# Overexpression of the Far Upstream Element Binding Protein 1 in Hepatocellular Carcinoma Is Required for Tumor Growth

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## Abstract

We identified the far upstream element binding protein 1 (FBP1), an activator of transcription of the proto-oncogene *c-myc*, in a functional yeast survival screen for tumor-related antiapoptotic proteins and demonstrated strong overexpression of FBP1 in human hepato-cellular carcinoma (HCC). Knockdown of the protein in HCC cells resulted in increased sensitivity to apoptotic stimuli, reduced cell proliferation, and impaired tumor formation in a mouse xenograft transplantation model. Interestingly, analysis of gene regulation in these cells revealed that *c-myc* levels were not influenced by FBP1 in HCC cells. Instead, we identified the cell cycle inhibitor *p21* as a direct target gene repressed by FBP1, and in addition, expression levels of the proapoptotic genes tumor necrosis factor *α,* tumor necrosis factor–related apoptosis-inducing ligand, Noxa, and Bik were elevated in the absence of FBP1.

**Conclusion—**Our data establish FBP1 as an important oncoprotein overexpressed in HCC that induces tumor propagation through direct or indirect repression of cell cycle inhibitors and proapoptotic target genes.

The human far upstream element (FUSE) binding protein 1 (FBP1) was originally identified as a factor that binds to the FUSE DNA sequence upstream of the *c-myc* proto-oncogene promoter.[1,2] As the central domain of FBP1 binds single-stranded nucleic acids of a specific sequence, melting of the cis-regulatory FUSE DNA element is required for FBP1 binding. The driving force for unwinding of the AT-rich FUSE sequence is torsional stress occurring during basal *c-myc* transcription due to counter-rotation of DNA and transcription machinery.[3] Through simultaneous interaction with transcription factor II H at the major P2

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promoter,[4] FBP1 action sharply raises *c-myc* transcription before the antagonistic FBP interacting repressor (FIR) binds to FBP1 and FUSE. FIR eventually replaces FBP1 in the DNA-containing complex and efficiently down-regulates *c-myc* transcription back to basal levels.[5] As a result, FBP1, FIR, transcription factor II H, and FUSE serve as regulatory machinery monitoring the intensity of ongoing *c-myc* transcription by RNA polymerase-induced torsional stress and programming a pulse of c-Myc expression required for cell cycle entry.[6,7] Apart from *c-myc*, no direct FBP1 target gene has been identified, although the protein binds to the promoters of several genes (unpublished data).

Recently, FBP1 was identified as one of many cellular factors associated with the 3′-nontranslated region of the hepatitis C virus genome.[8] It has been suggested that FBP1 is required for efficient replication of hepatitis C virus,[9] which is a chief cause for the formation of hepato-cellular carcinoma (HCC).

HCC is the fifth most common cancer worldwide and is the third leading cause of cancer mortality, with more than 500,000 deaths per year.[10] Because the symptoms of HCC often arise late during disease development, tumor formation is usually detected too late and clinical prognosis is poor. HCC rarely responds to chemotherapeutic treatment, and without therapy, the median survival for patients with unresectable liver tumors of advanced and late stages is 3 to 6 months.[11]

In the search for the molecular changes associated with HCC development, no consistent pattern of oncogene activation and/or tumor suppressor gene inactivation has emerged so far.[12] Therefore, the identification of molecular markers and targets for therapeutic approaches in HCC treatment remains an important challenge.

## Materials and Methods

Additional experimental procedures are described in the Supporting Material.

### Immunohistochemistry

Immunohistochemistry was performed on HCC Tissue Microarrays (BioCat, #BC03011) with a polyclonal antibody against FBP1 (Santa Cruz Biotechnology, sc-11098) at a concentration of 200 ng/mL. Slides were scanned with a ScanScope CS (Aperio).

### Lentiviral Knockdown of FBP1 Expression

Short hairpin RNA (shRNA) against *FBP1* was cloned into *pSEW* (provided by M. Grez), and the *pSEW-shLuc* control vector was provided by A. Weiss. Hep3B and HuH7 cells were transduced with lentivirus produced in 293T cells, and after three passages, transduction efficiencies were calculated by green fluorescent protein quantification using flow cytometry (see Supporting Material for details).

### Tumor Xenograft Experiments

For analysis of tumorigenicity, $7 \times 10^6$ Hep3B cells were subcutaneously injected into the flanks of 5- to 6-week-old non-obese diabetic/severe combined immunodeficient mice. Tumor volumes were monitored over a period of 4 weeks using calipers. Tumors were isolated and analyzed by way of hematoxylineosin histology and microscopy.

### Messenger RNA Quantification Using Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells with the High Pure RNA Isolation Kit and transcribed into complementary DNA (cDNA) with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time polymerase chain reaction (PCR) analysis was performed on a

LightCycler 480 (Roche) using Human Cell Cycle Regulation Panel 96 and Human Apoptosis Panel 384 (Roche). Relative messenger RNA (mRNA) levels were normalized to the expression of nine house keeping genes and calculated using the $2^{-\Delta\Delta Ct}$ method.

### Electrophoretic Mobility Shift Assay

Single-stranded oligonucleotide probes were generated from the *p21* promoter sequence (Supporting Table 1) and tested using FBP1 proteins purified from *Escherichia coli* or insect cells as described.[5]

### RNA Interference and Luciferase Reporter Assay

For transient down-regulation of FBP1, cells were transfected with small interfering RNAs (Stealth; Invitrogen) at 50 nM concentration using Lipofectamine 2000, and then transfected with the *p21* promoter-firefly luciferase reporters (*pGL3*; Promega) by Lipofectamine reagent after 2 days. Cells were harvested after another 1 or 2 days and analyzed by luciferase assay.

### Statistical Analysis

Statistical analysis was performed with *GraphPad Prism 5* software applying non-parametric Mann-Whitney test, two-way analysis of variance with Bonferroni posttest, or two-tailed *t* test.

## Results

### FBP1 Inhibits Apoptosis and Is Overexpressed in Human HCC

We developed a functional yeast survival screen to identify antiapoptotic proteins overexpressed in human tumors. In one of our screens, we transformed a *Schizosaccharomyces pombe* yeast strain that inducibly expresses the proapoptotic *Caenorhabditis elegans* protein CED4[13] with a human mamma carcinoma-derived cDNA library.[14] One isolated cDNA that was capable of suppressing CED4-induced yeast cell death coded for a C-terminal fragment of the human FBP1 protein, and by overexpression in the human colon carcinoma cell line RKO, we confirmed that full-length FBP1 also inhibits apoptosis in mammalian cells (data not shown).

Because FBP1 was identified as a positive transcriptional regulator of the potent proto-oncogene *c-myc*[1,15] and both *c-myc* expression and apoptosis inhibition contribute to tumor development, we speculated that FBP1 activity may be up-regulated in certain tumor entities. Therefore, we performed immunohistochemical stainings on multitumor tissue microarrays to investigate the expression levels of FBP1 in various tumor entities and corresponding normal tissues. Our data revealed that FBP1 was strongly expressed in 83% of the 109 HCC samples analyzed, whereas the protein was nearly undetectable in healthy liver tissue (Fig. 1).

### Loss of FBP1 Expression in the HCC Cell Line Hep3B Leads to Increased Apoptosis Sensitivity and to Decreased Proliferation

To test for functional implications of the observed FBP1 overexpression in HCC, we studied the function of FBP1 in the HCC cell line Hep3B. We stably transduced the cells with lentivirus containing an FBP1-specific shRNA, which resulted in efficient FBP1 knockdown compared to empty vector-transduced or luciferase control shRNA-transduced cells (Fig. 2A). The cells were then analyzed for their apoptosis sensitivity. Down-regulation of FBP1 expression led to increased cell death following ultraviolet irradiation or incubation with mitomycin C or doxorubicin compared to control cells (Fig. 2B).

The influence of FBP1 knockdown on proliferation of Hep3B cells was tested by bromodeoxyuridine (BrdU) pulse labeling and quantification of BrdU incorporation by way of fluorescence-activated cell sorting. FBP1 deficiency led to a significant decrease of BrdU-positive cells due to diminished proliferation compared with control cells with normal FBP1 expression (Fig. 2C,D).

## FBP1 Knockdown Abrogates Tumor Growth in an HCC Xenograft Transplantation Model

Because our *in vitro* studies suggested a functional role for FBP1 in HCC proliferation and apoptosis resistance, we investigated the contribution of FBP1 to HCC growth *in vivo*. For this purpose, Hep3B cells transduced with either empty vector control or *FBP1* shRNA-expressing lentivirus were subcutaneously injected into both flanks of immunocompromised non-obese severe combined immunodeficient (SCID) mice, and tumor growth was then monitored over a period of 25 days. Although control tumors reached a volume of 1 cm$^3$, corresponding FBP1 knockdown tumors displayed an average volume of 250 mm$^3$ and often had stopped expanding (Fig. 3A,B). Histological analysis of the SCID mouse tumors 25 days after injection exhibited significant differences in the extent of tumor cell proliferation and differentiation. Cells undergoing mitosis were quantified on 30 different paraffin-fixed and hematoxylin-eosin–stained tissue slides obtained from 10 different SCID mouse tumors. Strikingly, the mitotic index was twice as high in empty vector-transduced control tumors compared with *FBP1* shRNA-expressing Hep3B tumors (Fig. 3C). In addition, *FBP1* shRNA tumors displayed significantly fewer cells with an increased size ratio of nucleus and cytoplasm and a changed shape/appearance of the nucleus (Fig. 3D). These morphometric indices have been linked with a dedifferentiated (highly malignant) phenotype of HCC cells.[16]

## FBP1 Regulates the Expression of Cell Cycle Inhibitors and Proapoptotic Genes

To understand the mechanisms of FBP1-dependent HCC cell proliferation and survival, we compared the expression of 84 selected cell cycle–relevant and 350 apoptosis-relevant genes in Hep3B cells in the presence or absence of FBP1 using real-time PCR. FBP1 was initially described as a positive regulator of *c-myc* transcription in various cell lines.[2,7,17] Surprisingly, knockdown of FBP1 in Hep3B cells did not significantly influence the amount of *c-myc* mRNA (Fig. 4A) and protein (data not shown). Furthermore, we could not detect any positive correlation between FBP1 and c-Myc expression in HCC biopsies, suggesting that the regulation of the *c-myc* promoter might be uncoupled from FBP1 activity in HCC cells (data not shown). These results suggest that other unidentified FBP1 target genes may be responsible for the functions of FBP1 in HCC cells.

Our analysis revealed that the mRNA expression levels of several other known cell cycle regulators were affected by FBP1. The cell cycle inhibitors *p15* and *p21* were both up-regulated in the absence of FBP1 (Fig. 4A). In addition, *Cyclin D2* mRNA levels were diminished in the *FBP1* knockdown cells, whereas the amount of *Cyclin D1* mRNA remained unaffected.

Interestingly, we also observed the up-regulation of proapoptotic genes following *FBP1* knockdown in Hep3B cells (Fig. 4B). In particular, elevated expression of the Bcl-2 family members Bik and Noxa was detected. In addition, mRNA levels of the death ligands tumor necrosis factor (TNF) *α* and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) were significantly increased.

Using an antibody-spotted cytokine array, we quantified the amount of TNF-*α* secreted into the supernatant by Hep3B cells stably transduced with *FBP1* shRNA, and compared it with that of empty vector-transduced parental Hep3B cells. Consistent with the real-time PCR

data, a significant increase in TNF-$\alpha$ production was observed in FBP1-deficient cells also on protein level, and secretion of TNF-$\beta$/LTa3 was similarly increased (Supporting Fig. 1).

Our data demonstrate that in the absence of FBP1, an antiproliferative and proapoptotic status of the cell is established by up-regulation of several cell cycle inhibiting and apoptosis-inducing genes.

### FBP1 Directly Regulates the p21 Promoter

Because *p21* mRNA expression was increased 5.5-fold following stable *FBP1* knockdown in our experiments (Fig. 4A), we aimed to investigate the process by which this important cell cycle inhibitor is regulated by FBP1. Closer inspection of the human *p21* promoter (based on sequence similarity with the *c-myc* FUSE element) revealed four potential FBP1 binding sites within 3.2 kb upstream of the transcription start point (Fig. 5A). We designed short DNA oligonucleotides containing these potential FBP1 binding sites (Supporting Table 1) and tested for binding of recombinant FBP1. Oligonucleotide P3, located 2.7 kb upstream of the *p21* transcription start site, interacted strongly with FBP1, suggesting direct binding of FBP1 to this region of the *p21* promoter (Fig. 5A). Boundary mapping of the P3 FBP1 binding region using an electrophoretic mobility-shift assay allowed us to define a DNA sequence of 50 bp, the deletion of which completely abolished FBP1 binding activity in the P3 promoter region (Fig. 5B).

To test whether FBP1 regulates *p21* promoter activity, we constructed a luciferase reporter containing 3 kb of the wild-type *p21* promoter sequence, and a second construct in which this promoter region is lacking the 50 bp P3 FBP1 binding site. Luciferase assays were performed in HCC cells transiently transfected with *FBP1* small interfering RNA or with an unrelated control small interfering RNA. The results displayed in Fig. 5C reveal that knockdown of FBP1 expression led to a three- to four-fold decrease of luciferase activity of the reporter construct containing the *p21* wild-type promoter sequence. The corresponding *p21* promoter construct lacking the P3 FBP1 binding site revealed a greatly reduced luciferase activity in the presence of FBP1 compared with the wild-type promoter construct. We observed similar outcomes in Hep3B and HuH7 HCC cell lines stably transduced with *FBP1* shRNA (data not shown). Our results demonstrate that FBP1 not only binds to the *p21* promoter, but indeed directly regulates its activity.

## Discussion

Our study revealed strong overexpression of the single-stranded nucleic acid binding protein FBP1 in more than 80% of human HCC, whereas normal liver does not express detectable amounts of FBP1. Only one of six normal liver samples exhibited a weak positive immunohistochemical staining with the anti-FBP1 antibody and was scored positive.

Down-regulation of endogenous FBP1 in the HCC cell line Hep3B led to decreased proliferation and increased sensitivity for induction of apoptosis which are both oncogenic characteristics required for tumorigenesis.[18] Using a xenograft transplantation model in immunocompromised SCID mice, we found that tumor formation by injected Hep3B cells was nearly abolished upon FBP1 down-regulation. Histological analysis revealed that the mitotic index of tumor cells was reduced by 50% upon FBP1 knockdown. In addition, the number of dedifferentiated tumor cells transduced with *FBP1* shRNA was decreased to one-third of those observed in tumors with high endogenous FBP1 levels. Reduced proliferation and tumor cell dedifferentiation are both criteria of decreased malignancy of FBP1-deficient HCC cells, and these results clearly show a tumor-promoting potential for *FBP1 in vivo*.

FBP1 was initially described as a positive regulator of *c-myc* transcription in various cell lines.[2,7,17] However, we could not detect significant changes in *c-myc* mRNA and protein levels following *FBP1* knockdown in Hep3B cells, implying that the oncogenic antiapoptotic and pro-proliferative effect of FBP1 in HCC is mediated by target genes other than *c-myc*.

We found that increased apoptosis sensitivity of HCC cells in the absence of FBP1 correlates with up-regulation of the proapoptotic genes *TNF-α*, *TRAIL*, *Bik*, and *Noxa*. TRAIL and TNF-α both belong to the TNF superfamily and are capable of inducing apoptosis by activating their respective death receptors.[19] For TNF-α, complete regression of established murine HCC by *in vivo TNF-α* gene transfer was described.[20]

The influence of the TRAIL ligand on HCC cells is less clear. Although TRAIL potently induces apoptosis in many tumor cells, normal hepatocytes and HCC cells are relatively resistant to TRAIL-mediated cytotoxicity. However, adoptive transfer of TRAIL-expressing natural killer cells prevented recurrence of HCC after partial hep-atectomy,[21] and TRAIL-induced apoptosis in HCC cells was vastly augmented when the cells were simultaneously treated with chemotherapeutic drugs such as doxorubicin and camptothecin.[22]

Bik/Nbk and Noxa are members of the BH3 domain-only subgroup of the Bcl-2 family. As loss of Bik is a common feature of clear-cell renal cell carcinoma[23] and Noxa-induced apoptosis is defective in all p53-deficient tumors, both, Bik and Noxa, have been implicated as tumor suppressor molecules. In correlation with our results, c-Jun prevents apoptosis in chemically induced murine HCC by antagonizing p53-dependent Noxa activity.[24]

Our data demonstrate that down-regulation of FBP1 in liver cancer cells induces a proapoptotic milieu by up-regulating proteins that trigger the extrinsic and intrinsic apoptosis pathways.

In addition, we identified the three cell cycle–regulating genes *p21*, *p15*, and *CyclinD2* as differently regulated in HCC cells depending on the presence or absence of FBP1. In our analysis of *FBP1* shRNA-transduced Hep3B cells, we found that *p21* mRNA levels increase following *FBP1* knockdown, suggesting that FBP1 functions as a repressor of *p21*. Accordingly, when we hybridized the HCC tissue microarrays used for the original quantification of FBP1 expression by immunohistochemistry (IHC) with an anti-p21 antibody, we observed that the majority (65%) of tumors with high FBP1 expression displayed low p21 expression levels (data not shown).

In a recent study, a novel SELEX procedure was applied to define the optimal DNA binding site for FBP1.[25] According to these sequence criteria for an authentic FBP1 binding site, the *p21* FUSE element is a considerably better suited candidate for strong FBP1 binding than the *c-myc* FUSE element. The proof for binding of FBP1 to the p21 promotor as well as the regulation of p21 by FBP1 on the transcriptional level was provided by electrophoretic mobility-shift and luciferase reporter assays. Our results identify the tumor suppressor *p21* as the second direct FBP1 target gene in addition to the proto-oncogene *c-myc*.

Surprisingly, FBP1 acted as an activator of the *p21* promoter sequence that was tested in our luciferase assays. There are several possible explanations for this apparent contradiction. The reporter construct used in our assays consisted of the appropriate *p21* promoter sequences without any *p21* coding sequences. Such sequences, including additional binding sites for further FBP1-interacting proteins/repressors, may be important for the physiological p21 repressor function of FBP1. In addition, the *p21* promoter might be similarly regulated by FBP1 as *c-myc* transcription. Here, additional binding of the repressor FIR (and

ultimately, replacement of FBP1 by FIR) alters FBP1 activity from activation to repression.[26]

Several reports describe an inverse correlation between p21 expression and HCC tumorigenesis. p21 was identified as an independent survival prognostic factor for HCC patients after resection.[27] Notch 1 signaling inhibits HCC tumor growth through induction of apoptosis and cell cycle arrest, whereby the latter is mediated by up-regulation of p21 and down-regulation of positive cell cycle regulators.[28]

The cell cycle inhibitor p15 was also up-regulated upon FBP1 knockdown. A decrease in p15 levels in about 50% of all HCC tumor samples has been described as a consequence of 5′CpG island methylation in the *p15* promoter.[29] Our analysis of HCC cells after *FBP1* knockdown suggests that *p15* mRNA levels may also (directly or indirectly) depend on FBP1 activity.

A positive cell cycle regulator that we found down-regulated in Hep3B cells upon FBP1 inactivation was cyclin D2. While gene amplification was reported as a mechanism of aberrant *Cyclin D1* activation in HCC,[30] no such data exist for *Cyclin D2*. Numerous studies have classified D-type cyclins as cell cycle–promoting oncoproteins important for cellular transformation, and our results suggest that cyclin D2 is a candidate FBP1-regulated oncoprotein in HCC.

Both proproliferative and antiapoptotic properties of FBP1 are mediated by (direct or indirect) regulation of specific target genes. Together with the inhibition of apoptosis, repression of cell cycle inhibitors like p21 contributes to the oncogenic potential of FBP1 (Fig. 6). As targeting of FBP1 induces cell cycle arrest and apoptosis, inhibition of FBP1 activity in HCC may represent a promising therapeutic approach.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## Abbreviations

| | |
|---|---|
| **BrdU** | bromodeoxyuridine |
| **cDNA** | complementary DNA |
| **FBP1** | far upstream element binding protein 1 |
| **FIR** | far upstream element binding protein interacting repressor |
| **FUSE** | far upstream element |
| **HCC** | hepatocellular carcinoma |
| **mRNA** | messenger RNA |

| **PCR** | polymerase chain reaction |
| **SCID** | severe combined immunodeficient |
| **shRNA** | short hairpin RNA |
| **TNF** | tumor necrosis factor |
| **TRAIL** | tumor necrosis factor–related apoptosis-inducing ligand |

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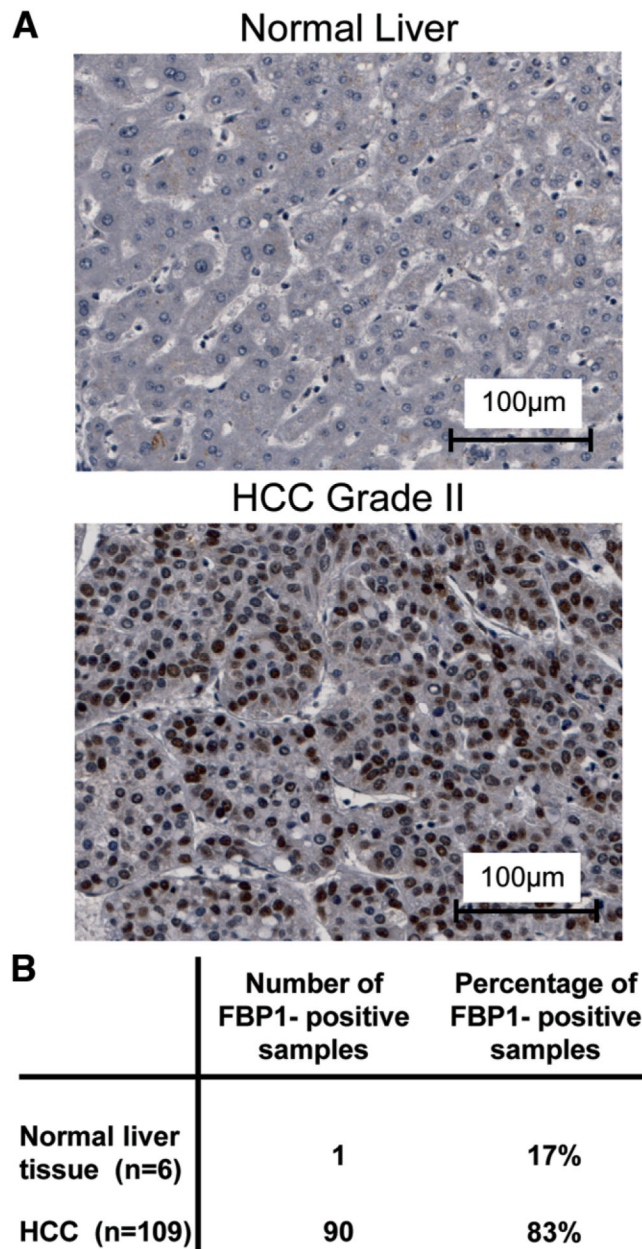
**A** Normal Liver



HCC Grade II



**B**

| | Number of FBP1- positive samples | Percentage of FBP1- positive samples |
|---|---|---|
| Normal liver tissue (n=6) | 1 | 17% |
| HCC (n=109) | 90 | 83% |

**Fig. 1.**
FBP1 is overexpressed in human HCC. (A) Tissue microarrays were incubated with anti-FBP1 antibody (sc-11098; Santa Cruz Biotechnology). Representative pictures for normal liver tissue and HCC are shown. The brown areas indicate positive anti-FBP1 staining in the cell nuclei. (B) Summary of immunohistochemical quantification of FBP1 expression in all HCC and normal liver samples. FBP1 was strongly expressed in 83% of 109 HCC samples analyzed in total, whereas only one out of six (17%) normal liver slides showed weak antibody staining.
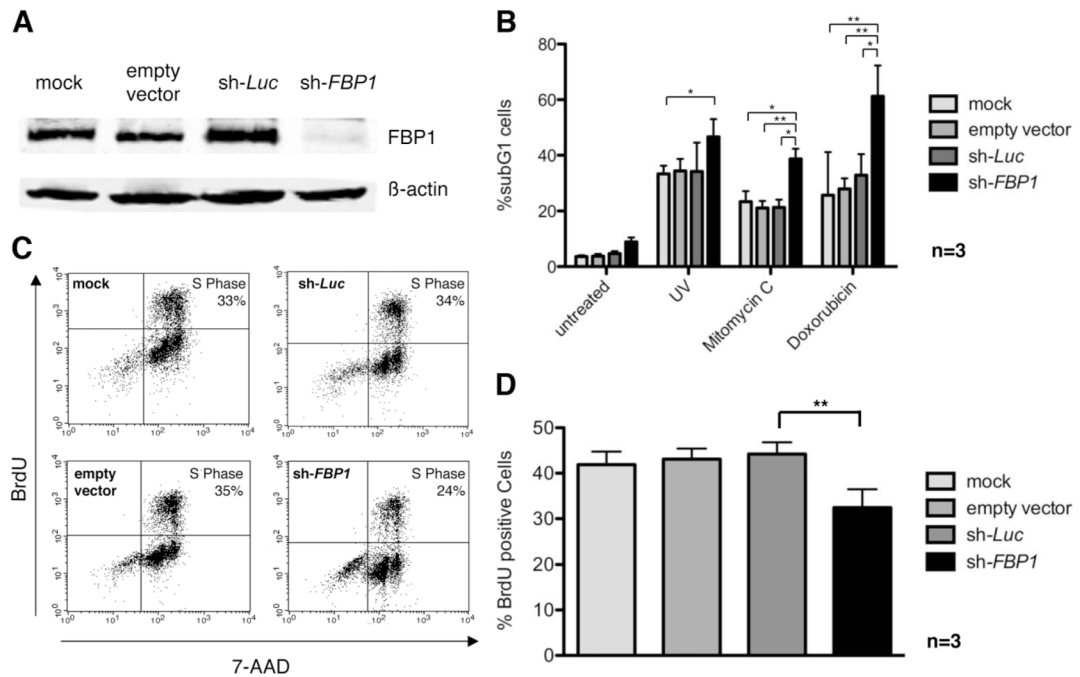
**Fig. 2.**
Knockdown of FBP1 in HCC cells leads to increased apoptosis sensitivity and reduced cell proliferation. (A) FBP1 was efficiently down-regulated by lentiviral shRNA expression. Hep3B cells were transduced with empty control vector, lentivirus containing *luciferase* shRNA, or lentivirus encoding *FBP1*-targeting shRNA. Total cell lysates were analyzed for FBP1 expression by immunoblotting. (B) FBP1 knockdown increased sensitivity of HCC cells to apoptotic stimuli. We treated $3 \times 10^5$ stably transduced Hep3B cells with either doxorubicin (10 $\mu$M), mitomycin C (40 $\mu$g/mL), or ultraviolet irradiation (120 mJ), and apoptosis was quantified by flow cytometry after 24 hours. Three independent experiments were performed, and bars represent the standard errors. $*P < 0.05$. $**P < 0.01$. (C) We treated $2 \times 10^5$ stably transduced Hep3B cells with 10 $\mu$M BrdU for 2.5 hours. After staining with allophycocyanin-labeled anti-BrdU antibody and 7-AAD, cells were analyzed using flow cytometry. One representative experiment is presented. (D) Summary of BrdU labeling experiments with stably transduced Hep3B cells. In the absence of FBP1, proliferation is reduced significantly ($**P < 0.01$). Data are expressed as the mean $\pm$ standard error of the mean for three independent experiments.
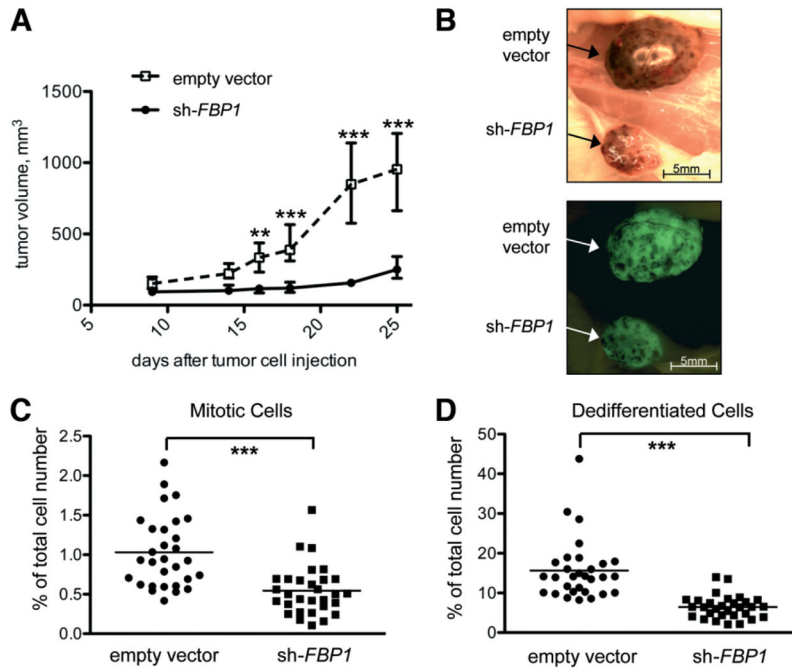
**Fig. 3.**
FBP1 is required for HCC tumor growth in a xenograft transplantation model. Hep3B cells were stably transduced with sh-*FBP1* or empty vector constructs. We injected $7 \times 10^6$ cells subcutaneously into the flanks of immunodeficient NOD/SCID mice. (A) Tumor volumes were measured over a period of 4 weeks using calipers (n = 10 for each cell type). Tumors arising from FBP1-deficient cells expanded significantly slower than control tumors. Data are expressed as the median ± standard error. \*\**P* < 0.01. \*\*\**P* < 0.001. (B) Twenty-five days after injection, tumors were excised for microscopic analysis. Representative bright field and green fluorescent protein micrographs of one empty vector-transduced and one sh-*FBP1*–transduced tumor are shown. (C) Knockdown of FBP1 significantly reduces the mitotic index of experimental Hep3B tumors. Mitotic cells were counted on 30 hematoxylin-eosin–stained tumor tissue slides for both empty vector-transduced and sh-*FBP1*–transduced tumors (3 slides/tumor). (D) FBP1-deficient tumors contain reduced numbers of dedifferentiated cells. Cells with an increased size ratio of nucleus and cytoplasm and a changed shape/appearance of the nucleus were counted on 30 hematoxylineosin–stained tumor tissue slides (3 slides/tumor). Statistical significance in (C) and (D) was calculated with a nonparametric Mann-Whitney *t* test. \*\*\**P* < 0.001.
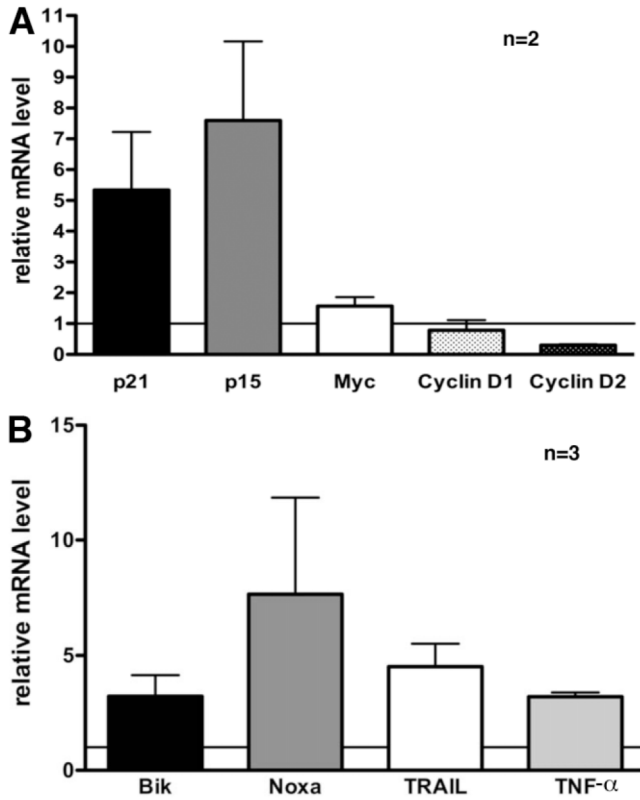
**Fig. 4.**
FBP1 down-regulates cell cycle inhibitors and proapoptotic genes. Expression analysis of (A) cell cycle–regulating and (B) apoptosis-regulating genes after *FBP1* knockdown. Total RNA prepared from sh-*FBP1*–transduced Hep3B cells was compared with RNA from sh-*luciferase*–transduced cells for expression of target genes by real-time PCR using the Cell Cycle Panel 96 (Roche) or the Apoptosis Panel 384 (Roche). Expression levels were normalized to nine housekeeping genes. Data are expressed as the mean ± standard error of the mean.
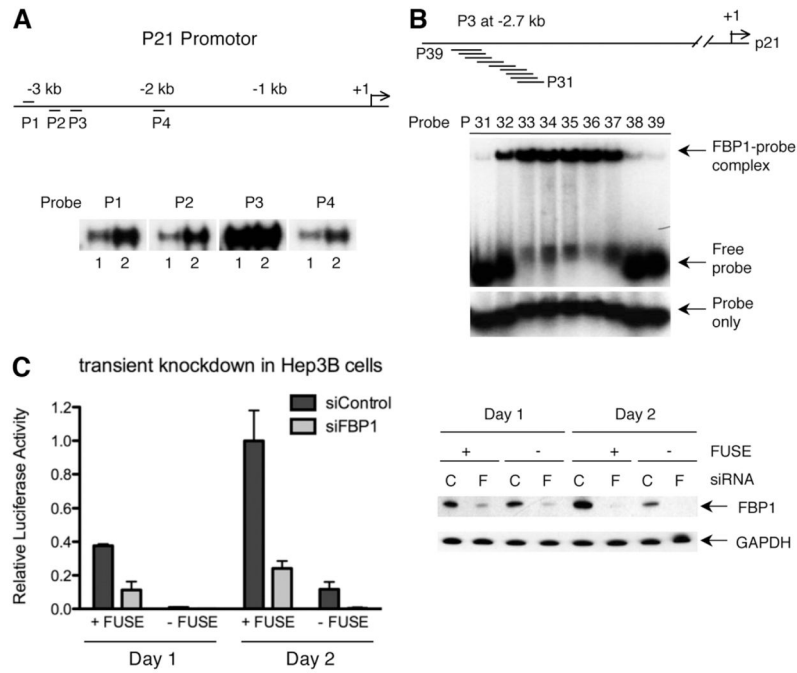
**Fig. 5.**
FBP1 directly regulates *p21* promoter activity. (A) Location of the putative FBP1 binding sites within the *p21* promoter. The *p21* promoter sequence, up to 3.2 kb upstream from the transcriptional start site, was examined for potential FBP1 binding sites. Four regions selected based on the FBP1 consensus[25,31] are shown on the top (P1) and the bottom strands (P2–P4). Single-stranded oligonucleotide probes were generated for the selected four regions and tested with the purified recombinant FBP1 DNA binding domain fused to GST. 0.5 pmole of each probe were incubated per reaction with 1 or 4 $\mu$g of GST-FBP KH3 + 4 (lanes 1 and 2, respectively). (B) Boundary mapping of the P3 FBP binding site on the *p21* promoter. Nine nested oligonucleotides covering the potential FUSE region (P31-P39) were tested for FBP1 binding using an electrophoretic mobility-shift assay. 0.5 pmole of each radioactively labeled oligonucleotide probe was incubated without (bottom) or with (top) 0.1 $\mu$g of the purified full-length FBP1 protein. Strong FBP1 binding was detected with the probes P32 to P36, which cover a region of 50 bp in total. (C) Effect of P3 FUSE deletion and FBP1 knockdown on the *p21* promoter activity. Luciferase activities driven by the *p21* promoter with or without the 50 bp P3 FBP binding region (+FUSE or –FUSE, respectively) are shown; each is accompanied by *FBP1* or control knockdown (si-*FBP1* or si-*Control*, respectively). Data points are the mean from triplicate transfections, normalized to the total protein concentration, and error bars show the standard error of the mean. One set of the triplicate cell lysates was analyzed by western blotting to confirm efficient FBP1 knockdown. C and F represent si-*Control* and si-*FBP1*, respectively.
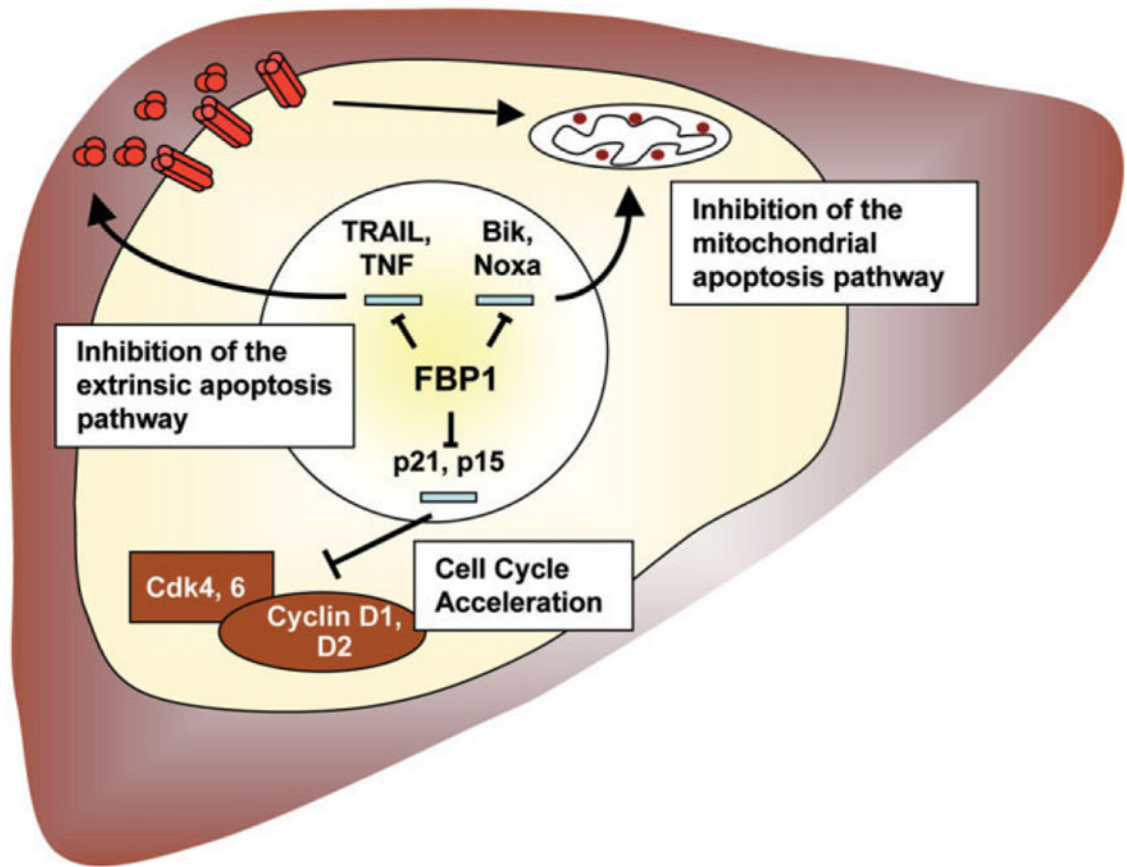
**Fig. 6.**
Model of the oncogenic FBP1 function in HCC.