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ORIGINAL ARTICLE

C/EBPαp30 plays transcriptional regulatory roles distinct from C/EBPαp42

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CCAAT/enhancer binding protein α (C/EBPα) is a transcriptional regulatory factor that inhibits cell proliferation, and alternative translational initiation produces two polypeptides, C/EBPαp30 and C/EBPαp42. By expression profiling, it was revealed that C/EBPαp30 specifically inhibited a unique set of genes, including MPP11, p84N5 and SMYD2, which were not affected by C/EBPαp42 in both QSG-7701 hepatocyte cell line and QGY-7703 hepatoma cells. Semi-quantitative RT-PCR analysis independently confirmed these results. Chromatin immunoprecipitation assay showed that C/EBPαp30 bound to the promoters of these genes more strongly than C/EBPαp42. In clinical hepatoma samples in which C/EBPα was downregulated, all three genes specifically inhibited by C/EBPαp30 were upregulated. However, repression of MPP11, p84N5 and SMYD2 genes might not be directly involved in C/EBPαp30-mediated growth inhibition. Our data suggest that C/EBPαp30 regulates a unique set of target genes and is more than a dominant-negative regulator of C/EBPαp42.

Keywords: C/EBPα, expression pattern, p84N5, MPP11, SMYD2

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Introduction

CCAAT/enhancer binding protein α (C/EBPα) is the prototype member of the C/EBP family, which belongs to the basic zipper class of transcription factors. C/EBPα is highly expressed in a variety of tissues including liver, lung, placenta, and white and brown adipose tissues [1, 2]. As a transcription factor, C/EBPα is a central regulator of energy metabolism [3]. It positively regulates a number of genes involved in integrative metabolic processes, including genes coding for phosphoenolpyruvate carboxykinase, tyrosine aminotransferase (TAT) and the 422 adipose P2 protein (aP2).

C/EBPα is a strong inhibitor of cell proliferation [4-6]. It initiates growth arrest by stabilizing p21, and by disrupting E2F transcriptional complexes during the G1 phase of the cell cycle [7-12]. It was suggested that C/EBPα might be a tumor suppressor gene. Downregulation of C/EBPα has been observed in breast cancer [13], lung cancer [14], skin carcinoma [15] and hepatocellular carcinoma (HCC) [16]. Mutations in the *C/EBP*α gene were found in human myeloid leukemia [17]. Expression of antisense C/EBPα RNA prevents both growth arrest and terminal differentiation of 3T3-L1 adipocytes [18]. In addition, C/EBPα is critical in regulating the differentiation of preadipocytes, myeloid cells, hepatocytes and pneumocytes [3, 5, 19-22].

C/EBPα can be translated into two proteins due to an inframe alternative translational initiation site of the same mRNA. Translation is initiated predominantly from the first and third ATG codons in the C/EBPα mRNA, resulting in C/EBPα isoforms with molecular masses of 42 and 30 kDa, respectively [23-25]. The two C/EBPα isoforms exhibit similar biological functions; for example, both C/EBPαp42 and C/EBPαp30 can transactivate promoters of aP2 and C/EBPα [23]. However, in certain cases C/EBPαp30 acts as a dominant-negative regulator of C/EBPαp42 [26, 27].

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The N-terminal part of C/EBPαp42, which is absent in C/EBPαp30, is required to bind retinoblastoma (Rb) and p21cdki [8-10]. Consequently, C/EBPαp30 lacks an anti-mitotic activity [23]. In clinical acute myeloid leukemia (AML), monoallelic mutant *C/EBPα* gene expressing only C/EBPαp30 can reduce the activity of C/EBPαp42 expressed from the wild-type allele by inhibiting its trans-activity on target genes [26].

There is evidence hinting that C/EBPαp30 might have functions different from or independent of that of C/EBPαp42. A recent paper indicated that C/EBPαp30 might limit the expression of the G-CSF receptor [28], whereas the C/EBPαp42 could enhance the G-CSF receptor expression [29]. We previously reported that C/EBPα was downregulated in HCC [16], and were interested in distinguishing the roles of the two C/EBPα isoforms in gene regulation and hepatocellular carcinogenesis. By monitoring the expression pattern of 18 000 human ESTs, we identified that p84N5, SMYD2 (SET and MYND domain containing 2) and MPP11 (M-phase phosphoprotein 11) genes were downregulated by C/EBPαp30, but were not affected by C/EBPαp42. All these genes were upregulated in clinical HCC, consistent with the fact that C/EBPα was downregulated in liver cancer [16]. Our data suggest that the C/EBPαp30 plays unique regulatory roles different from that of the larger isoform C/EBPαp42.

Materials and Methods

Plasmids construction

Plasmids pCMV-C/EBPα42 and pCMV-C/EBPα30 were gifts from Ueli Schibler [24]. The coding sequences of C/EBPαp30, C/EBPαp42, MPP11, p84N5 and SMYD2 were amplified from human liver tumor RNA using the primers as following:

MPP11 5' *Nhe*I: 5'-GCC CGC TAG CCA TCA TGC TGC TTC TGC-3';

MPP11 3' *BamH*I: 5'-GCG GGA TCC TTC TTG GCT CTA CTT GC-3';

p84N5 5' *Xho*I: 5'-GCC TCG AGC CAC CAT GTC TCC GAC GCC GCC GC-3';

p84N5 3' *BamH*I: 5'-GGG GAT CCC AAC TAT TTG TCT CAT TGT CAT TAG-3';

SMYD2 5' *Xho*I: 5'-CGC TCG AGC CCC GCC GCC ACC-3';

SMYD2 3' *EcoR*I: 5'-CCG AAT TCG GTG GCT TTC AAT TTC C-3';

C/EBPαp42 5' *Nhe*I: 5'-GGC GGC TAG CCA CCA TGG AAT CGG CTG ACT TCT ACG AGG CGG-3';

C/EBPαp30 5' *Nhe*I: 5'-GCG CTA GCA CCA TGC CAG GAG GAG CGC ACG GGC-3';

C/EBPα 3' *BamH*I: 5'-GGC GGG ATC CGC GCA AGT TGC CCA TGG C-3'.

All the cDNAs were cloned into pEGFP-N1 (6085-1, BD Biosciences) to generate the GFP fusion protein. The fractions of pGFP-C/EBPαp30, pGFP-C/EBPαp42, pGFP-MPP11, pGFP-p84N5 and pGFP-SMYD2 digested by *Nhe*I and *BamH*I were subcloned into pcDNA3.1B to generate the myc-tagged expression constructions, pC/EBPαp30-myc, pC/EBPαp42-myc, pMPP11-myc, pp84N5-myc and pSMYD2-myc.

Cell culture and transfection

Human HCC cell lines QGY-7703, BEL-7404 and normal hepatocyte line QSG -7701 (Type Culture Collection of Chinese Academy of Sciences, Shanghai) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 15% calf serum, 100 mg/ml streptomycin sulfate and 100 U/ml penicillin at 37 ºC in 5% CO_2. Plasmids pCMV-C/EBPα42, pCMV-C/EBPα30, pGFP-MPP11, pGFP-p84N5, pGFP-SMYD2, pGFP-C/EBPαp42, pGFP-C/EBPαp30, pcDNA3.1B, pC/EBPαp30-myc, pC/EBPαp42-myc, pp84N5-myc, pMPP11-myc and pSMYD2-myc were transfected into the cells using the LipofectAMINE PLUS^{TM} Reagent (Invitrogen). Stable SMYD2-myc-transfected cells were selected in the presence of 800 μg/ml G418 for 2 months.

RNA extraction, gene expression profiling and RT-PCR

Total RNA of transfected cells and HCC clinical samples were prepared by Trizol (Invitrogen). Preparation of cDNA array, expression profiling and data analysis were conducted as reported previously [16]. For RT-PCR, 1% of the reverse transcription reaction was amplified using *Taq* DNA polymerase for 1 min at 94 ºC, 1 min at 53 ºC and 1 min at 72 ºC for 27 cycles. The primers and genes were listed in the following:

C/EBPα RT 5': 5'-CGC CGC GCA CCC CGA CCT C-3';
C/EBPα RT 3': 5'-CAC CGC CTG GCC CCC TCA TCT TAG-3';
SMYD2 5': 5'-ATC AAG CCG GGA GAG GAG G-3';
SMYD2 3': 5'-GGA GGC CAC GTG AGG GAG-3';
MPP11 5': 5'-CTG AAT TAG AAG CTG CTC GGT TAG-3';
MPP11 3': 5'-AGC TTC TGT TCT TCT TGT GTC CA-3';
p84N5 5': 5'-GCT CCA ACA ACG TGC TCT ATT CC-3';
p84N5 3': 5'-AGT CCT CGG GTG CTG TTC TCT TC-3';
β-actin 5': 5'-GCT GGC CGG GAC CTG ACT GAC TAC-3';
β-actin 3': 5'-GGG GGC ACG AAG GCT CAT CAT T-3'.

Immunofluorescence

The pCMV-C/EBPα42- and pCMV-C/EBPα30-transfected liver cells were harvested after 48 h using 0.25% trypsin, and centrifuged onto slides. Then cells were fixed by 4% paraformaldehyde and permeated with permeabilizing solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 5 min on ice. The immunofluorescence was carried out with the primary antibody, anti-C/EBPα (sc-9314, Santa Cruz Biotechnology) and FITC-conjugated second antibody (sc-2348, Santa Cruz Biotechnology).

BEL-7404 cells were cultured on slides and transfected with plasmids, pGFP-MPP11, pGFP-p84N5, pGFP-SMYD2, pp84N5-myc, pMPP11-myc and pSMYD2-myc. The transfected cells were harvested after 48 h and fixed by 4% paraformaldehyde. The pp84N5-myc-, pMPP11-myc- and pSMYD2-myc-transfected cells were permeated with permeabilizing solution for 5 min on ice. After blocked in 3% BSA, the slides were incubated with anti-myc antibody (9E10, Wolwobiotech) and Cy3-conjugated anti-mouse IgG (115-165-003, Jackson ImmunoResearch). All the cells were counterstained with DAPI to visualize the nuclei.

Western blot

The transfected cells were lysed by the 1× SDS loading buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 20% glycerol; 0.25% bromophenol blue; 1.25% 2-mercaptoethanol). Western blot was carried

out with the primary antibodies, anti-C/EBPα (sc-9314, Santa Cruz Biotechnology), tubulin (T-6074, Sigma), c-myc (9E10, Wolwobiotech) and HRP-conjugated secondary antibodies (V8051, W402B, W401B, Promega).

Flow cytometry (FACS) and growth rate measurement

After transfected for 48 h, the cells were digested and fixed by 4% paraformaldehyde. Fixed cells were treated with RNaseA (10 μg/ml) for 30 min and stained with propidium iodide (50 μg/ml) for another 30 min. The cell cycle of GFP-positive transfected cells were analyzed by flow cytometry (Bectin Dickinson, San Jose, CA, USA). Growth rate of cultured cells was measured by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

Chromatin immunoprecipitation (ChIP)

BEL-7404 cells were transfected with plasmids pcDNA3.1B, pC/EBPαp30-myc or pC/EBPαp42-myc. ChIP was performed as the manual instructed (17-295, Upstate Biotechnology). Chromatin was immunoprecipitated using anti-myc antibody (sc-788, Santa Cruz Biotechnology). The primer pairs for PCR were used as following:

MPP11 -898 5': 5'-CTC ACC CCC AAC AGC AGT AT-3';

MPP11 -898 3': 5'-CTT TAA TTG TGA AAA TAT AAT GAA-3';

MPP11 -1196 5': 5'-CTG GGA TTA GAG GCG TGA GTC-3';

MPP11 -1196 3': 5'-AGG TTT GGG GCA GAA GAT TAG TT-3';

MPP11 -1450 5': 5'-GAG GCC GGG AGG TGG AGG TT-3';

MPP11 -1450 3': 5'-TTG AAT GGG AGG AGG TGT GAG GAA-3';

p84N5 -866 5': 5'-AGA GAC GTG GCC GAT GAG AGC-3';

p84N5 -866 3': 5'-TCG AAG AAG GGA ACA GAG TAC ACG-3';

p84N5 -1833 5': 5'-TGT TCT GGC CAA TTT GTA GTG AT-3';

p84N5 -1833 3': 5'-AAG TTA GAG GAG CGG CTA TGA AT-3';

SMYD2 -1330 5': 5'-GAC GGG TTA GAG GCT TAT TTA GTT-3';

SMYD2 -1330 3': 5'-GGA GGA TCA TTT GAG CCC AGG AA-3';

SMYD2 -1153 5': 5'-CAC CAC ACC CAG CTG TCA AAC AGT-3';

SMYD2 -1153 3': 5'-CTT CAA AAT AGG ACC TCA AAC AGT-3'.

Results

Expression profiling revealed a set of genes that are differentially regulated by the two isoforms of C/EBPα

Gene expression profiling was performed to search for genes that might be differentially regulated by the two isoforms of C/EBPα. The plasmids pCMV-C/EBPα30 and pCMV-C/EBPα42 that solely express C/EBPαp30 and C/EBPαp42, respectively, were transfected into a hepatoma line QGY-7703 and a hepatocyte line QSG-7701. The vacant vector pRC-CMV was transfected as the negative control. Transfection efficiencies were about 30% as estimated by immunostaining examination using an anti-C/EBPα antibody (Figure 1A). Gene expression patterns were measured in the cells 48 h post-transfection using a cDNA array representing 18 000 human ESTs [16].

Compared with cells transfected with a pRC-CMV vector, overexpression of C/EBPαp42 and C/EBPαp30 changed the expression levels of a set of genes in both cell lines tested (Table 1). Two hundred and eighty-eight genes were similarly regulated by the two C/EBPα isoforms in

Table 1 The number of genes with two-fold altered expression levels in response to the overexpression of C/EBPα isoforms

	Host cell	Two-fold altered gene
C/EBPαp42 vs vector	QSG-7701	2 039
	QGY-7703	1 194
	Similarly changed in both QSG-7701 and QGY-7703	678
C/EBPαp30 vs vector	QSG-7701	2 519
	QGY-7703	463
	Similarly changed in both QSG-7701 and QGY-7703	403
Similarly changed genes caused by both isoforms	QSG-7701	1 652
	QGY-7703	364
	Similarly changed in both QSG-7701 and QGY-7703	288[1]
C/EBPαp42 vs C/EBPαp30	QSG-7701	504
	QGY-7703	205
	Similarly changed in both QSG-7701 and QGY-7703	10[2]

[1]Genes in this section are shown in Figure 1C.

[2]Genes in this section are shown in Figure 1D.

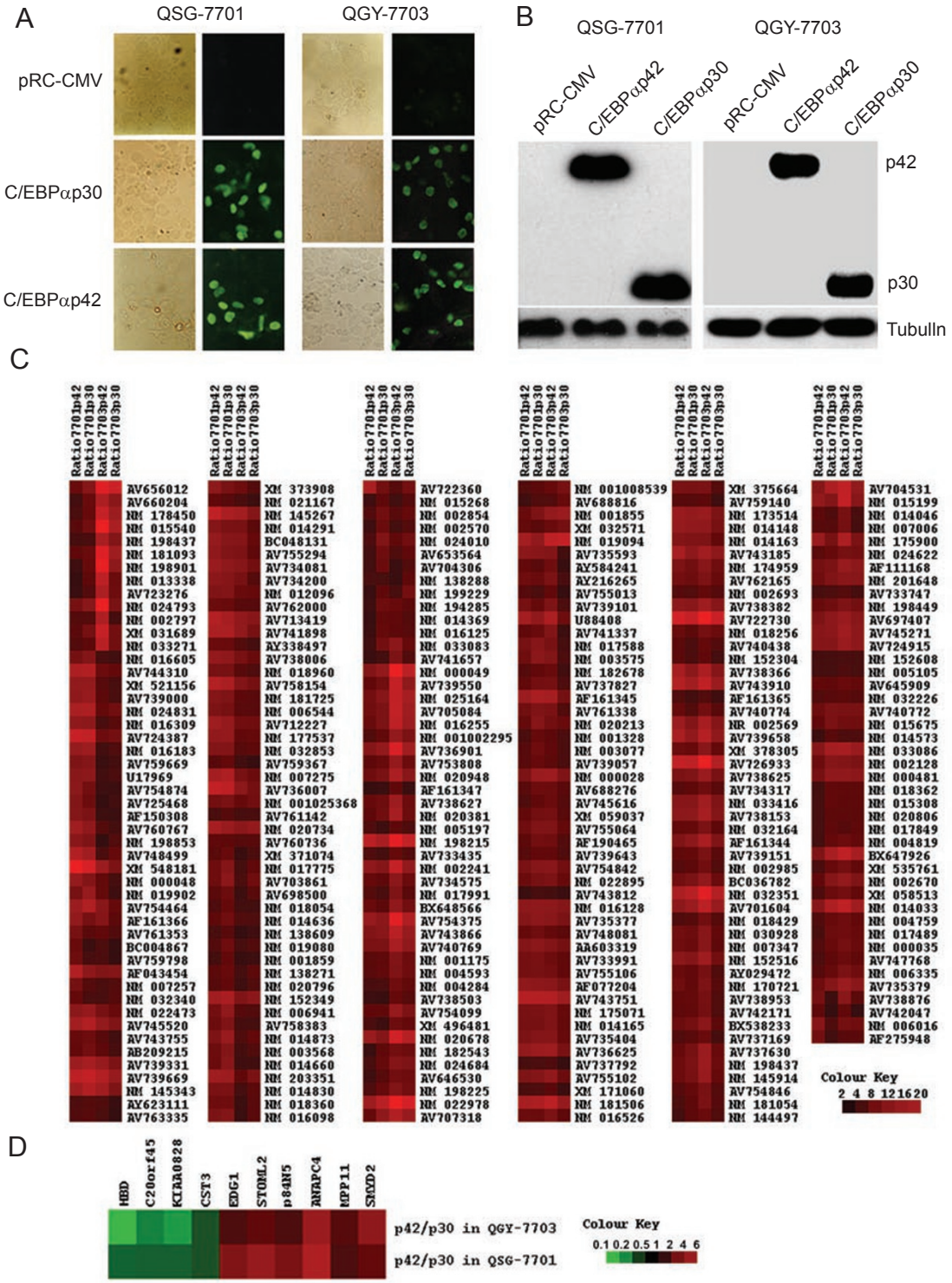

Figure 1 Expression profiling results. pRC-CMV, pCMV-C/EBPαp30 and pCMV-C/EBPαp42 were transfected into QSG-7701 and QGY-7703 cells for 48 h, respectively. **(A)** The cells transfected with indicated plasmids were stained with C/EBPα antibody. Transfection efficiency was defined as the number of the stained cells relative to the total cells. **(B)** Western blot of C/EBPαp30, C/EBPαp42 and α-tubulin from the transfected cells indicated the similar expression level of the two isoforms. **(C)** Two hundred and eighty-eight genes with two-fold altered expression levels in response to both C/EBPα isoforms in both tested cell lines were indicated using the Treeview program. The ratio of gene expression levels (C/EBPα vs vector control) was color coded as shown in the color bar. **(D)** Ten genes differentially regulated by the two isoforms of C/EBPα in both tested cells were indicated using the Treeview program. The ratio of gene expression levels (C/EBPαp42 vs C/EBPαp30) was color coded as shown in the color bar.

both cell lines, which were listed in Figure 1C using the Treeview program. Ten genes were differentially regulated by the two C/EBPα isoforms in both the liver originated cell lines (Figure 1D).

To confirm the expression profiling result, RNA was extracted from cells transfected with C/EBPαp30, C/EBPαp42 or the vacant vector. By semi-quantitative RT-PCR, it was confirmed that three genes, MPP11, p84N5 and SMYD2, were downregulated by C/EBPαp30, but were not affected by C/EBPαp42 in both cell lines (Figure 2A and 2B).

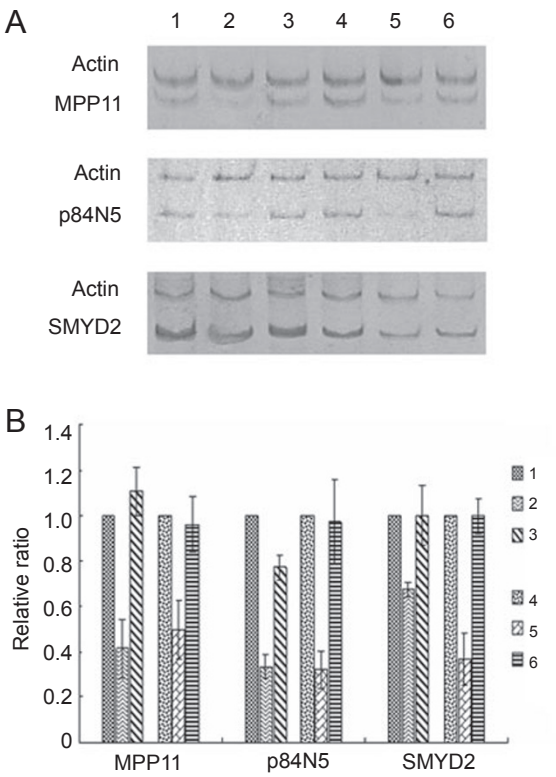

Figure 2 Semi-quantitative RT-PCR analyses of MPP11, p84N5 and SMYD2 expression in transfected cells. **(A)** pRC-CMV, pCMV-C/EBPαp30 and pCMV-C/EBPαp42 were transfected into QSG-7701 cells (samples 1-3) and QGY-7703 cells (samples 4-6) for 48 h, respectively. Lane 1: QSG-7701 cells transfected with pRC-CMV; lane 2: QSG-7701 cells transfected with pCMV-C/EBPαp30; lane 3: QSG-7701 cells transfected with pCMV-C/EBPαp42; lane 4: QGY-7703 cells transfected with pRC-CMV; lane 5: QGY-7703 cells transfected with pCMV-C/EBPαp30; lane 6: QGY-7703 cells transfected with pCMV-C/EBPαp42. **(B)** Densitometric analyses of RT-PCR results from **(A)** were illustrated in graph. Densitometric ratios of MPP11, p84N5 and SMYD2 mRNAs vs β-actin internal control in C/EBPαp42- and C/EBPαp30-transfected cells were normalized to that in vector-transfected cells. The numbers on the right of **(B)** indicated that the data were obtained by measuring the corresponding lanes in **(A)**.

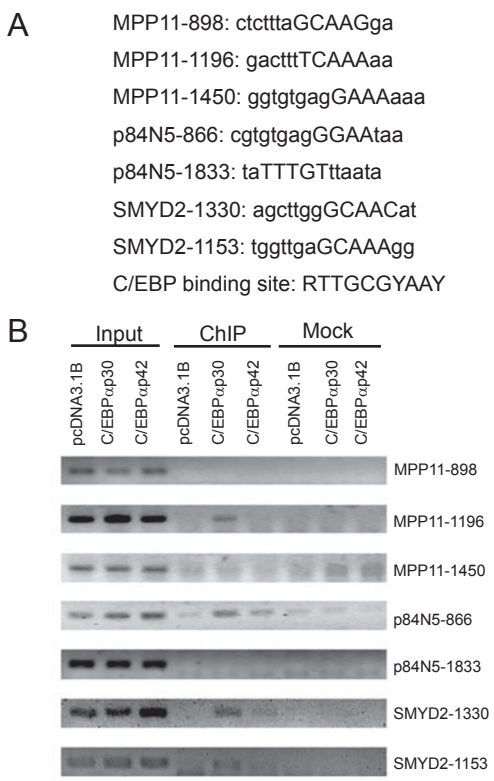

Figure 3 ChIP assay for detection of C/EBPα-binding sites on MPP11, p84N5 and SMYD2 promoters. **(A)** Seven C/EBP binding sites were predicted on the 2 000 bp upstream of the AUGs of MPP11, p84N5 and SMYD2 genes. The C/EBP binding consensus sequence was also shown. **(B)** Chromatin prepared from BEL-7404 cells transfected with pcDNA3.1B, pC/EBPαp30-myc or pC/EBPαp42-myc were immunoprecipitated with anti-myc antibody or without antibody (marked as mock). PCRs were performed with the primers specific to the predicted C/EBP binding sites indicated in (**A**).

Using the Match program (http://www.gene-regulation.com), a series of C/EBP binding sites were predicted in 2 000 bp regions upstream of the AUG start codons in MPP11, p84N5 and SMYD2 genes. Three sites in the MPP11 promoter and two sites in each of the p84N5 and SMYD2 promoters were examined by ChIP assays, as shown in Figure 3A. C/EBPα proteins bound to four out of the seven sites, including MPP11 -1196, p84N5 -866, SMYD2 -1330 and SMYD2 -1153 (AUG was numbered as 0) (Figure 3B). However, C/EBPαp30 bound to these promoters more strongly than C/EBPαp42, suggesting that C/EBPαp30 might directly inhibit the expression of these three genes.

Genes specifically regulated by C/EBPαp30 are upregulated in HCC tumors

Downregulation of C/EBPα was previously reported in

HCC [16]. It was interesting to study whether expressions of the three C/EBPαp30-inhibited genes were altered in HCC samples. mRNA levels of the MPP11, p84N5 and SMYD2 genes were measured using semi-quantitative RT-PCR in seven sets of HCC clinical samples, each including paired cancer and distal liver tissues from the same patient. Higher mRNA expressions of MPP11, p84N5 and SMYD2 were found in most of the cancer samples (marked with

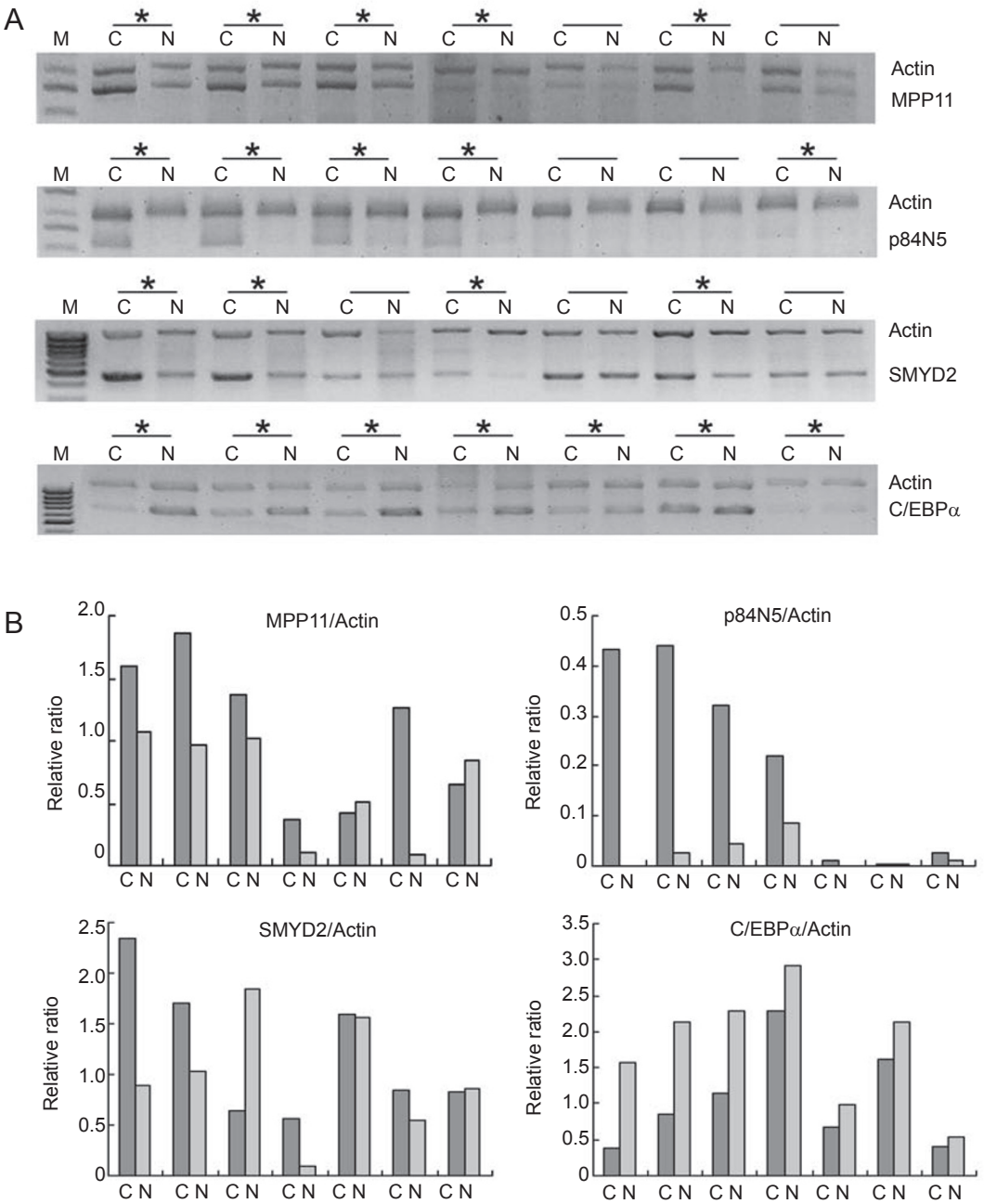

Figure 4 Semi-quantitative RT-PCR examinations of MPP11, SMYD2 and P84N5 transcripts in clinical HCC tumor samples. **(A)** Semi-quantitative RT-PCR examination showed the expression of MPP11, p84N5, SMYD2 and C/EBPα in clinical HCC tumor samples. Higher mRNA expression of MPP11, p84N5 and SMYD2 is marked with asterisk, compared with the paired non-tumorous samples. N: normal tissue; C: tumor; M: DNA marker. C/EBPα is downregulated in all the detected tumor samples. **(B)** Densitometric analysis of RT-PCR results from **(C)**, normalized to β-actin, in the corresponding samples. The order of the samples is identical to that in **(A)**.

asterisk), compared with the corresponding normal samples (Figure 4A and 4B). Thus, the downregulation of C/EBPα (Figure 4A and 4B) and upregulation of MPP11, p84N5 and SMYD2 in clinical HCC samples were well consistent with the discovery that C/EBPαp30 is a negative regulator of SMYD2, MPP11 and p84N5.

It was reported that MPP11 is highly expressed in head and neck squamous cell cancer (HNSCC) [30] and high mRNA expression of MPP11 is characterized in AML and chronic myeloid leukemia (CML) patients [31, 32]. Noticeably, the expression levels of p84N5 were very low in the non-tumorous liver tissues (Figure 4A and 4B), consistent with the report that p84N5 was upregulated in breast tumor but nearly undetectable in the normal breast tissue [33]. The potential roles of MPP11 and p84N5 genes in carcinogenesis need to be further studied.

p84N5 and MPP11 might affect cell cycle control

Subcellular localizations of the MPP11, SMYD2 and p84N5 gene products were examined by transfecting pGFP-MPP11, pMPP11-myc, pp84N5-myc, pGFP-p84N5, pGFP-SMYD2 and pSMYD2-myc plasmids into BEL-7404 cells. Forty-eight hours after transfection, both MPP11-GFP fusion protein and myc-tagged MPP11 protein were detected in the cytoplasm (Figure 5A), consistent with the previous report that MPP11 is a ribosome-tethered molecular chaperone [34]. SMYD2 was similarly localized in the cytosol. Both p84N5-GFP and myc-tagged p84N5 were visualized in nuclei (Figure 5A), consistent with the published results [33].

C/EBPα inhibits cell growth mainly in G1 phase in 3T3-L1 cells [18], Hep3B and Saos2 cells [4, 6], as well as in the HCC line BEL-7404 cells (Figure 5B). It was interesting to examine the effects of MPP11, p84N5 and SMYD2 on cell cycle progression. To this end, MPP11, p84N5 and SMYD2 were fused with GFP, transfected into BEL-7404 cells, and the cell cycle distribution of transfected cells was analyzed by flow cytometry [35]. Surprisingly, overexpression of MPP11-GFP and p84N5-GFP led to cell cycle arrest at G0/G1 phase (Figure 5B), despite the fact that C/EBPαp30 negatively regulated both MPP11 and p84N5. The data suggested that, although MPP11 and p84N5 were downregulated by C/EBPαp30, their effects on cell cycle control might not be directly related to the growth inhibitory function of C/EBPαp30.

We were unable to establish stable transfectants for C/EBPαp30, C/EBPαp42, MPP11 or p84N5 (data not shown), while control vectors could be stably transfected into BEL-7404 cells. This result was consistent with the growth inhibitory effects of MPP11 and p84N5.

Overexpression of SMYD2-GFP did not cause any significant changes in cell cycle (Figure 5B). Stable lines overexpressing myc-tagged SMYD2 (Figure 5C) had similar growth rates as the parental BEL-7404 cells according to MTT assay (Figure 5D), indicating that SMYD2 did not affect cell growth in the tested cells.

Discussion

C/EBPαp30 is more than a negative regulator of C/EBPαp42

C/EBPα mRNA generates two polypeptides by using two different AUGs within the same open reading frame [23]. The C/EBPαp30, compared with the full-length C/EBPαp42, lacks the N-terminal 117 amino acids, which is required by adipocyte differentiation, granulopoiesis [12, 22] and C/EBPα-Rb, C/EBPα-p21 interactions [8-10]. In liver cells, the expression ratio of C/EBPαp42 vs C/EBPαp30 is related to hepatocyte development [23].

By expression profiling and RT-PCR, we revealed that although C/EBPαp30 and p42 had similar effects on most of the C/EBPα-regulated genes, C/EBPαp30 did specifically regulate a unique gene set, which was not affected by C/EBPαp42. Moreover, these genes were upregulated in clinical HCC samples in which C/EBPα was downregulated. Identification of additional genes specifically regulated by C/EBPαp30 might help us to understand the roles of C/EBPαp30 different from that of C/EBPαp42.

The precise roles of C/EBPαp30 are to be further investigated

The roles of SMYD2, MPP11 and p84N5 genes in C/EBPαp30-mediated growth arrest are still unclear. Among these three genes, MPP11 is the human ortholog of ZUO1 and MIDA1 (mouse Id associate 1) [36] interfering with protein folding [34]. Upregulation of MPP11 is found in HNSCC [30], AML and CML [31, 32], as well as in HCC as we have observed. p84N5 binds the N-terminal domain of the Rb tumor suppressor [37], and is involved in transcriptional elongation and mRNA exportation [33]. p84N5 expression is increased in breast cancer [33] in which C/EBPα is downregulated [13], similar to what we have observed in HCC. However, both repression [33] and overexpression of p84N5 inhibited cell growth [38], and overexpression of MPP11 and p84N5 arrested cell cycle at G0/G1 phase despite the fact that these genes were downregulated by C/EBPαp30. This apparent inconsistency between C/EBPαp30 and its regulated SMYD2, MPP11 and p84N5 genes suggested that these three genes might be involved in the functions of C/EBPαp30 other than growth regulation.

The molecular mechanism of C/EBPαp30-specific regulation of MPP11, p84N5 and SMYD2 is not clear

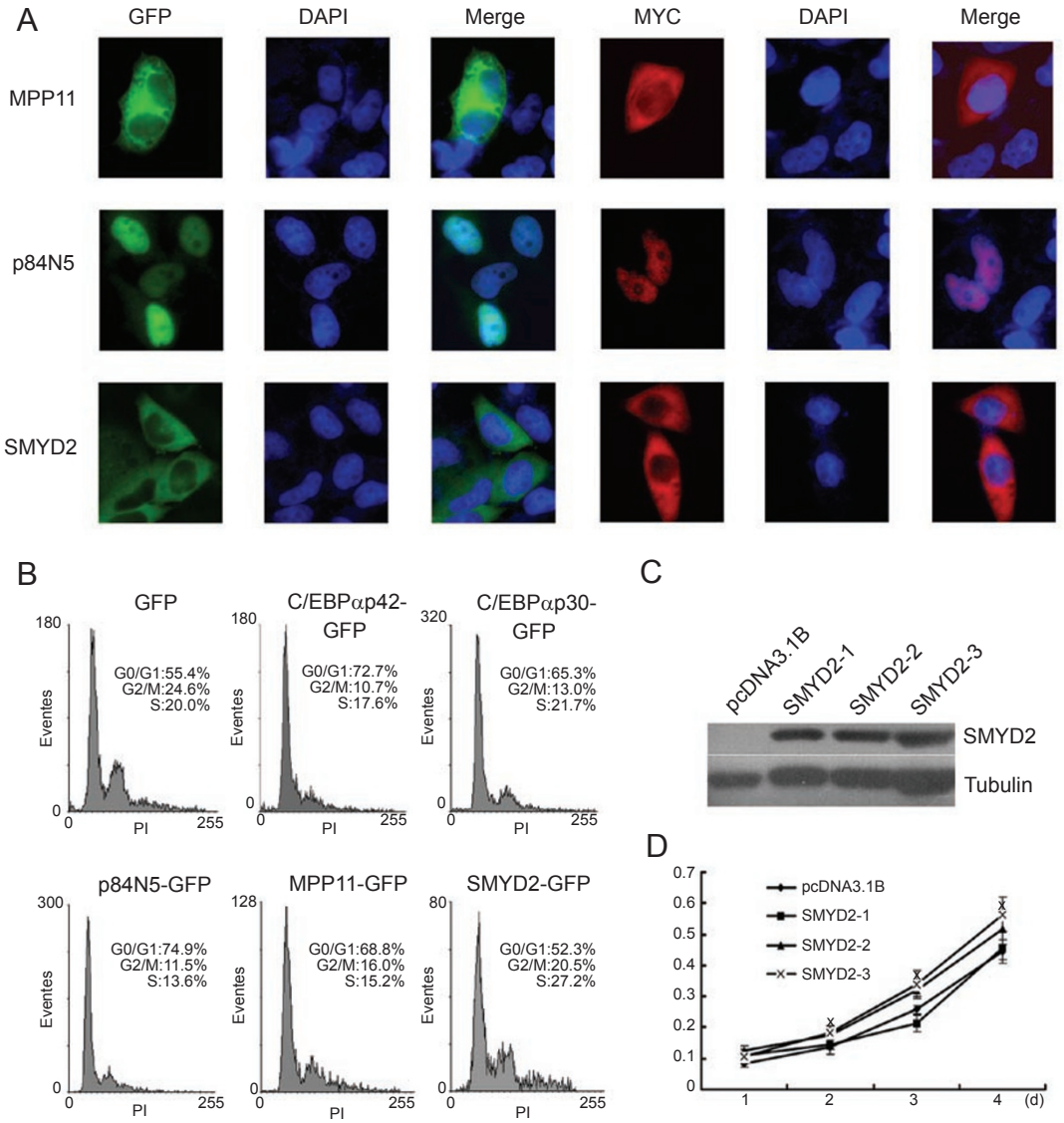

Figure 5 Effects of MPP11, p84N5 and SMYD2 overexpression on cell cycle progression and cell growth. **(A)** Subcellular localizations of MPP11, p84N5 and SMYD2 proteins were analyzed in BEL-7404 cells transfected with GFP-MPP11, GFP-p84N5, GFP-SMYD2, MPP11-myc, p84N5-myc and SMYD2-myc 48 h post-transfection. **(B)** BEL-7404 cells were transfected with pEGFP-N1, pGFP-C/EBPαp30, pGFP-C/EBPαp42, pGFP-p84N5, pGFP-MPP11 and pGFP-SMYD2. The distributions of GFP-positive cells at different cell cycle stages were analyzed using FACS 48 h post-transfection. **(C)** Western blot of SMYD2-myc in stably transfected BEL-7404 using the anti-myc antibody. The protein loading was controlled by reprobing with the anti-tubulin antibody. **(D)** Growth rate of cells stably transfected with SMYD2-myc or pcDNA3.1B (vector control) was measured by MTT assay.

yet. C/EBPαp30 lacks two of the three transactivation elements in C/EBPαp42 [39], and retains the negative regulatory region of C/EBPα [39-41], which might contribute to its unique regulatory function. ChIP assay revealed that C/EBPαp30 showed stronger binding ability to these promoters than C/EBPαp42, suggesting that C/EBPαp30 might regulate the transcription of these genes directly.

Efforts are being made to seek the detailed mechanism mediating the unique regulatory role of C/EBPαp30. Further distinguishing C/EBPαp30 function from C/EBPαp42 would help us to understand the role of this putative tumor suppressor in carcinogenesis.

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