptor Olr1347
GGTTTGACTTGGGTGGA	14	EX1	405221	Olr1598	Olfactory receptor Olr1598
CTGTCACCCTGCAAAGC	11	EX2	29256	Oprl1	Opioid receptor-like1
AATAGACTCTGCTCTGT	12	IN1	84587	Plcl1	130kDa-Ins(1,4,5)P3 binding protein
TGTTGTGATGGGTGTA	11	IN1	29340	Prkce	Protein kinase C, epsilon
AGGGTGACTTCTAACAG	3	IN25	60357	Prom1	Prominin 1
GTGGCTCCGGTGTTCCT	1	IN1	266976	Rims4	Regulating synaptic membrane exocytosis 4
GAGTGTGGCAATTATCT	1	IN4	29219	Suca	Synuclein, alpha
AAAGCTGCTGATGTCAG	8	5P	117556	Sv2b	Synaptic vesicle glycoprotein 2b
TGGCCTGTTTGGTTGC	1	IN2	66030	Synpr	Synaptoporin
ACTGTGAACAGGAGGG	1	3P	309082	Tmem12	Transmembrane protein 12
TGCCCCAGGGGCAGGCA	4	IN1	83529	Vdac1	Voltage-dependent anion channel 1
GTGCAAGGAAACGCCGC	17	3P	246772	Vps4a	Vacuolar protein sorting 4a

Abbreviations; EX: exon, IN: intron, 5P: 5' region, 3P: 3' region.

Table 2
List of C/EBP β target genes identified by both microarray and SACO analysis

Gene ID	Gene Symbol	Product	Fold change (FDR <0.02)
Downregulated Genes			
19378	Aldh1a2 [*]	aldehyde dehydrogenase family 1, subfamily A2	-1.73
64176	Sv2b	synaptic vesicle glycoprotein 2b	-2.97
Upregulated Genes			
67470	Abcg8	ATP -binding cassette, sub-family G	1.34
11308	Abi1	abl-interactor 1	1.49
280668	Adam1a	A disintegrin and metalloprotease domain 1a	1.23
11534	Adk	Adenosine Kinase	1.27
11975	Atp6v0a1	ATPase, H [*] transporting, lysosomal V0 subunit A1	1.23
16423	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	1.32
13030	Ctsb	cathepsin B	1.31
73649	Cybrd1	cytochrome b reductase 1	1.34
103967	Dnm3	dynamin 3	1.26
13528	Dtnb	dystrobrevin, beta	1.25
13649	Egfr	epidermal growth factor receptor	1.25
14205	Figf	c-fos induced growth Factor	1.60
71313	Fsip1	fibrous sheath-interacting protein 1	1.26
72258	Kcnk10	potassium channel, subfamily K, member 10	1.28
16562	Kif1c	kinesin family member 1C	1.31
214384	Myocd	myocardin	1.28
17933	Myt1l	myelin transcription factor 1-like	1.42
20320	Nptn	neuroplastin	1.43
18189	Nrxn1	Neurexin 1	1.25
18389	Opr1l	opioid receptor-like 1	1.30
19302	Pex2	peroxisome biogenesis factor 2	1.30
19229	Ptk2b	PTK2 protein tyrosine kinase 2 beta	1.26
24053	Sgcg	sarcoglycan, gamma (dystrophin-associated glycoprotein)	1.32
13487	Slc26a3	solute carrier family 26, member 3	1.25
105243	Slc9a3	solute carrier family 9 (sodium/hydrogen exchanger), member 3	1.27
24059	Slco2a1	solute carrier organic anion transporter family, member 2a1	1.25
20617	Snca [*]	synuclein, alpha	1.34
58504	Stk23	serine/arginine-rich protein specific kinase 3	1.26
72003	Synpr	synaptoporin	1.46
212070	Tmem12	clarin 3	1.26
94090	Trim9	tripartite motif-containing 9	1.49
232334	Vgll4	vestigial like 4 (Drosophila)	1.30

* denotes known C/EBP β target genes