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Human CCAAT/Enhancer-Binding Protein β Interacts with Chromatin Remodeling Complexes of the Imitation Switch Subfamily

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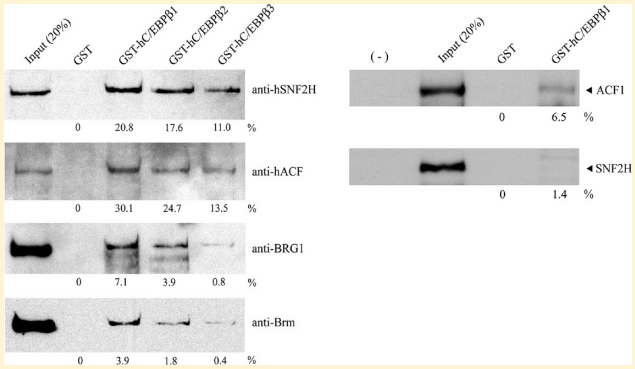
Supporting Information

ABSTRACT: Transcription factor C/EBPβ is involved in several cellular processes, such as proliferation, differentiation, and energy metabolism. This factor exerts its activity through recruitment of different proteins or protein complexes, including the ATP-dependent chromatin remodeling complex SWI/SNF. The C/EBPβ protein is found as three major isoforms, C/EBPβ1, -2, and -3. They are generated by translation at alternative AUG initiation codons of a unique mRNA, C/EBPβ1 being the full-length isoform. It has been found that C/EBPβ1 participates in terminal differentiation processes. Conversely, C/EBPβ2 and -3 promote cell proliferation and are involved in malignant progression in a number of tissues. The mechanisms by which C/EBPβ2 and -3 promote cell proliferation and tumor progression are not fully understood. In this work, we sought to identify proteins interacting with hC/EBPβ using a proteomics approach. We found that all three isoforms interact with hSNF2H and hACF, components of ACF and CHRAC chromatin remodeling complexes, which belong to the imitation switch subfamily. Additional protein−protein interaction studies confirmed this finding and also showed that hC/EBPβ directly interacts with hACF1. By overexpressing hC/EBPβ, hSNF2H, and hACF1 in HepG2 cells and analyzing variations in expression of cyclin D1 and other C/EBPβ target genes, we observed a functional interaction between C/EBPβ and SNF2H/ACF1, characterized mainly by suppression of C/EBPβ transactivation activity in the presence of SNF2H and ACF1. Consistent with these findings, induction of differentiation of HepG2 cells by 1% DMSO was accompanied by a reduction in the level of cyclin D1 expression and the appearance of hC/EBPβ, hSNF2H, and hACF1 on the promoter region of this gene.



Regulation of gene expression at the level of transcription involves the action of several proteins and protein complexes, which coordinately stimulate or repress specific genes in response to environmental stimuli. Transcription factors are some of the key elements participating in this process. These proteins are able to recruit different cofactors to particular gene regulatory regions. Among these cofactors are chromatin-modifying enzymes, which are required for chromatin remodeling events accompanying transcription activation or repression.[1] These modifiers are classified into two major groups: those that covalently modify histone proteins and those with ATP-dependent chromatin remodeling activity. The later comprise a large family of proteins and protein complexes divided into four subfamilies (SWI/SNF, ISWI, INO80, and CHD) according to particular structural motifs in their catalytic subunits.[2,3] The action of these complexes is required for processes such as transcription, DNA replication, and repair. All members of this family of chromatin modifiers are able to disrupt nucleosome structure in an ATP-dependent fashion, but the biochemical outcome of their activity differs from one subfamily to the other and even among members of a particular subfamily. Differences between the family members are also present at the level of biological function.[3,4] In humans, complexes containing BRG1 or hBRM as catalytic subunits are termed hSWI/SNF complexes and belong to the SWI/SNF subfamily. In terms of transcriptional regulation, these complexes have been observed to participate in both gene activation and repression. On the other hand, complexes of the ISWI subfamily, like ACF and CHRAC, have been mainly linked to gene repression and silencing.[2−6]

The CCAAT/enhancer-binding proteins (C/EBPs) make up a family of transcription factors composed of six members (α, β, γ, δ, ε, and ζ). C/EBPβ is encoded by an intronless gene, and

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the protein is found as three major isoforms, termed C/EBPβ1, -2, and -3 in humans (also known as LAP*, LAP, and LIP, respectively). These isoforms are generated as a consequence of translation initiation on three in-frame alternative start codons.[7,8] The transactivation domain of this protein is located in its N-terminal region. C/EBPβ1 is the full-length isoform; C/EBPβ2 is only 23 residues shorter than C/EBPβ1, while C/EBPβ3 lacks the whole transactivation domain and also regulatory domains present in the central region of the protein.[9] The control of gene expression exerted by this factor has been associated with several cellular processes, such as proliferation, differentiation, inflammation, and metabolism.[10] Depending on the cellular context, C/EBPβ isoforms have been found to act as transcriptional activators or repressors, although C/EBPβ3 has been observed to function mainly as a repressor.[8,10] In this regard, several studies have pointed out that, in the same cellular context, C/EBPβ isoforms exert differential roles in transcriptional regulation. Consistently, it has been observed that C/EBPβ1 activity is mainly linked to terminal differentiation processes, while C/EBPβ2 and -3 are involved in cell proliferation and tumor progression.[11−15]

The molecular mechanisms by which C/EBPβ influences several cell differentiation and tumor progression processes are not fully understood. Discovery of C/EBPβ-interacting proteins is crucial for improving our knowledge of these mechanisms. With this aim, we sought to identify new C/EBPβ-interacting proteins using a proteomics approach. Among the novel interactors found in this work are proteins belonging to chromatin remodeling complexes of the ISWI subfamily, including hSNF2H and hACF1. Our further studies confirmed the existence of physical interaction between hC/EBPβ and hSNF2H/hACF1, and a functional relationship of hC/EBPβ with hSNF2H and hACF1 proteins, in terms of transcriptional regulation.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies. The coding sequence for full-length human C/EBPβ was obtained by polymerase chain reaction (PCR) from human genomic DNA (Novagen, 69237), and the PCR product was inserted into the pGEX-4T1 bacterial expression vector. pGEX vectors encoding hC/EBPβ2 and -3 isoforms were obtained by PCR amplification of the corresponding DNA stretches using the pGEX-hC/EBPβ1 vector as a template, and further insertion into pGEX-4T1. For generation of C/EBPβ mammalian expression vectors, the DNA stretches corresponding to each isoform were cut from the pGEX plasmids and placed in pcDNA3.1-HisC. All plasmid constructions used in this work were confirmed by Sanger sequencing (Genytec Ltd.). Bacterial expression vectors containing coding sequences for rat C/EBPβ isoforms were a kind gift from L. Sealy (Vanderbilt University, Nashville, TN). pFastBac vectors containing the coding sequences for hSNF2H and hACF1 were obtained from G. Narlikar (University of California, San Francisco, CA). These sequences were subcloned into the pcDNA3 vector for studies requiring in vitro transcription/translation and transient transfection. Antibodies against C/EBPβ (sc-150 and sc-150X), Ini1 (sc-16189 and sc-13055), hSNF2H (sc-13054, sc-13054X, and sc-8759), hACF1 (sc-10627, sc-10627X, and sc-10629X), BAF-155 (sc-10756), actin (sc-1616), normal rabbit IgG (sc-2027), and normal goat IgG (sc-2028) were acquired from Santa Cruz Biotechnology. The anti-BRM antibody was acquired from BD Biosciences (610389) and the anti-six-His antibody from Clontech (631212). The anti-Brg1 antibody was a kind gift from W. Wang (National Institute on Aging, National Institutes of Health, Baltimore, MD).

Purification of Recombinant Proteins. hC/EBPβ isoforms were purified as glutathione *S*-transferase (GST) fusion proteins from *Escherichia coli* BL21 extracts using glutathione-Sepharose 4B resin (GE Healthcare, 17-0756-01) according to the manufacturer's instructions, with the following modifications: after the resin-bound fusion proteins had been washed with 1× PBS, elution buffers containing 10−15 mM glutathione were used as an additional washing step, which did not significantly remove C/EBPβ isoforms from the resin. These washes consisted of incubation with 1 volume of elution buffer at room temperature for 10 min (three times). Subsequently, three washes with 1× PBS were performed, and the resin-bound fusion proteins were stored at 4 °C as a 25% slurry in 1× PBS until they were used. Protease inhibitors (0.5 mM PMSF, 7 μg/mL TPCK, 10 μg/mL trypsin inhibitor, 5 μg/mL leupeptin, and 1 μg/mL pepstatin) were included in all buffers used for purification. For purification of rat C/EBPβ isoforms, 1−5 mM glutathione washes were used.

Cell Culture, Nuclear Extracts, and ChIP Assays. HepG2 and HeLa cells were grown in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, and 50 units/mL penicillin-streptomycin. Nuclear extracts were prepared according to the Dignam method.[16] Nuclear extracts from HeLa cells were dialyzed against 20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM β-mercaptoethanol, 20% glycerol, and 0.4 mM PMSF. For chromatin immunoprecipitation (ChIP) assays, 24 h after the dishes had been seeded (100 mm dishes) medium was replaced with fresh medium in the presence or absence of 1% (v/v) DMSO, continuing the culture for an additional 6 days. The same treatment was performed in separate dishes for reverse transcription PCR (RT-PCR) (22 mm dishes) and Western blot (100 mm dishes) analyses. Afterward, the ChIP assay was conducted as previously described.[17] DNA obtained from the immunoprecipitated material was analyzed by PCR amplification of residues −695 to −452 of the cyclin D1 gene.[9]

GST Pull-Down Assays. Typically, 2 μg of recombinant GST-hC/EBPβ1 protein and equimolar amounts of the other C/EBPβ isoforms and GST were used in the assays. For each pull-down reaction, 50 μg (10 μL) of HeLa cell nuclear extracts was precleared by combination with 30 μL of pull-down buffer for preclearing {PDB-P [20 mM Hepes (pH 7.9), 100 mM NaCl, 7.5% glycerol, 0.1% Tween 20, 1 mM EDTA, 1 mM DTT, and protease inhibitors]} and 10 μL (bed volume) of pre-equilibrated glutathione-Sepharose 4B resin, with incubation at 4 °C for 2 h under rotation. Precleared extract (40 μL) was mixed with resin (10 μL bed volume) containing the recombinant protein, with the incubation described above. Subsequently, the resin was washed three times with 20 volumes of PDB (same composition as PDB-P, but containing 150−180 mM NaCl and 10% glycerol) and protease inhibitors. The resin-bound proteins were eluted by being boiled in 1× loading buffer and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). C/EBPβ-interacting proteins were detected with a Western blot. hSNF2H and hACF1 proteins were produced using the TnT T7 Quick Coupled Transcription/Translation System (L1171, Promega), following the manufacturer's instructions and using L-[^{35}S]methionine for labeling (NEG709A, PerkinElmer). One microgram of hSNF2H (or hACF1) plasmid was used in a standard 50 μL reaction. Five microliters of this product was

used for the GST pull-down assay, which was conducted as described above for nuclear extracts. After SDS−PAGE, labeled hACF1 and hSNF2H proteins were detected by fluorography and additionally by exposure to a phosphor screen followed by screen scanning (Molecular Imager FX, Bio-Rad).

Mass Spectrometry. GST pull-down assays designed for mass spectrometry analysis were scaled up 8 times with respect to those performed for Western blot analysis using HeLa cell nuclear extracts. After the pull-down step, samples were washed four times with 5 volumes of PDB with 150 mM NaCl and protease inhibitors. Subsequently, resin-bound proteins were eluted by incubation twice for 5 min at room temperature with 2 volumes of 0.1% Sarkosyl (Sigma, L9150). Eluted proteins were precipitated by addition of 0.25 volume of 100% trichloroacetic acid (TCA) and kept on ice overnight. One-eighth of the sample was TCA-precipitated separately for silver staining to corroborate elution of C/EBPβ-interacting proteins with a low-level release of the GST fusion protein. After centrifugation (16000g for 15 min at 4 °C), precipitated proteins were washed twice with cold acetone, air-dried, and analyzed by multidimensional protein identification technology (MudPIT) as described previously.[18] SC (sequence coverage) corresponds to the percent of the protein sequence covered by detected peptides. NSAF (normalized spectral abundance factor) values are an indicator of the relative abundance of each protein in the whole mix of proteins detected for each pull-down assay.[19]

Co-Immunoprecipitation (CoIP) Assays. For CoIP assays using nuclear extracts from HeLa cells, each reaction used 20 μL (100 μg) of nuclear extract. This volume was mixed with 10 μL (2 μg) of the appropriate antibody (or normal rabbit IgG), with incubation at 4 °C overnight under rotation. Subsequently, 10 μL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, sc-2003) was added, and the mixture was incubated for 3 h at 4 °C. Afterward, the resin was washed three times with 10 volumes of buffer B [20 mM Hepes (pH 7.9), 250 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Tween 20, and protease inhibitors]. The resin-bound proteins were eluted by being boiled in 1× loading buffer, subjected to SDS−PAGE, and detected by Western blotting. In CoIP assays using HepG2 nuclear extracts, 20 μL (100 μg) of this extract was mixed with 64 μL of buffer C [20 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.1% Tween 20, and protease inhibitors] and 10 μL of antibody. Subsequent steps proceeded as described for HeLa cell nuclear extracts.

Transient Transfection and RT-PCR. HepG2 cells were transfected with vectors encoding each of the C/EBPβ isoforms, hSNF2H, and/or hACF1 using Satisfection (Stratagene, 204123), following the manufacturer's instructions. Twenty-four hours after transfection, total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, 15596-026). The interphase and the organic phase were processed according to the manufacturer's guidelines for protein overexpression analysis, using the alternative protocol that consists of dialysis against 0.1% SDS, and the resulting pellet was resuspended in 4 M urea and 0.5% SDS, with heating at 80 °C for 10 min. Aliquots of these samples were analyzed by Western blotting. In the case of mRNA analysis, for each condition, 1 μg of RNA was reverse transcribed using 0.25 μg of Anchored Oligo$(dT)_{20}$ Primer (Invitrogen, 12577-011) and M-MLV reverse transcriptase (Promega, M170A), in a final volume of 20 μL; 0.5 μL of this sample was analyzed by conventional PCR or by real-time PCR using Brilliant III QPCR Master Mix (Stratagene, 600882), according to manufacturer's instructions. Real-time PCRs were performed in a Mx3000p thermocycler (Stratagene). PPARγ and cyclin D1 mRNA levels were determined using the Standard Curve Method,[20] utilizing β-actin as a reference gene. Transfection efficiencies were comparable for the different combinations of C/EBPβ isoforms and hSNF2H, as determined by RT-PCR (see Figure S3 of the Supporting Information).

RESULTS

With the aim of detecting new C/EBPβ-interacting proteins, we established a GST pull-down protocol coupled to detection of all interacting proteins using MudPIT.[21] To do this, we purified the three human C/EBPβ isoforms as GST fusion proteins from bacteria, obtaining a high level of purity as judged by Coomassie staining (Figure 1A). Before performing our proteome-wide screening for C/EBPβ interactors, we wanted to test our fusion proteins with an already described interaction. It has been previously shown that C/EBPβ interacts with the SWI/SNF chromatin remodeling complex.[22] This study showed that, of the three isoforms, only C/EBPβ1 (LAP*) interacts with the complex. Given that the aforementioned study used the chicken version of these proteins, it was also worth studying this interaction with the human C/EBPβ isoforms. Therefore, we performed a GST pull-down assay coupled to detection of SWI/SNF core subunits[23] by Western blotting. As observed in Figure 1B, the two longer isoforms interact with SWI/SNF under our assay conditions. C/EBPβ3 shows a much weaker binding or no interaction with the complex. Overall, interaction of C/EBPβ2 with the SWI/SNF complex seems to be weaker than that found for the full-length isoform, but affinities appear to be on the same order of magnitude. Similar data were obtained using the rat C/EBPβ isoforms (Figure S1 of the Supporting Information). These results differ from those found for chicken C/EBPβ, for which interaction with SWI/SNF was found only for the C/EBPβ1 isoform.[22]

Once the previously described C/EBPβ−SWI/SNF interaction was confirmed under our assay conditions, we performed GST pull-down assays coupled to mass spectrometry detection of C/EBPβ-interacting proteins. To do this, we scaled up the pull-down assays eight times with the aim of obtaining a sample sufficient for mass spectrometry detection. A number of proteins were detected at very high levels, relative to other proteins identified in the screening. A list of proteins including hSWI/SNF core subunits and other proteins detected at high levels, as well as proteins displaying differential interaction with C/EBPβ isoforms, is provided in Table 1 (additional tables of mass spectrometry results are provided as Supporting Information). This table depicts the relative abundance of C/EBPβ-interacting proteins in terms of SC and NSAF (see the footnotes of Table 1 for a description of these parameters). As observed in the table, C/EBPβ2 seems to have an affinity for components of human SWI/SNF complexes slightly weaker than that of C/EBPβ1, consistent with our Western results described above. Remarkably, hSNF2H and hACF1, which are components of chromatin remodeling complexes of the ISWI subfamily, were detected with a relative abundance even higher than that observed for any component of hSWI/SNF complexes. Additionally, the ATPases INO80 and HLTF were detected at a relatively high abundance in the mass

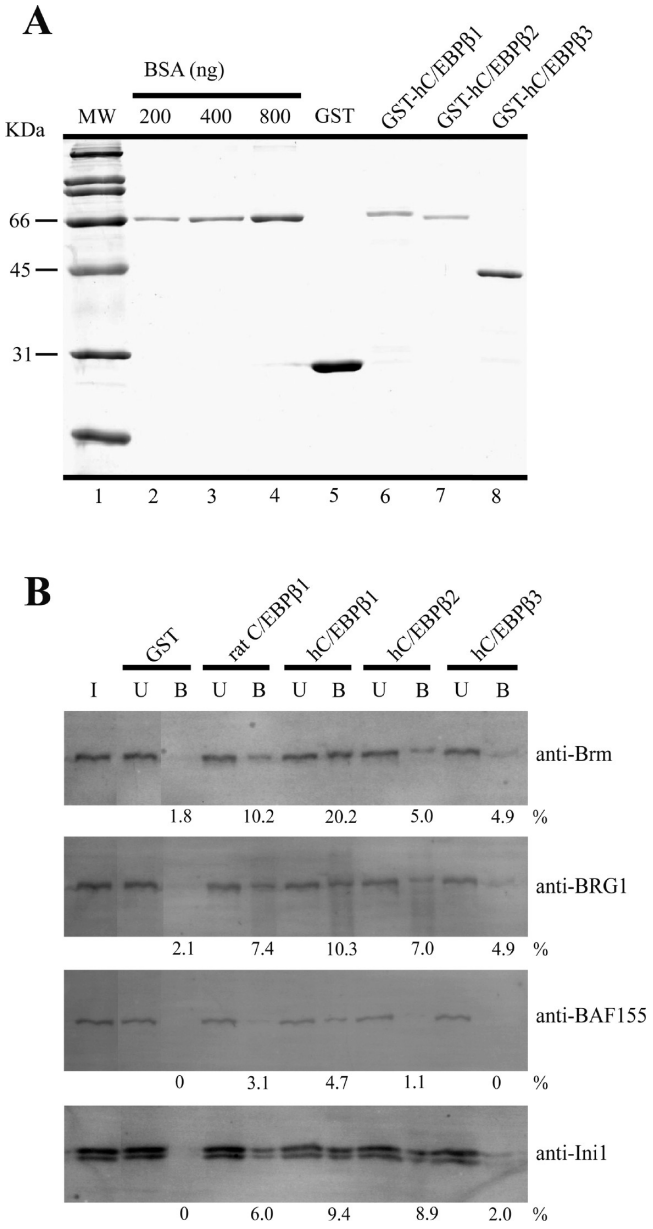

Figure 1. Interaction of mammalian C/EBPβ isoforms with human SWI/SNF complexes. (A) SDS–PAGE analysis for purification of the human C/EBPβ isoforms, obtained as GST fusion proteins. The picture shows Coomassie staining of a 12% gel. The identity of each sample is indicated at the top. (B) GST pull-down assay performed with resin containing purified C/EBPβ isoforms as GST fusion proteins and nuclear extracts obtained from HeLa cells. The proteins used in the assay are indicated at the top and the antibodies used in immunodetection at the right. Numbers below each image indicate the percentage of input protein (prey) bound to each C/EBPβ isoform or GST (bait). Abbreviations: I, input (20%); U, unbound supernatant (20%); B, bound proteins.

spectrometry analysis. A number of proteins for which interaction with C/EBPβ has been already described were also detected (see Table S1 of the Supporting Information).

The strong detection of hSNF2H and hACF1 suggested a link between C/EBPβ and the ACF and CHRAC complexes. The ACF complex is composed of hACF1 and hSNF2H. Both proteins are also components of the CHRAC complex, which also contains CHRAC15 and CHRAC17.[24,25] Notably, the relative abundance of CHRAC15 in our MudPIT screening is one of the highest among all proteins detected. In contrast, CHRAC17 was detected at relatively low levels. It has been reported that CHRAC15 and -17 constitute a histone fold pair inside the CHRAC complex.[26] Complexes containing one of these proteins associated with a different histone fold protein have been described previously,[27] although we did not find in our mass spectrometry screening other histone fold proteins with a relative abundance in the range observed for CHRAC15.

The involvement of ACF and CHRAC complexes in transcriptional regulation and other nuclear processes that require chromatin remodeling[3,6,28,29] was of particular interest to us, as the main focus in our study is to shed light on the mechanisms involved in the role played by C/EBPβ at the level of transcriptional regulation. To confirm the existence of these protein–protein interactions, we performed pull-down assays using the C/EBPβ isoforms as GST fusion proteins and looked for hACF1 and hSNF2H by immunodetection. As observed in Figure 2A, pull-down assays using HeLa cell nuclear extracts result in interaction of all C/EBPβ isoforms with these proteins to a similar extent. As a control, we also performed detection of Brg1 and BRM proteins in these assays, for which C/EBPβ3 showed little or no interaction (bottom panels in Figure 2), which is consistent with our previous results (Figure 1B and Table 1). Additionally, the immunodetection of hACF1 and hSNF2H was systematically higher than that of Brg1 and BRM, in terms of the percentage of input protein bound to C/EBPβ (Figure 2A). In relation to these studies, it has been pointed out that the presence of nucleic acids in protein–protein interaction assays, such as GST pull-down assays, could produce the appearance of false positives.[30] Considering this issue, we performed GST pull-down assays incorporating a micrococcal nuclease digestion step to eliminate contaminant nucleic acids that could be present in the protein preparations.[30] In these assays, we obtained the same result of interaction of all three C/EBPβ isoforms with SNF2H and ACF1 (Figure S2 of the Supporting Information). To ascertain whether hC/EBPβ directly interacts with hSNF2H and/or hACF1, we produced these two proteins separately by in vitro transcription/translation and performed GST pull-down assay using GST-C/EBPβ1 as bait. As observed in Figure 2B, we clearly detected association of GST-hC/EBPβ1 with hACF1 protein, but not in the case of hSNF2H. For this protein, a faint but specific band reflecting binding to GST-hC/EBPβ1 was observed by film overexposure (data not shown). According to phosphor image quantification, the percentage of input protein bound by C/EBPβ1 was ~5 times higher for hACF1 than for hSNF2H. This result indicates that interaction of hC/EBPβ with the ACF complex proceeds mainly through direct physical interaction with hACF1.

Until this point, our data confirming the interaction of C/EBPβ with hSNF2H and hACF1 were based on the use of recombinant C/EBPβ proteins produced in bacteria. To confirm the existence of these interactions between endogenous proteins, we performed CoIP assays using nuclear extracts obtained from HeLa and HepG2 cells. Importantly, these assays were performed with nuclear extracts derived from non-transfected cells; i.e., none of the proteins under study were overexpressed in the cells used for nuclear extract preparation. Immunoprecipitation of C/EBPβ with an antibody recognizing all three isoforms resulted in a strong detection of hACF1 (Figure 3A). Consistent with results observed in our previous protein–protein interaction studies, detection of hACF1, in terms of the percentage of input immunoprecipitated, was

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Table 1. C/EBPβ-Interacting Proteins Identified by GST Pull-Down Assays Coupled to Multidimensional Protein Identification Technology (MudPIT)[a]

		C/EBPβ1		C/EBPβ2		C/EBPβ3		GST		
protein	protein ID (NP)	SC	NSAF	SC	NSAF	SC	NSAF	SC	NSAF	description
BRG1	003063.2	15.3	0.00118	11.4	0.00063	5.1	0.00011	1.6	0.00008	core components of hSWI/SNF complexes
BRM	620614.2	5.9	0.00043	3.4	0.00031	0	0	0	0	
BAF155	003065.2	16.5	0.00129	10.7	0.00079	6.4	0.00072	3.7	0.00012	
Ini1a	003064.2	25.1	0.00057	11.9	0.00044	4.4	0.00013	0	0	
hSNF2H	003592.2	34.7	0.00200	28.6	0.00256	22.5	0.00220	2.9	0.00022	components of ACF and CHRAC complexes
ACF1	038476.2	32.9	0.00250	29.2	0.00233	21.9	0.00327	0	0	
CHRAC15	059140.1	45.8	0.00607	45.8	0.00864	45.0	0.00589	0	0	components of CHRAC complex
CHRAC17	059139.2	17.7	0.00014	17.7	0.00039	17.7	0.00105	0	0	
INO80	060023.1	18.8	0.00100	13.1	0.00096	16.4	0.00097	0	0	catalytic subunit of INO80 complex
HLTF	620636.1	34.5	0.00313	25.2	0.00216	22.3	0.00377	0	0	chromatin remodeling enzyme of the SWI/SNF family
DNA-PKcs	008835.5	59.4	0.03501	55.0	0.02687	46.2	0.07127	11.9	0.00075	catalytic subunit of the DNA-dependent protein kinase (DNA-PK)
USF1	009053.1	23.6	0.00147	22.9	0.00183	9.4	0.01178	0	0	transcription factor, bHLH-ZIP family
TFAP4	003214.1	39.9	0.00417	38.8	0.00586	27.2	0.00982	0	0	transcription factor, bHLH-ZIP family
BEND3	001073919.1	41.8	0.00281	54.5	0.00492	37.8	0.00814	0	0	KIAA1553 (XP_166320.4)
TFCP2	005644.2	31.7	0.00241	29.5	0.00225	29.1	0.00830	16.1	0.00060	α-globin transcription factor CP2
NR2F2	066285.1	27.5	0.00350	21.7	0.00232	24.6	0.00777	0	0	TFCOUP2
JUNB	002220.1	42.9	0.00269	39.2	0.00375	40.6	0.00601	8.1	0.00009	transcription factor jun-B
LIG3	039269.2	23.7	0.00217	28.5	0.0025	25.2	0.00594	0	0	involved in DNA excision repair
SKP1A	733779.1	52.8	0.00549	30.7	0.00417	30.7	0.00268	0	0	component of SCF complexes
PNKP	009185.2	23.0	0.00095	25.9	0.00125	31.3	0.00469	0	0	involved in DNA repair
ATF7	006847.1	29.2	0.00337	34.2	0.00363	31.3	0.00336	0	0	cyclic AMP-dependent transcription factor ATF-7
XRCC1	006288.1	30.0	0.00207	30.5	0.00246	28.0	0.00341	0	0	involved in repair of DNA single-strand breaks
ESRRA	004442.3	35.5	0.00216	13.7	0.00114	9.0	0.00274	0	0	nuclear receptor that is closely related to the estrogen receptor
TERF2IP	061848.2	44.4	0.00199	31.3	0.00248	26.6	0.00251	7.5	0.00017	TERF2-interacting telomeric protein 1
NR2C2	003289.2	32.5	0.00136	28.1	0.00120	40.8	0.00230	0	0	member of the nuclear hormone receptor family
RFXANK	003712.1	32.7	0.00183	42.7	0.00207	16.2	0.00139	0	0	binds to the X box motif of certain MHC class II gene promoters
RFC3	002906.1	42.1	0.00151	42.7	0.00207	16.0	0.00079	0	0	component of the RFC (replication factor C) complex
NAP1L1	631946.1	35.3	0.00158	17.7	0.00058	0	0	4.4	0.00017	member of the nucleosome assembly protein (NAP) family
NFYC	055038.2	7.5	0.00113	7.5	0.00130	7.5	0.00015	0	0	component of a trimeric transcription factor
MEIS1	002389.1	31.3	0.00102	8.2	0.00051	5.4	0.00007	0	0	homeodomain-containing protein
SSBP1	003134.1	22.3	0.00040	27.7	0.00134	0	0	0	0	involved in the maintenance of genome stability
NFIC	995315.1	16.6	0.00028	23.3	0.00108	5.0	0.00010	0	0	CCAAT-binding transcription factor
RXRB	068811.1	14.1	0.00071	16.3	0.00060	0	0	0	0	member of the retinoid X receptor (RXR) family
DDX49	061943.2	17.8	0.00078	9.5	0.00030	5.8	0.00005	0	0	DEAD box polypeptide 49
RFX1	002909.3	18.4	0.00055	12.4	0.00040	3.6	0.00008	0	0	member of the regulatory factor X gene family
RHOA	001655.1	12.4	0.00031	7.8	0.00060	0	0	0	0	ras homologue gene family, member A
HMGB2	002120.1	7.2	0.00038	6.2	0.00010	0	0	0	0	high-mobility group protein
DEK	003463.1	6.9	0.00016	6.7	0.00020	0	0	0	0	binds to cruciform and superhelical DNA
RAD21	006256.1	12.5	0.00016	6.0	0.00005	0	0	0	0	involved in DNA repair
CDK2AP1	004633.1	11.3	0.00242	11.3	0.00025	0	0	0	0	negatively regulates CDK2 activity
POLR2L	066951.1	35.8	0.00178	0	0	0	0	0	0	subunit of RNA polymerase II
RBM7	057174.1	30.1	0.00105	6.8	0.00011	0	0	0	0	RNA binding motif protein 7
RPA2	002937.1	19.6	0.00103	5.9	0.00031	0	0	0	0	replication protein A2
ZBTB7A	056982.1	13.0	0.00044	4.9	0.00005	0	0	0	0	involved in different types of cancer
PAXIP1	031375.3	12.6	0.00042	1.5	0.00003	1.5	0.00004	0	0	involved in maintenance of genome stability
SHPRH	775105.1	5.4	0.00011	1.6	0.00003	0	0	0	0	functional homologue of *Saccharomyces cerevisiae* RAD5
SMARCAL1	054859.2	4.7	0.00006	0	0	0	0	0	0	member of the SWI/SNF family of proteins

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Table 1. continued

[a]The first section of the table lists the values for detection of proteins that are components of ATP-dependent chromatin remodeling complexes, including core components of SWI/SNF and subunits of ACF and CHRAC complexes. The second section lists proteins that were detected at relatively high levels. The other sections list proteins that exerted differential interaction with C/EBPβ isoforms. SC (sequence coverage) is the percent of the protein sequence covered by detected peptides. NSAF (normalized spectral abundance factor) is an indicator of the relative abundance of each protein in the whole mix of proteins detected for each pull-down assay (see Experimental Procedures for more details). Additional proteins detected through this screening are listed in the tables of the Supporting Information.

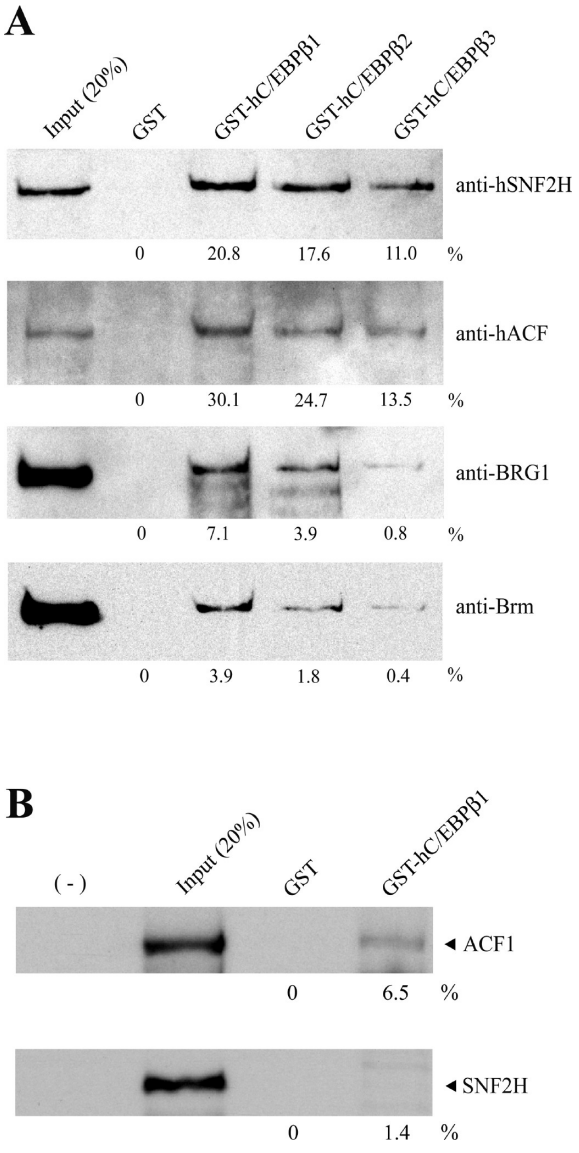

Figure 2. Interaction of C/EBPβ with major subunits of ACF/CHRAC complexes. (A) GST pull-down assay performed with resin containing purified hC/EBPβ isoforms as GST fusion proteins and nuclear extracts obtained from HeLa cells. The fusion proteins used as bait in the assay are indicated at the top and the antibodies used in immunodetection at the right. (B) GST pull-down assay performed using GST-C/EBPβ1 or GST (bait) and hACF1 (top) or hSNF2H (bottom), produced by in vitro transcription/translation (prey). The images show fluorographic detection of the proteins translated in vitro after the pull-down assay and SDS–PAGE. In (A) and (B), the numbers below each image indicate the percentage of input protein (prey) bound to each C/EBPβ isoform or GST.

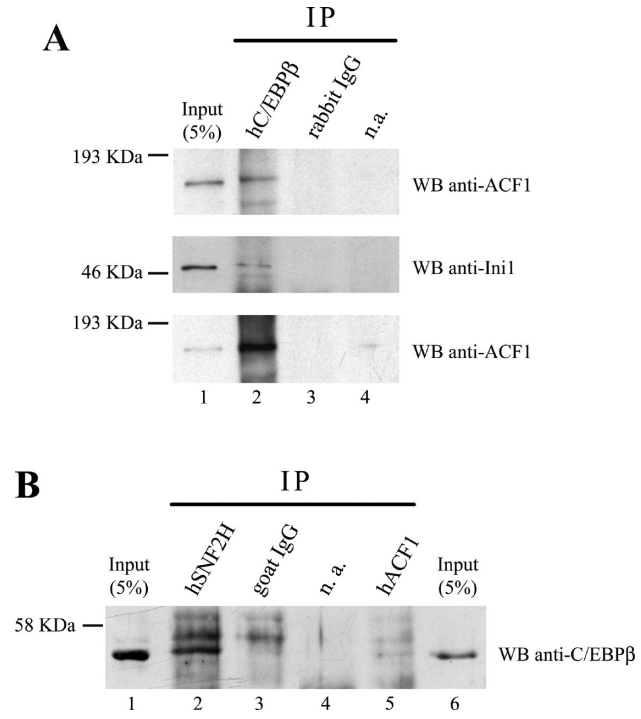

Figure 3. Interaction of hC/EBPβ with hACF1/hSNF2H in nuclear extracts from human cell lines. (A) Nuclear extracts from non-transfected cells were used in co-immunoprecipitation (CoIP) assays. The immunoprecipitation (IP) was performed against C/EBPβ. ACF1 or Ini1 was then detected by Western blotting. The top and middle panels show CoIP assays conducted using nuclear extracts from HeLa cells. The bottom panel shows CoIP using nuclear extract from HepG2 cells. n.a., no antibody used in the IP; rabbit IgG, normal rabbit IgG. (B) Reciprocal CoIP. Nuclear extracts from HeLa cells were used for hSNF2H or hACF1 IP, and then C/EBPβ was detected by Western blotting. n.a., no antibody used in the IP; goat IgG, normal goat IgG.

stronger than detection of hSWI/SNF components [represented here by Ini1 detection (Figure 3A)]. To further demonstrate the existence of physical interaction of hC/EBPβ with components of the ACF and CHRAC complexes, we performed the reciprocal CoIP, using antibodies against hACF1 and hSNF2H. As shown in Figure 3B, immunoprecipitation of hSNF2H or hACF1 results in detection of a band with an apparent molecular mass of near 55 kDa, which corresponds to the migration characterized for hC/EBPβ1.[9] We did not observe interaction with the shorter C/EBPβ isoforms. This was expected as we detect these shorter isoforms by Western blotting when analyzing amounts of HeLa or HepG2 nuclear extracts significantly larger than those used in Western blots derived from our CoIP assays (data not shown).

Given that our different approaches pointed to the existence of physical interaction of hSNF2H and hACF1 with hC/EBPβ, we wanted to test the functional relevance of these interactions. Several studies have implicated ACF and CHRAC complexes in chromatin remodeling processes determining gene activation or repression.[6,28,29,31,32] To date, there are no studies linking C/EBPβ to complexes of the ISWI subfamily in relation to

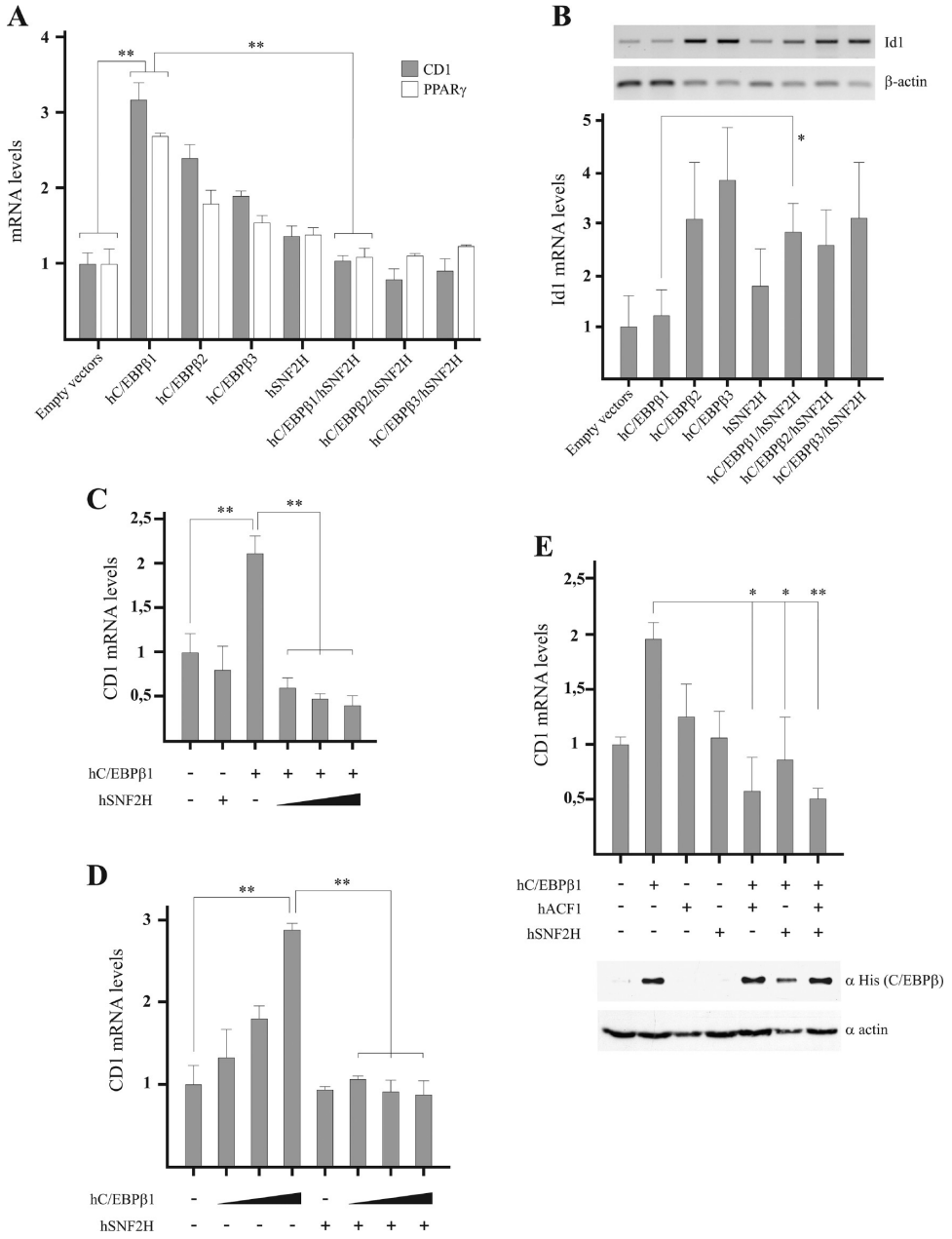

Figure 4. Effect of overexpression of C/EBPβ, hSNF2H, and hACF1 on mRNA levels of C/EBPβ target genes. HepG2 cells were transfected with expression vectors encoding hC/EBPβ isoforms or cotransfected with each of these vectors and vectors encoding hSNF2H or hACF1, as depicted in the bottom parts of the graphs. Twenty-four hours after transfection, total RNA was extracted from these cells and mRNA levels were measured by qRT-PCR (PPARγ and cyclin D1) or conventional PCR (Id1). For each condition, the target gene mRNA:β-actin mRNA ratio was calculated. These values are expressed in the graphs relative to the "empty vector" condition. Error bars for qRT-PCR data represent one standard deviation (SD) of a representative assay performed in triplicate. For conventional RT-PCR, error bars correspond to one SD from PCR product quantification of three separate cotransfection experiments. Asterisks denote statistically significant differences (*$p < 0.05$; **$p < 0.01$), as deducted from the t test. (A and B) Cotransfection experiments performed for all three C/EBPβ isoforms (0.6 μg) and hSNF2H (1.5 μg) expression vectors. (C) Cotransfection assay performed with the C/EBPβ1 expression vector (0.6 μg) and increasing amounts (0.5−1.5 μg) of the hSNF2H vector. (D) Cotransfection assay performed with a fixed amount (1 μg) of the hSNF2H vector and increasing amounts (0.25−1 μg) of the hC/EBPβ1 vector. (E) Cotransfection of C/EBPβ1 (0.6 μg), hSNF2H (1 μg), and hACF1 (1 μg). The bottom panel corresponds to a Western blot analysis of transfected C/EBPβ levels (using anti-His) for each of the transfection combinations shown in the graph. The first column in panels C−E corresponds to transfection with empty vectors.

transcriptional regulation of any particular gene. Thus, we decided to analyze the influence of C/EBPβ and hSNF2H on the transcriptional activity of known target genes of C/EBPβ, such as cyclin D1,[9,10] peroxisome proliferator-activated receptor γ (PPARγ),[33] and inhibitor of DNA binding 1 (Id1).[34] To do this, we transiently transfected HepG2 cells for 24 h with mammalian expression vectors encoding hC/EBPβ isoforms and hSNF2H, separately or combining each C/EBPβ isoform with hSNF2H, and looked for changes in mRNA levels of these target genes using real-time RT-PCR (qRT-PCR) or conventional RT-PCR (Id1). As observed in Figure 4A, overexpression of C/EBPβ1 resulted in an increase in the levels of cyclin D1

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and PPARγ mRNAs, suggesting transcriptional stimulation of these genes. The mRNA levels for both genes were also increased in the presence of the shorter C/EBPβ isoforms, but to a lesser extent. However, none of the C/EBPβ isoforms drove an increase in the levels of cyclin D1 and PPARγ mRNA in the presence of hSNF2H. Overexpression of hSNF2H alone did not reduce the basal level of cyclin D1 or PPARγ mRNA but specifically reduced the level of C/EBPβ-driven expression. Similar results were obtained when using shorter and longer transfection periods (Figure S3 of the Supporting Information). In the case of the Id1 gene, an increase in mRNA levels was observed in response to transfection with C/EBPβ2 and -3 vectors, but not with C/EBPβ1. Interestingly, cotransfection of C/EBPβ1 and hSNF2H vectors resulted in increased Id1 mRNA levels (Figure 4B). For C/EBPβ2 and -3, cotransfection with hSNF2H did not change mRNA levels for this gene, compared to overexpression of these C/EBPβ isoforms alone. Cotransfection assays in titration of C/EBPβ1 or hSNF2H vector further confirmed abrogation of C/EBPβ1-mediated transcriptional stimulation by hSNF2H (Figure 4C,D). We wanted to analyze whether overexpression of hACF1 has the same effect observed with hSNF2H. Thus, we performed cotransfection assays including combinations of C/EBPβ with hSNF2H and hACF1. As observed in Figure 4E, both hACF1 and hSNF2H abrogate C/EBPβ1-mediated stimulation of cyclin D1 expression. In some of the several cotransfection assays performed, we observed reduction of cyclin D1 mRNA below basal levels when cotransfecting C/EBPβ and hSNF2H/hACF1 (see Figure 4C,E). C/EBPβ overexpression was not affected by either hSNF2H or hACF1 cotransfection, as determined by Western blotting (Figure 4E).

Considering that hSNF2H and hACF1 abrogated C/EBPβ-mediated stimulation of the cyclin D1 gene in our cotransfection experiments, we wanted to analyze whether these proteins are present at the promoter region of this gene, especially under conditions negatively affecting cyclin D1 expression in HepG2 cells. It has been previously shown that treatment with 1% DMSO induces differentiation processes on HepG2 cells and other cell lines.[35,36] Thus, we compared the presence of hC/EBPβ, hSNF2H, and hACF1 on the cyclin D1 gene promoter in HepG2 cells after a 7 day culture in the presence or absence of 1% DMSO. We observed a reduction in the levels of cyclin D1 mRNA upon DMSO treatment. Protein levels of hC/EBPβ, hSNF2H, and hACF1 did not change with this treatment (Figure 5A). Reduction of cyclin D1 mRNA levels correlated with an increase in the level of hC/EBPβ, hACF1, and hSNF2H at the promoter region of this gene (Figure 5B).

Taken together, the data from our cotransfection and ChIP assays suggest the existence of a functional connection between C/EBPβ- and SNF2H-containing complexes, at least at the level of transcriptional regulation.

DISCUSSION

In this work, we searched for human C/EBPβ-interacting proteins not currently described, by using a proteomics approach. A fraction of the proteins identified by mass spectrometry was detected at high levels of relative abundance (NSAF) and sequence coverage (SC). Remarkably, hSNF2H and hACF1 proteins appeared at NSAF and SC values higher than those found for components of human SWI/SNF complexes, for which interaction with C/EBPβ has been previously described.[22] Consistently, further GST pull-down

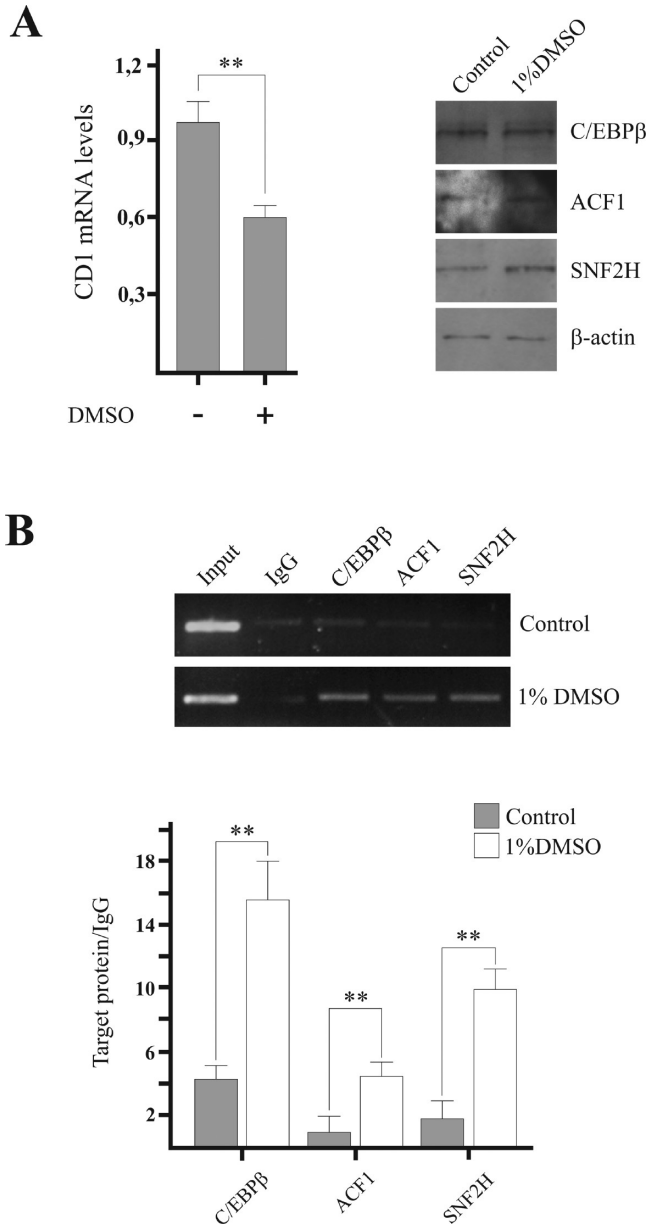

Figure 5. Reduction of cyclin D1 levels in HepG2 cells correlates with an increase in the level of C/EBPβ, hSNF2H, and hACF1 on the promoter region of the cyclin D1 gene. HepG2 cells were cultured in the presence or absence of 1% DMSO for 6 days, and then Western blot analyses, RT-PCR, and ChIP assays were performed. (A) Determination of cyclin D1 mRNA levels by qRT-PCR (left). Values in the graph correspond to the target gene mRNA:β-actin mRNA ratio. Error bars represent one standard deviation (SD) of a representative assay performed in triplicate. Western blot analysis of protein levels in nuclear extracts derived from cells cultured in the absence or presence of DMSO (right). (B) ChIP assay performed with antibodies against C/EBPβ, SNF2H, and ACF1. The top panels show the products of conventional PCR amplification between positions −695 and −452 of the cyclin D1 gene. These photographs show the products of PCRs using 5% immunoprecipitated material and 0.2% input material. The graph shows quantification of the amplified DNA for each immunoprecipitation, relative to IgG. Error bars correspond to one SD from three PCRs of a representative ChIP assay. Asterisks denote statistically significant differences (**$p < 0.01$), as deducted from the t test.

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assays pointed to a physical interaction of hC/EBPβ with hSNF2H and hACF1 apparently stronger than the interaction with hSWI/SNF. In our mass spectrometry screening, proteins belonging to other complexes of the ISWI subfamily were detected at levels generally 5−10 times lower than those of hACF1 and hSNF2H (see Table S2 of the Supporting Information). This observation is consistent with an association of C/EBPβ with ACF/CHRAC complexes through direct interaction with ACF1 (Figure 2B). These differences in detection levels might also be caused by differences in the abundance of the different SNF2H-containing complexes. We cannot rule out the possibility that association of C/EBPβ with the CHRAC complex could also proceed through the CHRAC15 or CHRAC17 protein. However, the low NSAF obtained for CHRAC17 in our mass spectrometry analyses makes interaction through this protein seem unlikely. It has been demonstrated that CHRAC17 interacts with hSNF2H and hACF1 through CHRAC15, which might be the reason for its detection at levels lower than those found for the other components of the CHRAC complex.[26]

Complexes of the ISWI subfamily are involved in transcriptional activation and repression, with ACF and CHRAC complexes being mainly linked to repression.[3,6] Recruitment of these complexes by C/EBPβ would then be expected to trigger transcriptional repression. In the case of the cyclin D1 and PPARγ genes, although generally we did not observe repression of basal transcription levels when cotransfecting hC/EBPβ1 and hSNF2H, hC/EBPβ1-mediated transcriptional stimulation was completely abrogated. Other studies have found that C/EBPβ1 acts as a repressor of cyclin D1 transcription.[9,37] This difference in C/EBPβ1 effect might rely on the use in our assays of cell types other than those used in the cited studies. HepG2 cells are highly proliferative and resemble poorly differentiated hepatocyte cells. Interestingly, we observed stimulation of Id1 gene expression when cotransfecting C/EBPβ1 and hSNF2H, which suggest that the combined action of C/EBPβ and ACF/CHRAC complexes may positively affect expression in a subset of C/EBPβ target genes.

In our cotransfection experiments, we observed transcriptional stimulation of cyclin D1 and PPARγ by C/EBPβ3. This isoform has mainly been associated with transcriptional repression. However, it has been observed that in some cellular contexts this isoform can act as a transcriptional activator. It is thought that C/EBPβ3-mediated transcriptional activation might rely on heterodimer formation of this isoform with factors such as glucocorticoid receptor, Runx2, and NF-κB.[10]

Although there are no studies connecting C/EBPβ to complexes of the ISWI subfamily in terms of regulation of gene expression, a number of independent studies raise the possibility of a combined action of hC/EBPβ and hSNF2H-related complexes in transcriptional regulation of particular genes. Indeed, Talianidis and co-workers found hSNF2H physically present in the core promoter region of the albumin gene in G0/G1-enriched HepG2 cells.[38] Early studies of C/EBPβ function identified the albumin gene as a target of this factor.[39] Later studies have suggested that C/EBPβ1 and -2 act as activators of this gene, while C/EBPβ3 acts as a repressor, antagonizing the action of the longer isoforms.[40] Additionally, SNF2H has been recently shown to act as a repressor of the interleukin-2 (IL-2) gene in mouse T cells,[41] and C/EBPβ has been previously found to impair IL-2 induction in these cells.[42] Future studies aimed at determining the functional relationship of the different C/EBPβ isoforms and SNF2H in relation to transcriptional regulation of these genes will be of significant interest.

An additional set of proteins detected with a significant relative abundance in our mass spectrometry screening correspond to components of the INO80 complex (see Table 1 and Table S3 of the Supporting Information). Future studies will be required to confirm physical interaction between C/EBPβ and INO80 and to ascertain whether there is a functional connection between these proteins.

Although most of the protein−protein interactions described for C/EBPβ in previous studies and most of those found at high detection levels in our study point to the involvement in transcriptional regulation, some of the proteins detected in our screening might relate C/EBPβ to processes involved in the response to DNA damage. In fact, the catalytic subunit of the DNA-dependent protein kinase complex (DNA-PKcs) corresponds to the protein detected at the highest levels in our screening (see Table 1). Additionally, we observed that XRCC1, LIG3, XRCC4, PAXIP1, and other proteins known to be involved in DNA repair[43,44] were detected at high relative abundance values. In the same context, we detected HLTF protein at high abundance levels (see Table 1). This protein is an ATPase enzyme of the SWI/SNF family of chromatin remodelers, less characterized than the other members of the family, and a role in DNA repair has been ascribed to it.[45,46] Recent studies have assigned a role for C/EBPβ in DNA damage response,[47,48] although it is currently unknown whether this factor plays a direct role in DNA repair.

In summary, the finding of novel C/EBPβ-interacting proteins will be significantly important for unraveling the mechanisms by which this factor regulates transcription of a number of its target genes. It is also important for gaining insight into the role of C/EBPβ in cellular processes such as cell differentiation and malignant transformation.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1−S4 listing additional proteins detected by MudPIT, Figures S1 and S2 showing additional GST pull-down assays, and Figure S3 showing control analyses for the cotransfection experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ACF, ATP-utilizing chromatin assembly and remodeling factor; BRG1, Brahma-related gene 1; BRM, Brahma; C/EBPβ, CCAAT/enhancer-binding protein β; CHD, chromodomain, helicase, DNA binding; CHRAC, chromatin accessibility factor; Id1, inhibitor of DNA binding 1; INO80, inositol requiring 80; ISWI, imitation switch; MudPIT, multidimensional protein identification technology; NSAF, normalized spectral abundance factor; PPARγ, peroxisome proliferator-activated receptor γ; SC, sequence coverage; SNF2H, sucrose nonfermenting 2 homologue; SWI/SNF, switching defective/sucrose nonfermenting.

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