PLOS ONE

RESEARCH ARTICLE

CCAAT-enhancer binding protein-α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) regulate expression of the human fructose-1,6-bisphosphatase 1 (FBP1) gene in human hepatocellular carcinoma HepG2 cells

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OPEN ACCESS

Citation: Wattanavanitchakorn S, Rojvirat P, Chavalit T, MacDonald MJ, Jitrapakdee S (2018) CCAAT-enhancer binding protein-α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) regulate expression of the human fructose-1,6-bisphosphatase 1 (FBP1) gene in human hepatocellular carcinoma HepG2 cells. PLoS ONE 13(3): e0194252. https://doi.org/10.1371/journal.pone.0194252

Editor: Gulam Waris, Rosalind Franklin University of Medicine and Science, UNITED STATES

Received: January 16, 2018

Accepted: February 27, 2018

Published: March 22, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by an international research network (grant IRN 59W0003), the Thailand Research Fund. S.W. was supported by an RGJ-PHD scholarship (4CMU56010), the Thailand Research Fund. The

Abstract

Fructose-1,6-bisphosphatase (FBP1) plays an essential role in gluconeogenesis. Here we report that the human FBP1 gene is regulated by two liver-enriched transcription factors, CCAAT-enhancer binding protein-α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) in human hepatoma HepG2 cells. C/EBPα regulates transcription of FBP1 gene via binding to the two overlapping C/EBPα sites located at nucleotide -228/-208 while HNF4α regulates FBP1 gene through binding to the classical H4-SBM site and direct repeat 3 (DR3) located at nucleotides -566/-554 and -212/-198, respectively. Mutations of these transcription factor binding sites result in marked decrease of C/EBPα- or HNF4α-mediated transcription activation of FBP1 promoter-luciferase reporter expression. Electrophoretic mobility shift assays of -228/-208 C/EBPα or -566/-554 and -212/-198 HNF4α sites with nuclear extract of HepG2 cells overexpressing C/EBPα or HNF4α confirms binding of these two transcription factors to these sites. Finally, we showed that siRNA-mediated suppression of C/EBPα or HNF4α expression in HepG2 cells lowers expression of FBP1 in parallel with down-regulation of expression of other gluconeogenic enzymes. Our results suggest that an overall gluconeogenic program is regulated by these two transcription factors, enabling transcription to occur in a liver-specific manner.

Introduction

The liver plays an important role in maintaining glucose homeostasis [1, 2]. During feeding, an increase in blood glucose triggers pancreatic beta cells to release insulin which acts on the liver to stimulate glycogen synthesis while inhibiting gluconeogenesis. In contrast, during fasting, low plasma glucose stimulates pancreatic alpha cells to release glucagon which acts on the

funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

liver by suppressing glycolysis and stimulating glycogen breakdown and gluconeogenesis [3, 4]. The latter pathway constitutes > 90% of hepatic glucose production during prolonged fasting [5]. Fructose-1,6-bisphosphatase (FBP) is one of the four gluconeognic enzymes. FBP catalyzes the dephosphorylation of fructose-1,6-bisphosphate to fructose-6-phosphate. Fructose-6-phosphate is then converted to glucose-6-phosphate immediately before being terminally converted to glucose by glucose-6-phosphtase. FBP activity is allosterically inhibited by AMP and fructose-2,6-bisphosphate [6,7]. The latter allosteric molecule is produced by the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PFK2/FBP2) when the level of insulin is high [8]. FBP is comprised of two isoforms, FBP1 and FBP2 [9, 10]. FBP1 is expressed in gluconeogenic tissues while FBP2 is expressed in skeletal muscle where it supports glycogen synthesis [11, 12]

Several studies in rodent models show that increased FBP1 is associated with diabetes [13–17]. Similar findings were also reported for humans with type 2 diabetes [18]. In addition to diabetes, FBP1 was also reported to be a tumor suppressor gene. Loss of function expression of FBP1 in many cancers results in accumulation of fructose-1,6-bisphosphate, increasing the levels of glycolytic intermediates which in turn drives the Warburg effect [19–22]. In the past decade, FBP1 draws an attention of being an attractive anti-diabetic drug because several FBP1 inhibitors have been reported, many of which can reduce plasma glucose during fasting and postprandial periods in obese and non-obese type 2 diabetic animals [23–28]. Although accumulating data show that overexpression of FBP1 is associated with hyperglycemia in diabetic patients, little is known how FPB1 expression is regulated at transcriptional level. Although the human FBP1 gene promoter has been cloned and some *cis*-acting elements that mediate basal transcription of FBP1 have been reported [29–30], neither of the study identified the transcription factors that implicate in hepatocyte-specific or energy metabolism. Here we identified for the first time that the hepatocyte nuclear factor 4α (HNF4α) and CCAAT-enhancer binding protein-α (C/EBPα) are important transcriptional regulators for FBP1 expression in HepG2 cells.

Materials and methods

Generation of human FBP1 promoter-luciferase reporter constructs and site-directed mutagenesis

The 886 nucleotides upstream of transcription start site together with the first 20 nucleotides downstream of transcription site of human FBP1 gene (-886/+20) were cloned from genomic DNA by a PCR technique using the hFBP1 forward and reverse primers (sequences shown in Table 1) designed from human FBP1 gene sequence deposited at NCBI (accession no. NT_008470). Five 5'-truncated hFBP1 gene promoter fragments consisting of 520, 420, 320, 220 and 120 nucleotides were generated by PCR using forward oligonucleotide primers that direct to different nucleotide positions at the 5'-end of the FBP1 promoter and the common reverse primer (hFBP1-Rev) using full length (906 bp hBP1) as a template. Oligonucleotide primers used to generate these mutants are shown in Table 1. The PCR was carried out in a 50 μl reaction mixture containing 1x PCR reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.5 μM each primer, 50 ng genomic DNA and 2.5 units *Taq* DNA polymerase in an automated thermal cycler MJ Mini (Bio-Rad). The PCR profile consisted of initial denaturation at 94˚C for 5 min followed by 30 cycles of denaturation at 94˚C for 45 sec, annealing at 60˚C for 45 sec and extension at 72˚C for 2 min, and final extension at 72˚C for 10 min. The PCR products were digested with *Sac*I and *Xho*I before ligation into *Sac*I and *Xho*I sites of the pGL4 luciferase reporter vector (Promega).

Table 1. Oligonucleotides used for generating wild type and mutant reporter constructs, EMSA and qPCR.

Oligonucleotide	Sequence (5'-3')	Construct/Gene name
5'-truncation constructs		
hFBP1-F	GAGCTCAAGCTTTTACTGAGGCCTCTGC	pGL4-886 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
-500hFBP1-F	GAGCTCCTCACATCTTGGAAATTCAAATACT	pGL4-500 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
400hFBP1-F	GAGCTCAGAAA CGGGGACTCTGTGTC	pGL4-400 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
-300hFBP1-F	GAGCTCCACGGGGCAGGAGCTGCA	pGL4-300 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
-200hFBP1-F	GAGCTCACTTCCGTT TTATGATTTTGGAGG	pGL4-200 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
-100hFBP1-F	GAGCTCGGGTGTGTGTGGGGGGCG	pGL4-100 hFBP1
hFBP1-R	CTCGAGGCTCCGCCTGCTTGGATCT	
Site-directed mutagenesis		
MutC/EBPα1 (-233/-204)-F	GGTGGCACATATGTTGTTACTTAACCTTTC	ΔC/EBPα1–300 hFBP1
MutC/EBPα1(-233/-204)-R	GAAAGGTTAAGTAACAACATATGTGCCACC	
MutC/EBPα2 (-228/-199)-F	CATTGAGCAAGCATA TGTTCCTTTCTGAAC	ΔC/EBPα2–300 hFBP1
MutC/EBPα2 (-228/-199)-R	GTTCAGAAAGGAACATATGCTTGCTCAATG	
MutC/EBPα1 and 2 (-228/-199)-F	CACATATGTTGCATATGTTCCTTTCTGAAC	ΔC/EBP1αΔC/EBPα2–300 hFBP1
MutC/EBPα1 and 2 (-228/-199)-R	GTTCAGAAAGGAACATATGCAACATATGTG	
MutHNF4α1 (-577/-548)-F	GTGGAGCCCTCTCATATGTGTGTGGTAGCC	ΔHNF4α1–886 hFBP1
MutHNF4α1 (-577/-548)-R	GGCTACCACACACATATGAGAGGGCTCCAC	
MutHNF4α2 (-367/-337)-F	AGAAGGGCCAGGCATATGCTTAGGCAGAGTG	ΔHNF4α2–886 hFBP1
MutHNF4α2 (-367/-337)-R	CACTCTGCCTAAGCATATGCCTGGCCCTTCT	
MutHNF4α3 (-222/-189)-F	GCAAGTTACTTAACCATATGGAACTTCCGTTTTA	ΔHNF4α3–886 hFBP1
MutHNF4α3 (-222/-189)-R	TAAAACGGAAGTTCCATATGGTTAAGTAACTTGC	
EMSA		
C/EBPα (-228/-208)-F*	GGTGGCATTGAGCAAGTTACTTAACCTTTCT	Probe and wild type competitor
C/EBPα (-228/-208)-R	AGAAAGGTTAAGTAACTTGCTCAATGCCACC	
C/EBP consensus-F*	CTCGCCTATTGCGCAAGGGGCCGGATC	
C/EBP consensus-R	GATCCGGCCCCTTGCGCAATAGGCGAG	
HNF4α1 (-569/-549)-F*	CCTCTGGCCTTTGTGTGGTAG	
HNF4α1 (-569/-549)-R	CTACCACACAAAGGCCAGAGG	
HNF4α2 (-361/-341)-F*	GGCCAGGTGACAGGCCAGGCA	
HNF4α2 (-361/-341)-R	TGCCTGGCCTGTCACCTGGCC	
HNF4α3 (-216/-194)-F*	TACTTAACCTTTCTGAACTTCCG	
HNF4α3 (-216/-194)-R	CGGAAGTTCAGAAAGGTTAAGTA	
qPCR		
Exon3 hFBP1-F	AGCCTTCTGAGAAGGATGCTC	FBP1
Exon3 hFBP1-R	GTCCAGCATGAAGCAGTTGAC	
PC-For	GATGACTTCACAGCCCAG	PC
PC-Rev	GGGCACCTCTGTGTCCAG	
PEPCK-C-F	CCACAGCGGCTGCAGAACAT	PEPCK-C
PEPCK-C-R	GAAGGGCCGCATGGCAA	
G6PC-F	GGGAAAGATAAAGCCGACCTAC	G6Pase
G6PC-R	CAGCAAGGTAGATTCGTGACAG	

(*Continued*)

Table 1. (Continued)

Oligonucleotide	Sequence (5'-3')	Construct/Gene name
HNF4-F	CAGGCTCAAGAAATGCTTCC	HNF4α
HNF4-R	GGCTGCTGTCCTCATAGCTT	
C/EBPα -F	TGGACAAGAACAGCAACGAGTA	C/EBPα
C/EBPα-R	ATTGTCACTGGTCAGCTCCAG	

*3′ labeled with biotin

Underline; restriction enzyme site

https://doi.org/10.1371/journal.pone.0194252.t001

The binding sites of C/EBPα (-218/-208 and -228/-218) or HNF4α (-212/-198, -359/-346 and -566/-554) in the hFBP1 promoter-luciferase reporter were mutated by Quick change site-directed mutagenesis (Stratagene Agilent Technologies) using the 320 nucleotides fragment of hFBP1 or the 906 bp nucleotides of hFBP1 promoter-reporter construct as template. The mutagenesis reaction was performed by PCR as described previously [31]. The primers used for site-directed mutagenesis are shown in Table 1. The clones containing corrected mutations were verified by nucleotide sequencing (Macrogen, South Korea).

Construction and expression of plasmids overexpressing C/EBPα or HNF4α protein

The full coding sequence of C/EBPα cDNA was PCR-amplified from the rat C/EBPα cDNA clone [32] using C/EBPα forward (5′-AAGCTTATGGAGTCGGCCGACTTCTAC-3′) and reverse primers (5′-CTCGAGTCACGCGCAGTTGCCCATGGC-3′) using the PCR conditions as described above. The PCR products were then digested with *Hind*III and *Xho*I before ligation into *Hind*III and *Xho*I sites of the pcDNA3 expression vector (Invitrogen). A bacterial and mammalian expression plasmid for 6Histidine tagged human HNF4α was used to prepare human HNF4α as described previously [31].

Cell culture, transient transfection and reporter assays

The human hepatocellular carcinoma cell line, HepG2 cells (ATCC: HB-8065) were obtained from Professor John Wallace, University of Adelaide, Australia. Cells were maintained in a complete medium [high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco)] supplemented with 28 mmol/l $NaHCO_3$, 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 100 units/ml penicillin-streptomycin (Gibco) at 37˚C in 5% CO_2 atmosphere. For transient transfection, $1x10^5$ cells were seeded into a 24-well cell culture plate containing 0.5 ml of antibiotic free-complete DMEM. After 24 h, cells were transfected with 0.2 pmol each of the hFBP1 promoter-luciferase reporter constructs and pRSV-β-gal vector expressing β-galactosidase and 0.1 pmol of plasmid encoding rat C/EBPα (pcDNA3-C/EBPα) or human HNF4α (pcDNA3-HNF4α) or empty vector (pcDNA3) using Lipofectamine^{TM} 2000 reagent (Invitrogen) as described previously [31]. The transfected cells were incubated at 37˚C in 5% CO_2 for 48 h prior to harvesting for subsequent analysis. Luciferase enzyme activity was measured using luciferase assay reagent (Promega) and β-galactosidase activity was assayed using ONPG as substrate as described previously [31].

For overexpression studies, $5x10^5$ HepG2 cells were seeded into a 6-well cell culture plate before transfection with 1.6 pmol of C/EBPα-pcDNA3, hHNF4α-pcDNA3 or empty vector in the presence of 10 μl of Lipofectamine 2000 reagent. The transfected cells were maintained as described above before being harvested for Western blot analysis and EMSA.

Electrophoretic mobility-shift assays (EMSAs) and ChIP assays

Nuclear proteins were prepared from HepG2 cells overexpressing C/EBPα. Cells were washed once with cold PBS, scraped off and centrifuged at 3000 x g at 4˚C for 5 min. The cell pellet was resuspended in 1 ml of cold buffer 1, containing 10 mM HEPES-KOH buffer, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1x protease inhibitor (Roche) and incubated on ice for 15 min. Fifty microlitres of 10% (v/v) NP-40 was added to the cell suspension before centrifugation at 3000x g at 4˚C for 3 min. The pellet was resuspended in 50 μl of cold buffer 2 containing 20 mM HEPES-KOH buffer, pH 7.9, 25% (v/v) glycerol, 0.2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$ and 420 mM NaCl, and incubated on ice for 40 min. After centrifugation at 12,000 x g at 4˚C for 15 min, the supernatant was collected and stored at -80˚C until used. The 3'-labeled biotinylated oligonucleotides (Biobasic, Canada) were annealed with their unlabeled complementary strands in 1x annealing buffer (10 mM Tris pH 7.4, 1 mM EDTA and 50 mM NaCl). The oligonucleotides used in EMSA are shown in Table 1. The DNA-protein binding reaction was performed in a 20 μl-reaction mixture containing 1x binding buffer (15mM HEPES pH 7.9, 50mM KCl, 1mM $MgCl_2$), 2 μg of poly (dI-dC), 0.05% NP-40, 2.5% (v/v) glycerol, 60–480 fmole of biotinylated labeled double-stranded oligonucleotide probe and 5 μg of nuclear extract or 0.2 μg of purified 6xHis hHNF4α [31] at 4˚C for 30 min. For the competition assays, excess amounts of unlabeled double stranded oligonucleotide (competitor) were included in the binding reaction mixture at 4˚C for 30 min before adding probe. For supershift assays, 0.2 μg of rabbit anti-C/EBPα (sc-61) polyclonal antibody (SantaCruz Biotech) or anti-HNF4α (sc-8987) polyclonal antibody (Santa Cruz Biotech) was included in the reaction mixture at 4˚C for 30 min before adding probe. The DNA-protein complexes were analyzed by 5% non-denaturing polyacrylamide gel electrophoresis followed by electroblotting. The DNA-protein complexes were then detected using Lightshift Chemiluminescent EMSA kit (Pierce) and visualized by Gel Doc System (GeneTools).

siRNA transfections

5 x 10^6 HepG2 cells were transfected with 5 nM siRNA target to human C/EBPα or human HNF4α (Qiagen) using X-treamGene siRNA transfection reagent (Roche). At 48 h post-transfection, the transfected cells were harvested and total RNAs were extracted. Expression of C/EBPα, HNF4α, pyruvate carboxylase (PC), PEPCK-C, G6Pase1 and FBP1 were analyzed by qPCR as described above. The PCR primers used for detection expression of these genes are shown in Table 1.

Quantitative real time RT-PCR (qPCR)

Total RNA was isolated from HepG2 or Huh7 cells using TRIzol®Reagent (Gibco) according to manufacturer's instructions. Reverse transcription was performed using the Improm-II™ Reverse Transcription system (Promega) following manufacturer's instructions in which 20 μl of reaction mixture contained 2 μg of total RNA, 0.2 μg random hexamers,1x ImProm-II™ reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTP mix and 1 μl of ImProm-II™ reverse transcriptase. The reaction was incubated at 25˚C for 5 min before shifting to 42˚C for 60 min and terminated at 70˚C for 15 min. The cDNA was stored at -20˚C until used.

Quantitative gene expression analysis was performed using an Mx3000P™ Real-Time PCR System. Each amplification reaction was performed in a 12 μl of reaction mixture containing 1x Master mix (KAPA™SYBR®Fast), 0.2 μM each of primer, 2 μl of cDNA. Thermal profiles consisted of an initial denaturation at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec and dissociation at 95˚C for 1 min, 55˚C for 30 sec and 95˚C for 30 sec. Expression of 18s rRNA gene was used

to normalize the expression of FBP1. Expression data were calculated from the cycle threshold (Ct) value using the ΔCt method of quantification. Oligonucleotides used for qPCR are listed in Table 1.

Western blot analysis

50 μg of nuclear proteins were separated by 10% discontinuous SDS–PAGE under reducing conditions [33]. Proteins were transferred to a PVDF membrane using semi-dry blotting and subjected to immunological detection. The C/EBPα band was detected by rabbit anti-C/EBPα polyclonal antibody while the HNF4α band was detected by anti-HNF4α polyclonal antibody. Anti-β-actin monoclonal antibody was used for normalizing protein loading. The immunoreactive bands were visualized upon adding mouse anti-rabbit IgG or ratbbit anti-mouse IgG secondary antibodies followed using the enhanced chemiluminescence detection system (Pierce).

Statistical analysis

All data are presented as the means ± SD from three independent experiments. Statistical significance between samples was determined by using one way analysis of variance, Sigma Stat 3.5.

Results

Induction of human FBP1 expression by C/EBPα and HNF4α in HepG2 cells

A previous study identified some ubiquitous transcription factors such as Sp1, USF1, USF2 and NF-κB that bind to their cognate sequences (Fig 1A) located in the proximal region of the human FBP1 promoter [30]. However, the transcription factors that regulate liver specific energy metabolism were not identified. Using PROMO [34] and JASPAR databases [35], which predict regulatory elements in eukaryotic promoters, we were able to identify two putative binding sites for the CCAAT-enhancer binding protein-α (C/EBPα), located at nucleotides -228/-218 (designated C/EBPα1 site: 5′-ATTGAGCAAG-3′) and -218/-208 (C/EBPα2 site: 5′-GTTACTTAAC-3′), and three binding sites for hepatocyte nuclear factor-4α, located at nucleotides -556/-554 (designated HNF4α1 site: 5′-TGGCCTTTGTGTG-3': antisense strand), (HNF4α2 site: 5′-AGGTGACAGGCCA-3': sense strand) and the -212/-198 (HNF4α site3: 5′-TAACCTTTCTGAACT-3′: antisense strand) (Fig 1A).

To examine whether these two transcription factors can regulate FBP1 expression, C/EBPα or HNF4α was overexpressed in hepatocyte HepG2 cells. As shown in Fig 1B, both transcription factors were successfully overexpressed in HepG2, resulting in 3-fold and 4-fold increase in the levels of hFBP1 mRNA expression (Fig 1C), respectively. Similar results were obtained when HNF4α or C/EBP1α was overexpressed in Huh7, another human hepatocyte cell line (Fig 1D). These results indicate that both HNF4α and C/EBPα can act as activators of FBP1 transcription in human hepatocytes.

C/EBPα regulates expression of human FBP1 through two C/EBPα binding sites

To further investigate whether this positive effect on FBP1 expression is mediated through the above C/EBPα binding sites, we performed transactivation assays in which the -886 human FBP1 promoter-luciferase reporter construct containing the first 886 nucleotides of hFBP1 promoter (pGL4-886hFBP1) was co-transfected with plasmid overexpressing C/EBPα into

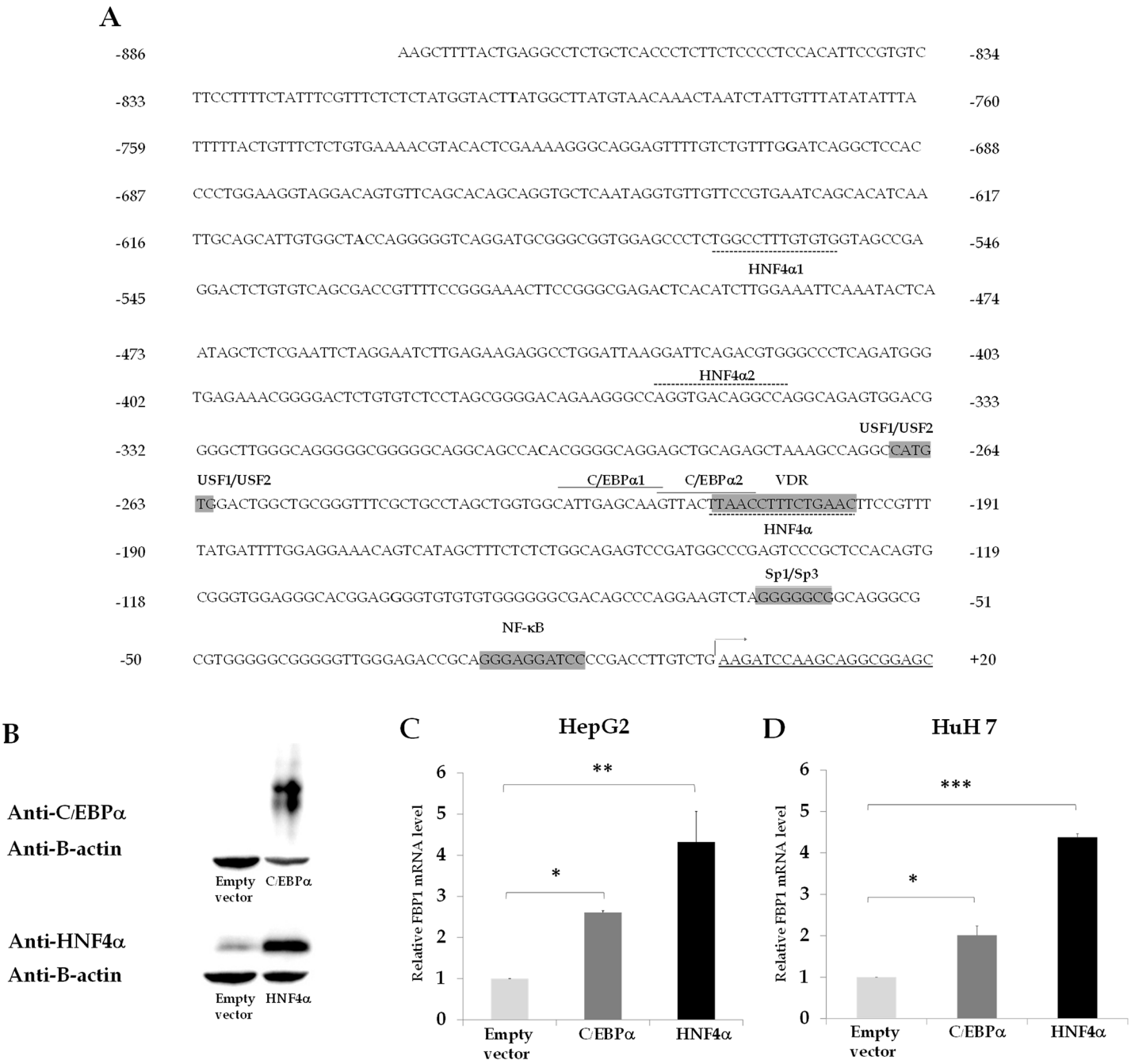
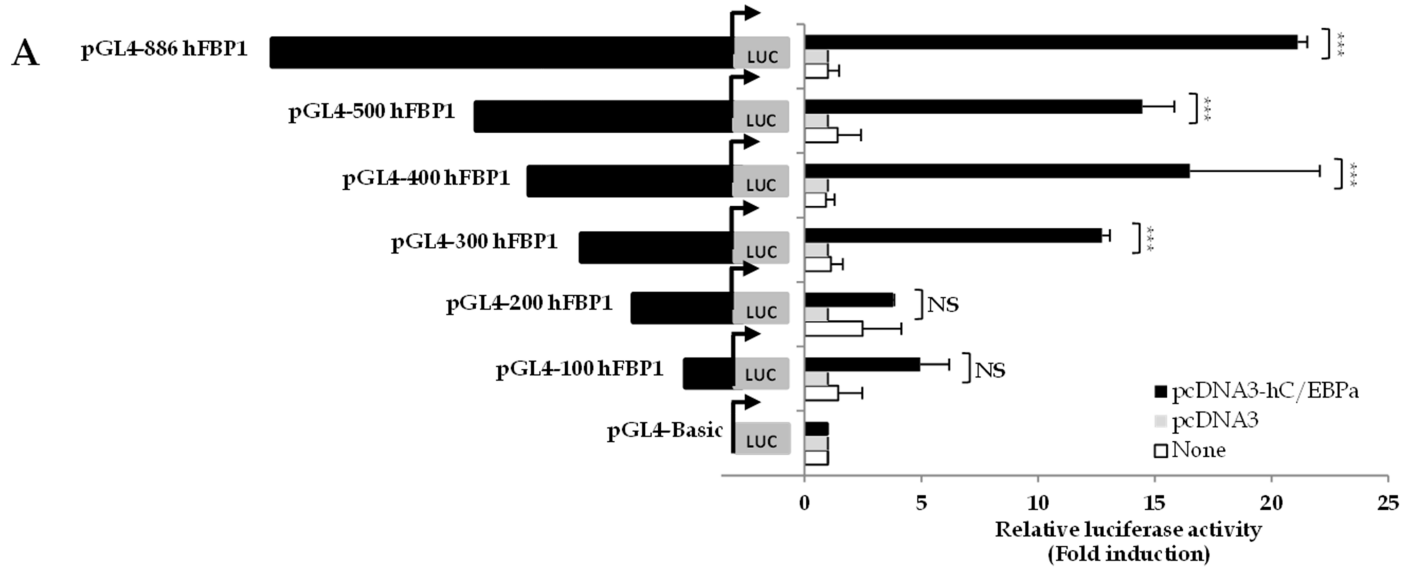


Fig 1. Human FBP1 gene promoter and induction of hFBP1 expression in HepG2 by C/EBPα or HNF4α. (A) Nucleotide sequence of human FBP1 promoter with various putative transcription factor binding sites identified by PROMO [34] and JASPAR databases [35]. The binding sites of previously identified transcription factors including Sp1, USFs, NF-κB [31] and VDRE [36, 37] are also included. (B) Western blot analysis of HepG2 cells transfected with pcDNA3 (empty vector), pcDNA3-C/EBPα or pcDNA3-HNF4α using anti-C/EBPα or anti-HNF4α antibodies. Control loading was assessed by probing the blot with anti-β-actin antibody. (C) qRT-PCR of FBP1 expression of HepG2 cells transfected with empty vector, C/EBPα or HNF4α. Human FBP1 mRNA expression was normalized with that of 18s rRNA, and shown as relative expression. (D) qRT-PCR of FBP1 expression in Huh7 cells transfected with empty vector, C/EBPα or HNF4α. The values obtained from cells transfected with pcDNA3-C/EBPα or pcDNA3-HNF4α were relative to that obtained from cells transfected with empty vector, which was arbitrarily set as 1. The values shown are means ± standard deviation of three independent experiments (n = 3). The statistical analysis was conducted by ANNOVA test where $^{*}p < 0.01$ and $^{**}p < 0.05$.

https://doi.org/10.1371/journal.pone.0194252.g001

HepG2 cells. As shown in Fig 2A, overexpression of C/EBPα resulted in a 20-fold increase in the luciferase activity from the FBP1 promoter-luciferase reporter construct. Truncations of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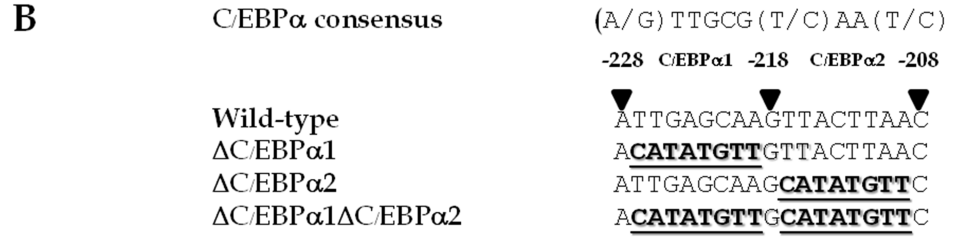
pGL4-886 hFBP1
pGL4-500 hFBP1
pGL4-400 hFBP1
pGL4-300 hFBP1
pGL4-200 hFBP1
pGL4-100 hFBP1
pGL4-Basic
LUC
***
***
***
***
NS
NS
pcDNA3-hC/EBPa
pcDNA3
None
0
5
10
15
20
25
Relative luciferase activity
(Fold induction)


B
C/EBPα consensus
(A/G)TTGCG(T/C)AA(T/C)
-228
C/EBPα1
-218
C/EBPα2
-208
Wild-type
ATTGAGCAAGTTACTTAAC
ΔC/EBPα1
ACATATGTTGTTACTTAAC
ΔC/EBPα2
ATTGAGCAAGCATATGTTC
ΔC/EBPα1ΔC/EBPα2
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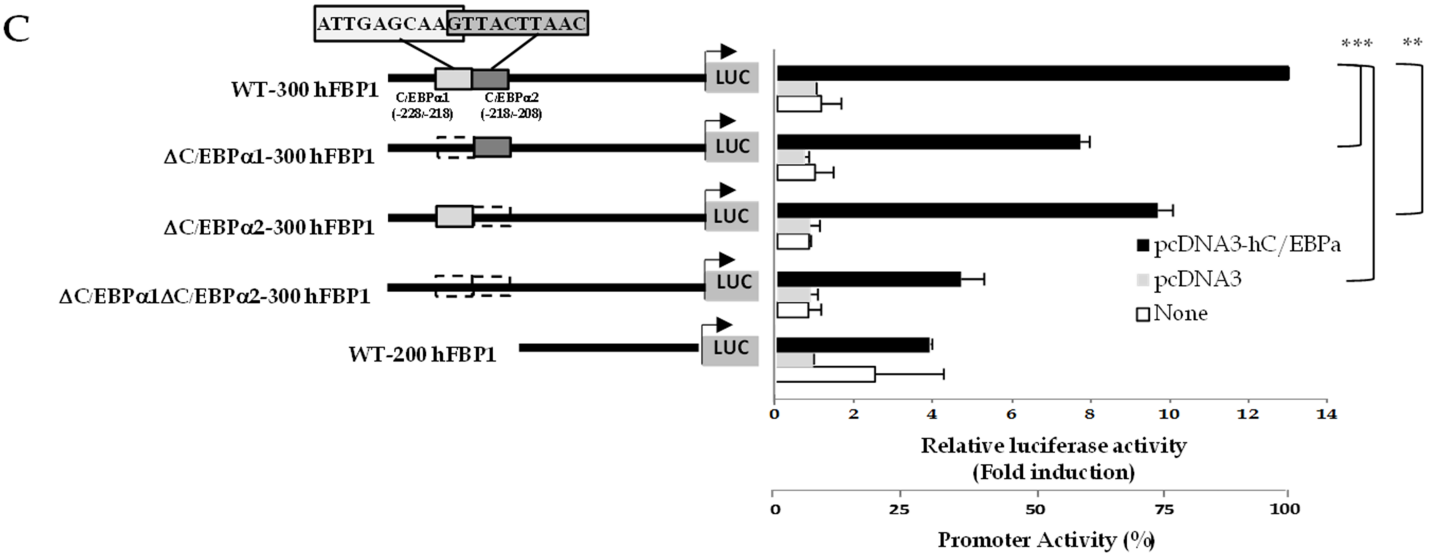
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WT-300 hFBP1
C/EBPα1
(-228/-218)
C/EBPα2
(-218/-208)
ΔC/EBPα1-300 hFBP1
ΔC/EBPα2-300 hFBP1
ΔC/EBPα1ΔC/EBPα2-300 hFBP1
WT-200 hFBP1
LUC
***
**
pcDNA3-hC/EBPa
pcDNA3
None
0
2
4
6
8
10
12
14
Relative luciferase activity
(Fold induction)
0
25
50
75
100
Promoter Activity (%)

Fig 2. Identification of functional C/EBPα binding sites in human FBP1 promoter. (A) Transactivation of 5'-truncated hFBP1 promoter-luciferase reporter construct by C/EBPα in HepG2 cells. The 886 nucleotides-hFBP1 promoter-luciferase reporter gene or its 5'-truncated constructs (500, 400, 300, 220 and 100 nucleotides) were transiently co-transfected with empty vector (pcDNA3; grey bar) or plasmid overexpressing C/EBPα (pcDNA3-C/EBPα; black bar) into HepG2. The luciferase activity of wild type or mutant construct was normalized with β-galactosidase activity shown as relative luciferase activity. Relative luciferase activity obtained from the cells transfected with the hFBP1-promoter-luciferase constructs and plasmid encoding C/EBPα protein was presented as "fold change" relative to those transfected with hFBP1-promoter-luciferase construct and empty vector, which was arbitrarily set as 1. (B) Nucleotide sequences of two overlapping C/EBPα binding designated C/EBPα1 (-227/-218) and C/EBPα2 (-218/-209) in hFBP1 promoter and its mutagenic sequences in ΔC/EBPα1 and ΔC/EBPα2 or double mutant ΔC/EBPα1 ΔC/EBPα2. Underline indicates nucleotide changes of each mutant. (C) Effect of mutations of two overlapping C/EBPα binding sites on C/EBPα transactivation of FBP1 promoter activity. Single or double mutations of C/EBPα1 and C/EBPα2 sites in 300 hFBP1 promoter-reporter construct and co-transfected with empty vector (pcDNA3; grey bar) or vector containing C/EBPα (pcDNA3-C/EBPα; black bar) into HepG2 cells. The luciferase activity of each construct was normalized to β-galactosidase activity and expressed as relative luciferase activity. Relative luciferase activity obtained from cells transfected with WT or mutated FBP1 promoter-luciferase and plasmid overexpressing C/EBPα was presented as fold change relative to those transfected with the parental or mutated FBP1-luciferase reporter and pcDNA empty vector, which was arbitrarily set as 1. The values shown are means ± standard deviation of three independent experiments (n = 3). The statistical analysis was conducted by ANNOVA test where $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$.

https://doi.org/10.1371/journal.pone.0194252.g002

the upstream sequences of FBP1 promoter to nucleotide positions -500 (pGL4-500hFBP1), -400 (pGL4-400hFBP1) and -300 (pGL4-300hFBP1) resulted in the further reduction of C/EBPα-mediated activation of luciferase activity to about 12-15- fold. However, further deletion of the 5'-end to nucleotide positions -200 (pGL4-200hFBP1) and -100 (pGL4-100hFBP1) resulted in only 3-fold induction by C/EBPα, suggesting that the major determinant for C/EBPα response is located between nucleotides -300 and -200, corresponding to the two putative C/EBPα sites at -228/-218 (C/EBPα1) and -218/-208 (C/EBPα2). To further examine which of these two sites confers the C/EBPα response, we mutated C/EBPα site 1 or 2 to unrelated sequences (Fig 2B) in the pGL4-300hFBP1 construct that contains these two C/EBPα sites and co-transfected them with a plasmid encoding C/EBPα. As shown in Fig 2C, mutations of the C/EBPα1 (pGL4-ΔC/EBPα1) or C/EBPα2 (pGL4-ΔC/EBPα2) decreased the promoter activity by 40% and 25%, respectively, while double mutation of both sites (ΔC/EBPα1ΔC/EBPα2) further reduced the promoter activity by 65%. The marked reduction of C/EBPα response in the double mutation construct was also similar to the pGL4-200hFBP1 that lacks both C/EBPα binding sites, suggesting that both C/EBPα1 and C/EBPα2 sites act cooperately to maximize FBP1 expression in HepG2 cells.

To confirm whether C/EBPα indeed binds to any of these two C/EBPα sites, EMSA was performed using double stranded oligonucleotide spanning these two sites (C/EBPα-hFBP1), compared with a consensus C/EBPα binding site [38] (see sequence in Fig 3A) and a nuclear extract of HepG2 cells overexpressing C/EBPα. As shown in Fig 3B, the FBP1 probe harboring both C/EBPα sites produced a predominant DNA-protein complex (lane 1). Addition of 5x, 10x and 50x unlabeled C/EBPα-FBP1 probe gradually decreased the complex formation. Incubation of the binding reaction in the presence of anti-C/EBPα antibody markedly prevented the complex formation concomitant with the formation of a supershifted band (lane 5). A similar pattern of DNA-protein binding was observed when the consensus C/EBPα probe was incubated with the nuclear extract of HepG2 cells overexpressing C/EBPα (lane 6). Similar to C/EBPα -FBP1 probe, addition of an unlabeled consensus C/EBPα probe or the C/EBPα -FBP 1 probe (lanes 7 and 8) in the binding reaction eliminated the formation of DNA-protein complex and addition of anti-C/EBPα antibody produced a supershifted band (lane 9).

HNF4α regulates expression of human FBP1 through an HNF4-specific binding motif (H4SBM) and direct repeat DR3

We next investigated whether the stimulatory effect of HNF4α on endogenous FBP1 expression is mediated through the three putative HNF4α binding sites shown in Fig 2. We performed 5'- truncation analysis of the FBP1 promoter to localize the *cis*-acting element(s) that mediates HNF4α activation. The same set of 5'-truncated FBP1 promoter mutant constructs

A

Probe / Competitor

C/EBP consensus CTCGCCTATTGCGCAAGGGGCCGGATC

C/EBPα-hFBP1 GGTGGCATTGAGCAAGTTACTTAACCTTTCT

Site 1 (-228/-218)

Site 2 (-218/-208)

B

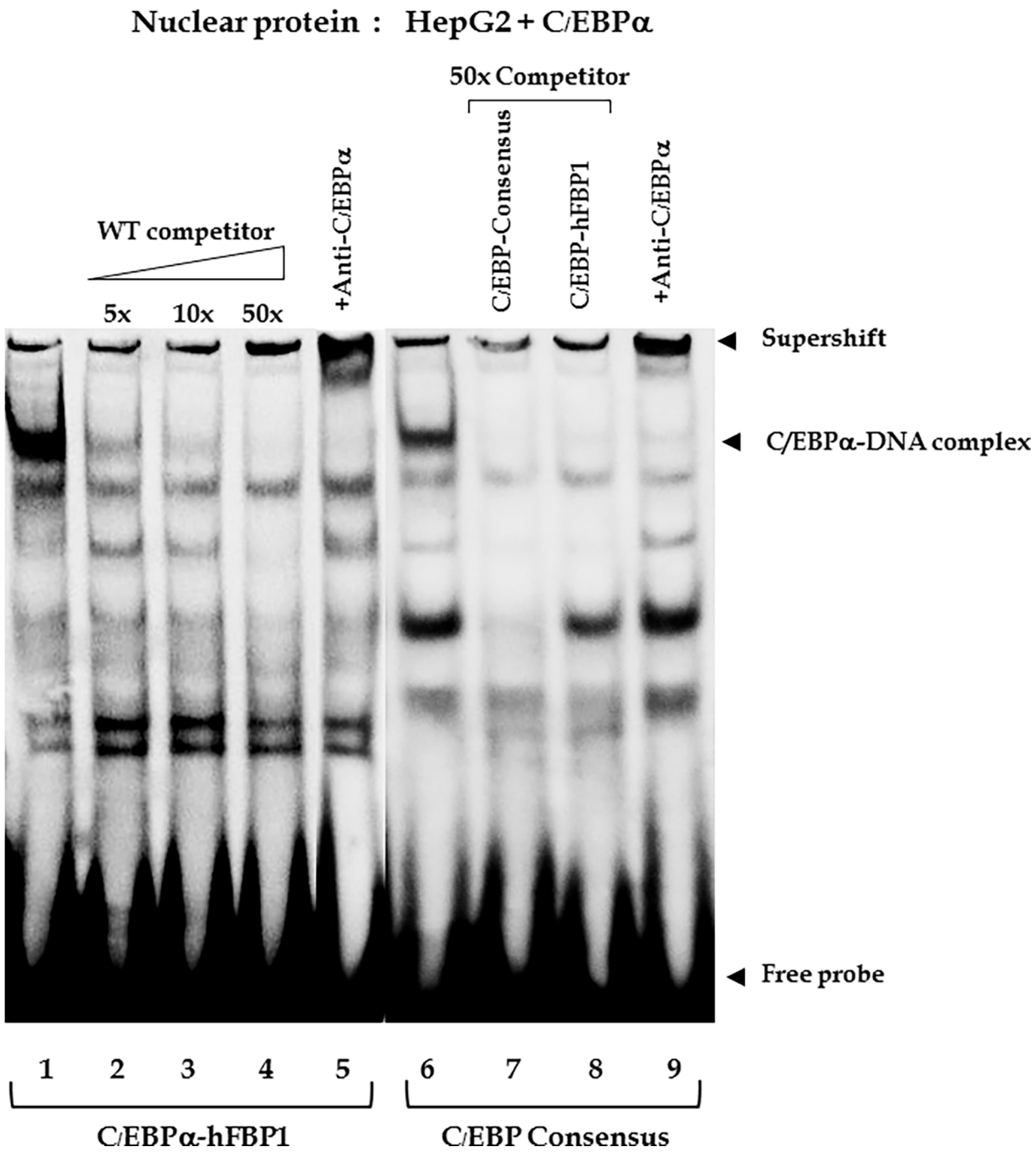
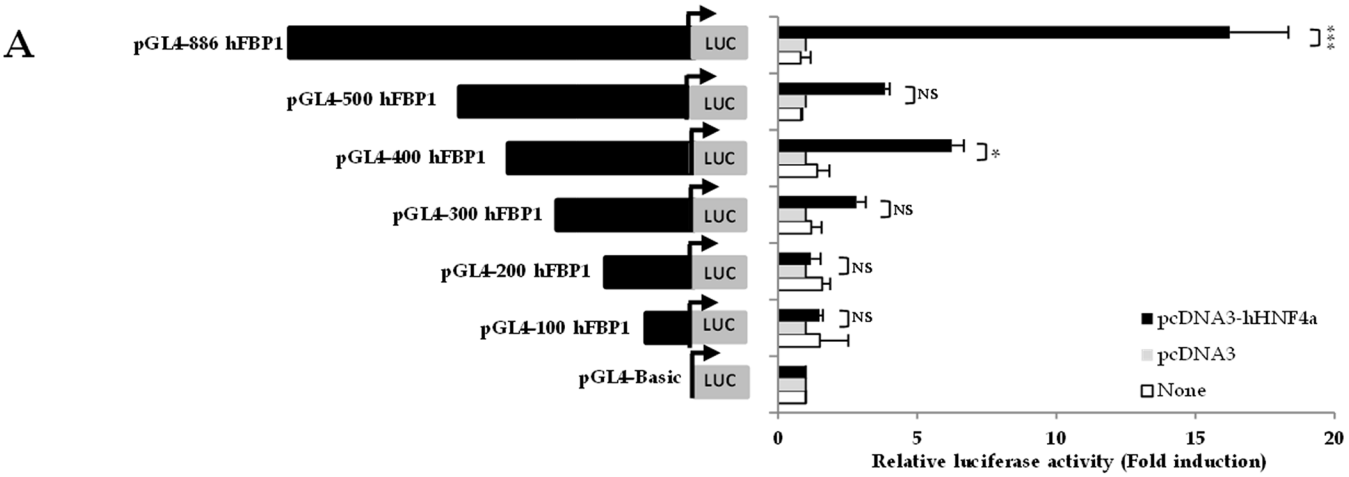


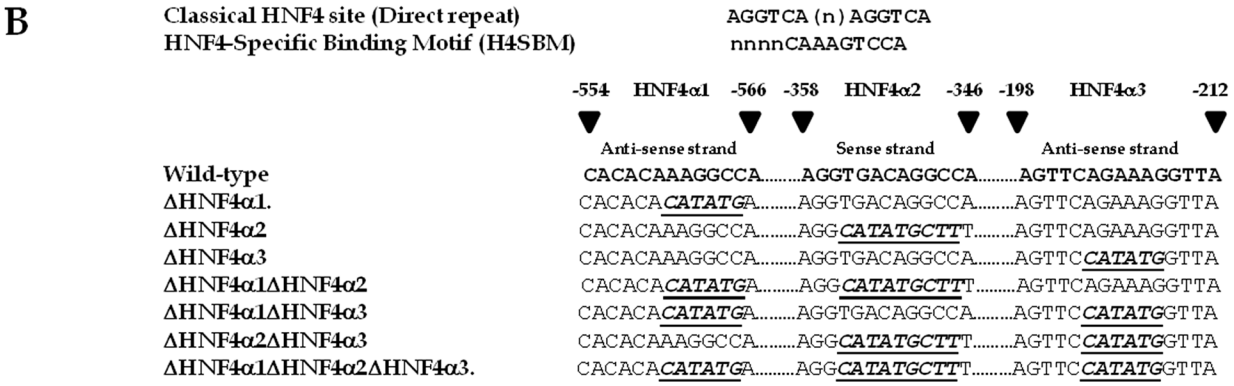
Fig 3. EMSA of C/EBPα binding site in humanFBP1 promoter. (A) Oligonucleotide sequences of C/EBPα consensus binding site and two overlapping C/EBPα binding sites (-227/-218 and -218/-209) in hFBP1promoter used in EMSA. Underline shows the core binding site. (B) EMSA of -227/-218 and -218/-209 C/EBPα binding site of FBP1 promoter comparing with consensus C/EBPα binding site or C/EBPα-hFBP1 site. Biotin-labeled double stranded containing two overlapping C/EBPα binding sites of FBP1 with nuclear extract of HepG2 cells overexpressing C/EBPα (lanes 1 and 5), together with 5x, 10x, 50x excess unlabeled C/EBPα-FBP1 probe (lanes 2–4), or with xx unlabeled consensus C/EBPα probe (lane 6) or with anti-C/EBPα antibody (lane 7). Biotin-labeled C/EBPα consensus sequence was also used as a positive control of which this probe was incubated with nuclear extract of HepG2 cells overexpressing C/EBPα alone (lane 8) or together with unlabeled consensus C/EBPα (lane 9), or unlabeled C/EBPα-FBP1 (lane 10) or with anti-C/EBPα antibody (lane 11).

https://doi.org/10.1371/journal.pone.0194252.g003

that were used to identify C/EBPα binding sites were co-transfected with a plasmid overexpressing HNF4α. As shown in Fig 4A, truncation of the FBP1 promoter from nucleotide positions -886 to -500 resulted in a marked reduction of luciferase activity (65%), indicating the presence of the first HNF4α-responsive sequence between these nucleotides. However, further deletion to -400 resulted in a slight increase of the reporter activity while further deletion to -300 resulted in 60% reduction of luciferase activity, suggesting the presence of a second HNF4α-responsive element. Likewise, further truncation to -200 lowered the reporter activity by 50%, indicating the presence of the third HNF4α-responsive element, locating between nucleotides -300 to -200. The presence of three HNF4α-responsive regions between nucleotides -856 to -500, -400 to -300 and -300 to -100 is consistent with the three HNF4α binding sites shown in Fig 1. It is noted that the -556/-554 HNF4α (HNF4α1 site: 5′-TGGCCTTTG TGTG-3′: antisense strand) resembles the HNF4-specific binding motif (H4SBM: 5′-NNN NCAAAGTCCA-3′) as described by Fang *et al*., 2012 [39] except it contains one nucleotide divergent from the H4SBM consensus sequence (underlined). In contrast, the -359/-346 (HNF4α2 site: 5′-AGGTGAcAGGCCA-3′: sense strand) and the -212/-198 (HNF4α site3: 5′-TAACCTttcTGAACT-3′: antisense strand) are similar to the classical direct repeat 1 (DR1) and DR3, respectively for tor the nuclear receptor [5′-AGGTCA$(N)_{1\text{-}3}$AGGTCA-3′] for the nuclear receptors (NR) [40]. However, both HNF4α2 and HNF4α3 sites contain two nucleotides different (underlined) from the DR motif. We next confirmed the functional importance of these sites by mutating them to the sequences shown in Fig 4B and used them for the transactivation assay with HNF4α. As shown in Fig 4C, single mutation of HNF4α1, HNF4α2 and HNF4α3 site decreased HNF4α mediated transactivation of FBP1 promoter activity by 75%, 35% and 50%, respectively. Mutations of HNF4α1 together with HNF4α2 or HNF4α3 decreased the reporter activity that was similar to the single HNF4α1 mutation while double mutation of HNF4α2 and HNF4α3 sites lowered reporter activity by 50%. Mutations of three HNF4α binding sites resulted in 80% reduction of HNF4α-mediated transactivation activity. These results indicated that the functional importance of HNF4α binding sites with respect to HNF4α- transactivation activity being HNF4α1 > HNF4α3 > HNF4α2, respectively.

To confirm binding of HNF4α to these three sites, electrophoretic mobility shift assays (EMSA) were performed by incubating various concentration of oligonucleotide probes harboring HNF4α1, HNF4α2 or HNF4α3 sites (60, 120, 240, 360 and 480 fmol) with a limited amount (200 ng) of purified hHNF4α (Fig 5A). Quantification of the HNF4α-DNA complex bands in Fig 5B demonstrated that HNF4α bound to site 3 with slightly higher affinity than to site 1 while its affinity for site 2 was much lower. Although HNF4α binds to HNF4α1 and HNF4α3 sites with slightly different affinities, it seems that both sites are important for HNF4α-mediated activation of hFBP1 promoter activity as indicated by mutation of either sites produced a great effect on expression of the reporter gene in Fig 4. Conversely, this EMSA showed the poor binding of HNF4α to the HNF4α2 site, suggesting that HNF4α2 site is the insignificant binding site for HNF4α. Analysis of chromatin immunoprecipitation sequence (ChIP seq) of HNF4α in genomic DNA of adult liver cells mapped by ENCODE


A
pGL4-886 hFBP1
pGL4-500 hFBP1
pGL4-400 hFBP1
pGL4-300 hFBP1
pGL4-200 hFBP1
pGL4-100 hFBP1
pGL4-Basic
LUC
***
NS
*
NS
NS
NS
pcDNA3-hHNF4a
pcDNA3
None
0
5
10
15
20
Relative luciferase activity (Fold induction)


B
Classical HNF4 site (Direct repeat)
AGGTCA (n) AGGTCA
HNF4-Specific Binding Motif (H4SBM)
nnnnCAAAGTCCA
-554
HNF4α1
-566
-358
HNF4α2
-346
-198
HNF4α3
-212
Anti-sense strand
Sense strand
Anti-sense strand
Wild-type
CACACAAAGGCCA.....AGGTGACAGGCCA.....AGTTCAGAAAGGTTA
ΔHNF4α1.
CACACACATATGA.....AGGTGACAGGCCA.....AGTTCAGAAAGGTTA
ΔHNF4α2
CACACAAAGGCCA.....AGGCATATGCTTT.....AGTTCAGAAAGGTTA
ΔHNF4α3
CACACAAAGGCCA.....AGGTGACAGGCCA.....AGTTCCATATGGTTA
ΔHNF4α1ΔHNF4α2
CACACACATATGA.....AGGCATATGCTTT.....AGTTCAGAAAGGTTA
ΔHNF4α1ΔHNF4α3
CACACACATATGA.....AGGTGACAGGCCA.....AGTTCCATATGGTTA
ΔHNF4α2ΔHNF4α3
CACACAAAGGCCA.....AGGCATATGCTTT.....AGTTCCATATGGTTA
ΔHNF4α1ΔHNF4α2ΔHNF4α3.
CACACACATATGA.....AGGCATATGCTTT.....AGTTCCATATGGTTA

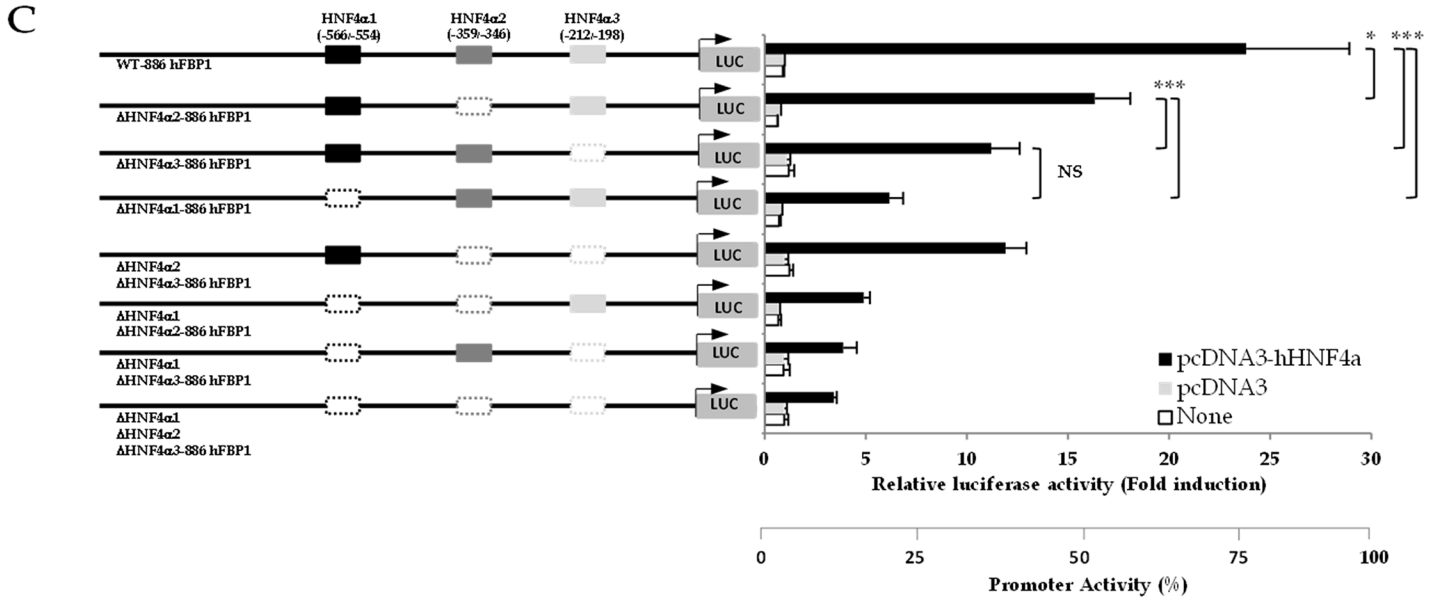

C
HNF4α1
(-566/-554)
HNF4α2
(-359/-346)
HNF4α3
(-212/-198)
WT-886 hFBP1
ΔHNF4α2-886 hFBP1
ΔHNF4α3-886 hFBP1
ΔHNF4α1-886 hFBP1
ΔHNF4α2
ΔHNF4α3-886 hFBP1
ΔHNF4α1
ΔHNF4α2-886 hFBP1
ΔHNF4α1
ΔHNF4α3-886 hFBP1
ΔHNF4α1
ΔHNF4α2
ΔHNF4α3-886 hFBP1
LUC
*
***
***
NS
pcDNA3-hHNF4a
pcDNA3
None
0
5
10
15
20
25
30
Relative luciferase activity (Fold induction)
0
25
50
75
100
Promoter Activity (%)

Fig 4. Identification of HNF4α binding sites in hFBP1 promoter. (A) Transactivation of 5'-truncated hFBP1 promoter-luciferase reporter construct by HNF4α in HepG2 cells. The 886 nucleotides-hFBP1 promoter-luciferase reporter gene or its 5'-truncated mutants (500, 400, 300, 200 and 100) were transiently co-transfected with empty vector (pcDNA3; grey bar) or plasmid overexpressing HNF4α (pcDNA3-HNF4α; black bar) into HepG2. The luciferase activity of wild type or mutant construct was normalized with β-galactosidase activity and shown as relative luciferase activity. Relative luciferase activity obtained from cells co-transfected with the hFBP1-promoter-luciferase constructs and plasmid encoding HNF4α was presented as "fold change" relative to those transfected with hFBP1-promoter-luciferase construct and empty vector, which was arbitrarily set as 1. (B) Nucleotide sequences of consensus HNF4α binding sites including classical DR1 and H4-SBM and three HNF4α binding sites (HNF4α1 (-566/-554, antisense strand), HNF4α2 (-358/-346; sense strand) and HNF4α3 site (-212/-198; antisense strand)) in hFBP1 promoter also shown (C) Effect of mutations of three HNF4α binding sites on FBP1 promoter activity. Mutations of the HNF4α1, HNF4α2 and HNF4α3 sites were introduced in the 886 FBP1 promoter-reporter construct and co-transfected with empty vector (pcDNA3; grey bars) or vector containing human HNF4α (pcDNA3-HNF4α; black bars) into HepG2 cells. The luciferase activity of each construct was normalized to β-galactosidase activity and expressed as relative luciferase activity. The values obtained from mutated constructs are expressed relative to the corresponding parental or mutant construct which was arbitrarily set as 100%. The values shown are means ± standard deviation (n = 3). $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$.

https://doi.org/10.1371/journal.pone.0194252.g004

project [41] reveals the presence of one broad peak covering the transcription start site to the first 600 nucleotides upstream of FBP1 gene which includes HNF4α1(-566/-554, antisense strand), HNF4α2 (-358/-346) and HNF4α3 (-212/-198) (Fig 5C) sites, confirming the prediction by JASPAR prediction, reporter assay and EMSA.

siRNA-suppression of HNF4α and C/EBPα lowered expression of FBP1 and other gluconeogenic enzymes

We next asked whether down-regulation of C/EBPα or HNF4α expression would affect expression of FBP1 expression in HepG2 cells. HepG2 cells were transiently transfected with siRNAs targeted to C/EBPα and HNF4α and the expression of FBP1 and other gluconeogenic enzymes including PC, PEPCK-C, and G6PaseI were measured by qPCR. As shown in Fig 6, knocking down of C/EBPα expression by 80% resulted in 35%, 55% and 65% reduction of FBP1, PEPCK-C and G6PaseI mRNA expression, respectively while minimally affected expression of PC mRNA. In contrast, siRNA-mediated suppression of HNF4α resulted in simultaneous down-regulation of FBP1, PC, PEPCK-C and G6Pase1 by 45%, 30%, 60% and 40%, respectively. These data indicate that HNF4α have a strong influence on regulation of all four gluconeogenic enzymes while C/EBPα also regulates expression of most gluconeogenic enzymes except PC.

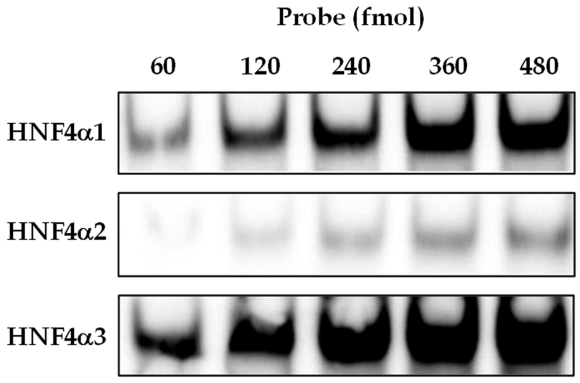
Discussion

Our result shows for the first time that human FBP1 is regulated by two important transcription factors, C/EBPα and HNF4α. C/EBPα is a member of the basic region leucine zipper family that binds to a sequence motif 5′-(A/G)TTGCG(C/T)AA(C/T)-3′ [38]. C/EBPα regulates transcription by forming a complex with two transcription co-activators, p300 or CBP and enhances RNA polymerase II basal transcriptional activity [42, 43]. Regarding its targets, C/EBPα regulates transcription of genes whose products are involved in development and central metabolism [44, 45]. Since C/EBPα expression is most abundant in liver, abrogation of its expression severely affected hepatocyte development and metabolism [46, 47]. Previous studies show that null or liver-specific C/EBPα knockout mice develop severe fasting hypoglycemia due to impaired postnatal gluconeogenesis accompanied by a marked reduction of expression of PEPCK-C and G6Pase1 but no data were available regarding regulatory role of C/EBPα on other gluconeogenic enzymes [47, 48]. The only evidence that shows that C/EBPα may be involved in regulation of FBP1 expression come from studies by He et al. [49, 50] demonstrating that loss of function of CBP, a transcription co-activator of C/EBPα resulted in impaired fasting-induced gluconeogenesis accompanied with reduced expression of PEPCK-C, FBP1 and G6Pase, underscoring the regulatory role of this transcription factor in controlling gluconeogenesis. Here we show that overexpression of C/EBPα results in up-

A

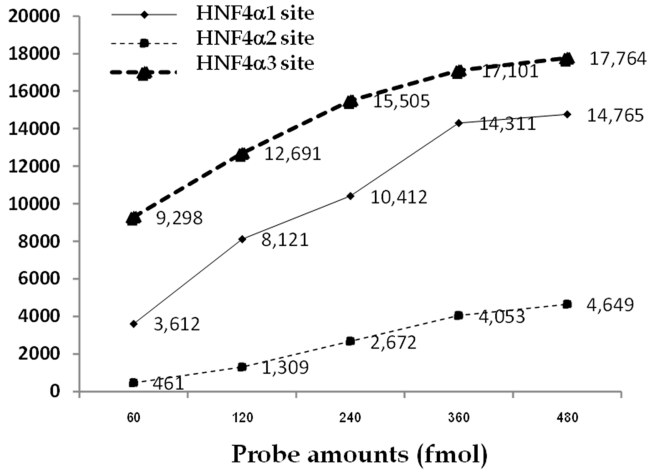
Probe / WT Competitor

HNF4α1-hFBP1 CCTC**TGGCCTTTGTGTG**GTAG
HNF4α2-hFBP1 GGCC**AGGTGACAGGCCA**GGCA
HNF4α3-hFBP1 TACT**TAACCTTTCTGAACT**TCCG



B

Integrated unit



C

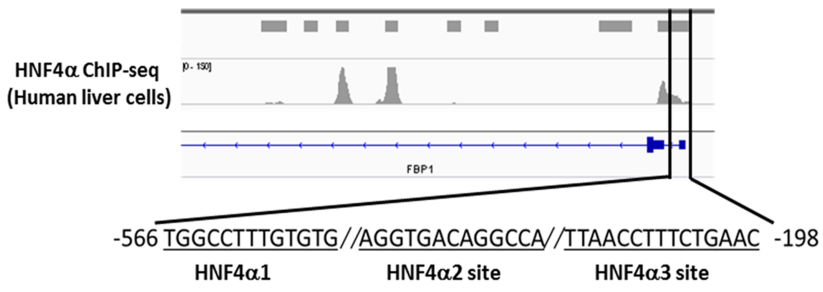


Fig 5. Interaction of HNF4α to three HNF4α binding sites in human FBP1 promoter. (A) Biotin-labelled double stranded nucleotides corresponding to three HNF4α binding sites in the hFBP1 promoter used for EMSA. The core HNF4α binding sites in each probe are highlighted. (B) EMSA of various amounts of FBP1 probes (60, 120, 240, 360 and 280 fmole) harboring different HNF4α binding site in the presence of 200 ng of purified HNF4α. (C) The intensities of the HNF4α-bound complexes were plotted against the amounts of each probe. (D) HNF4α binding site in FBP1 promoter mapped by ChIP seq in human adult liver cell.

https://doi.org/10.1371/journal.pone.0194252.g005

regulation of endogenous FBP1 in HepG2 cells. This transcriptional activation is mediated through the two overlapping C/EBPα binding sites, located at nucleotides -227/-218 (C/EBPα1, 5′-ATTGAGCAAG-3′) and -218/-209 (C/EBPα2, 5′-GTTACTTAAC-3′). Both of which contain two nucleotides (underlined) different from the consensus sequence [(A/G)TTGCG(T/C)AA(T/C)] [38]. These two overlapping C/EBPα binding sites appear to work in concert because mutating either one of these two sites produced only marginal or moderate reduction of C/EBPα-mediated transcriptional activation of the hFBP1 promoter while double mutation produced a more pronounced effect. EMSA using an hFBP1 probe containing these C/EBPα sites with a nuclear extract of HepG2 cells overexpressing C/EBPα clearly confirmed its binding to the these two C/EBPα binding sites. Although we were able to show that C/EBPα binds to these two overlapping C/EBPα sites by EMSA, we could not detect *in situ* interaction of C/EBPα in HepG2 cells using ChiP assay. This failure may suggest a relative poor binding of C/EBPα to its cognate sequence in FBP1 promoter *in vivo*. Nevertheless we were able to show that down-regulation of C/EBPα by siRNA produces a great impact on the expression of FBP1 together with other gluconeognic enzymes except PC. Collectively, our data demonstrate that C/EBPα plays an important role in programming the gluconeogenic pathway in human liver through PEPCK-C, FBP1 and G6Pase1. It is noted that C/EBPα expression is also under hormonal control. Rat hepatoma cells treated with dexamethasone or cAMP show a marked increase in the expression of C/EBPα mRNA while insulin opposes this effect [51]. It would be obvious that during caloric deprivation, gluconeogenic enzymes would

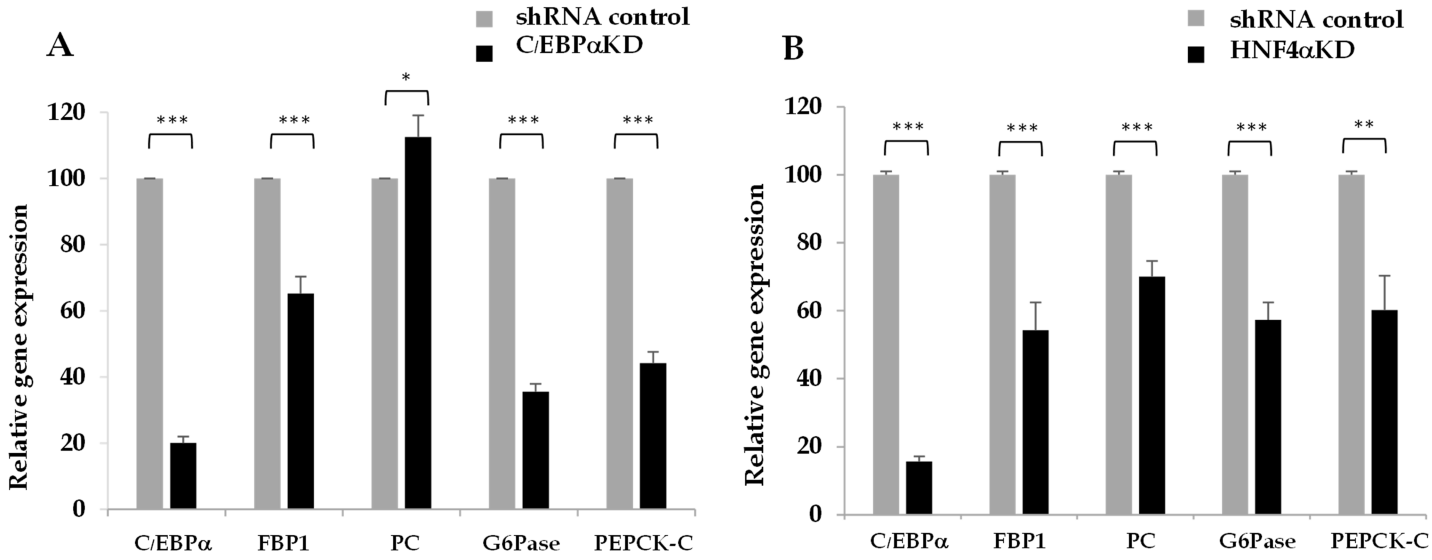


Fig 6. Suppression of C/EBPα or HNF4α lowered expression of gluconeogenic enzyme mRNAs. (A) Suppression of C/EBPα siRNA (B) Suppression of HNF4α siRNA. The expression of PC, PEPCK-C, FBP1 and G6PC1 was measured by quantitative real time PCR and normalized to that of 18s rRNA. The values obtained from C/EBPα or HNF4α knockdown HepG2 cells is expressed relative to that obtained from the scramble control group (shRNA control) which was arbitrarily set as 100%. Statistical analysis was performed by ANNOVA test where $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$.

https://doi.org/10.1371/journal.pone.0194252.g006

be upregulated via cAMP-induced C/EBPα expression. This is also observed in the liver of the C/EBPα knockout mouse that showed impaired cAMP-induced PEPCK-C transcription [52].

HNF4α is a liver-enriched transcription factor, belonging to member of hormone nuclear superfamily and highly expressed in liver as is C/EBPα [53]. In liver, HNF4α plays a role in regulating expression of several genes involved in metabolic pathways including carbohydrate, lipid and bile acid metabolism [54]. Mutations of HNF4α gene in humans cause maturity onset diabetes of the young 1 (MODY1) [55]. Here we showed that human FBP1 expression is regulated by HNF4α as in PC, PEPCK-C and G6PaseI [31, 56, 57]. Overexpression of this transcription factor in HepG2 cells resulted in 4-fold increase in FBP1 mRNA expression. This transcriptional activation by HNF4α is mediated through three HNF4α binding sites in the FBP1 promoter, located at -556/-554 (HNF4α1), -359/-346 (HNF4α2) and the -212/-198 (HNF4α3). HNF4α1 resembles the H4-SBM which is specific for binding by HNF4α while HNF4α2 and HNF4α3 resemble the classical direct repeat, binding site for several nuclear receptors. Mutational analysis demonstrates that these three sites confer differential responses to HNF4α transactivation being site 1 > site 3 > site 2, respectively. The use of these three HNF4α binding sites for HNF4α transactivation of FBP1 expression was also confirmed by HNF4α-ChiP seq data of adult liver cells demonstrating binding of HNF4α across these three binding sites.

HNF4α has previously been reported as the vitamin D3-responsive element (VDRE)/retinoic acid responsive element (RAR) that mediates vitamin-D/retinoic acid–induced FBP1 expression in monocytes [36, 37]. It is possible that vitamin D receptor and HNF4α may share the same responsive element for transcription activation of FBP1 expression in monocytes and hepatocytes, respectively. Sharing a common responsive element for mediating transcriptional activation by two distinct nuclear receptors in different tissues is not uncommon [58, 59]. One example is for PC, one of the four gluconeogenic enzymes in which its promoter contains DR1 that enables HNF4α or PPARγ to bind to and mediate its expression in hepatocytes and adipocytes, respectively [31, 60]. Regulation of FBP1 expression by HNF4α is probably associated with the peroxisome proliferator activated receptor 1α (PGC1α) that plays a key role in coordinating gluconeogenic enzyme levels in liver. Previous studies showed that fasting-induced PGC1α expression or ectopic expression of PGC1α in primary rat or mouse hepatocytes induced expression of PEPCK-C, G6Pase1 and FBP1, concomitant with increased hepatic gluconeogenesis [61, 62]. This transcriptional activation of PGC1α on gluconeogenic genes is mediated through the physical interaction with HNF4α [63]. Our finding that human FBP1 expression is regulated by HNF4α provides a further link between PGC1α and FBP1 expression during fasting. Lastly, we showed that suppression of HNF4α expression in HepG2 cells resulted in a simultaneous down-regulation of all gluconeogenic enzymes similar to what is observed in rodents models in which ablation of this transcription factor *in vivo* or in cultured hepatocytes also affect expression of all gluconeogenic enzymes [31, 64]

In summary we show that C/EBPα and HNF4α are the two important transcription factors that regulate expression of human FBP1 expression in HepG2 cells. This transcriptional activation is mediated through binding to -227/-218 and -218/-209 C/EBPα binding sites and through -566/-554, and -212/-198 HNF4α binding sites, respectively. Suppression of expression of both transcription factors results in a marked decrease in expression of all gluconeogenic enzymes.

Acknowledgments

We are grateful to Dr. Justin J. Rochford, University of Aberdeen, Scotland for providing pBluescript-C/EBPα vector.

Author Contributions

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Funding acquisition: Sarawut Jitrapakdee.

Investigation: Siriluck Wattanavanitchakorn, Tanit Chavalit.

Supervision: Pinnara Rojvirat, Michael J. MacDonald, Sarawut Jitrapakdee.

Writing – original draft: Siriluck Wattanavanitchakorn.

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