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PI3K/AKT/mTOR-Dependent Stabilization of Oncogenic Far-Upstream Element Binding Proteins in Hepatocellular Carcinoma Cells

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Abstract

Transcription factors of the far-upstream element-binding protein (FBP) family represent cellular pathway hubs, and their overexpression in liver cancer (hepatocellular carcinoma [HCC]) stimulates tumor cell proliferation and correlates with poor prognosis. Here we determine the mode of oncogenic FBP overexpression in HCC cells. Using perturbation approaches (kinase inhibitors, small interfering RNAs) and a novel system for rapalog-dependent activation of AKT isoforms, we demonstrate that activity of the phosphatidylinositol-4,5-biphosphate 3-kinase/AKT pathway is involved in the enrichment of nuclear FBP1 and FBP2 in liver cancer cells. In human HCC tissues, phospho-AKT significantly correlates with nuclear FBP1/2 accumulation and

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Supporting Information

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expression of the proliferation marker KI67. Mechanistic target of rapamycin (mTOR) inhibition or blockade of its downstream effector eukaryotic translation initiation factor 4E activity equally reduced FBP1/2 concentrations. The mTORC1 inhibitor rapamycin diminishes FBP enrichment in liver tumors after hydrodynamic gene delivery of AKT plasmids. In addition, the multikinase inhibitor sorafenib significantly reduces FBP levels in HCC cells and in multidrug resistance 2-deficient mice that develop HCC due to severe inflammation. Both FBP1/2 messenger RNAs are highly stable, with FBP2 being more stable than FBP1. Importantly, inhibition of phosphatidylinositol-4,5-biphosphate 3-kinase/AKT/mTOR signaling significantly diminishes FBP1/2 protein stability in a caspase-3/-7-dependent manner.

Conclusion—These data provide insight into a transcription-independent mechanism of FBP protein enrichment in liver cancer; further studies will have to show whether this previously unknown interaction between phosphatidylinositol-4,5-biphosphate 3-kinase/AKT/mTOR pathway activity and caspase-mediated FBP stabilization allows the establishment of interventional strategies in FBP-positive HCCs.

Signaling pathways transfer exogenous stimuli through activation of a distinct number of transcription factors, which can in turn induce a variety of biological effects. In this context, transcription factors integrate different input information to achieve a coordinated and temporal cellular response under physiological conditions. Robustness and flexibility of these biological networks are usually achieved through implementation of a so-called bow tie topology.[1] However, under pathophysiological conditions altered expression or aberrant activation of transcription factors, which represent core elements of these bow tie modules, are frequently observed in, e.g., tumorigenesis and inflammatory diseases.

The far-upstream element (FUSE)-binding proteins (FBPs) consist of three family members (FBP1/ FUBP1, FBP2/KHSRP, FBP3/FUBP3), which recognize single-stranded nucleic acid. It has been demonstrated that sequence-specific binding of FBPs to the so-called FUSE site in the promoter of the c-MYC proto-oncogene is important for its proper transcriptional regulation after serum stimulation.[2] For this the activation domain of FBP1 interacts with TFIIH subunits (e.g., p89/XPB) to initiate the escape of RNA polymerase II from the c-MYC promoter. Together with the FBP-interacting repressor (FIR; synonyms PUF60, SIAHBP1), which binds FUSE/FBP and negatively regulates target gene expression, the FUSE/FBP/FIR complex represents a sensitive molecular tool for the fine-tuned regulation of transcriptional targets.[3] In addition, FBP family members affect messenger RNA (mRNA) abundance in a transcription-independent manner. For example, FBP2 destabilizes interleukin-8 mRNA, while FBP1 is involved in splicing of MDM2.[4,5]

Overexpression of FBPs (and here especially FBP1) has been described for many malignancies such as hepatocellular carcinoma (HCC),[6,7] non-small-cell lung cancer,[8] and colorectal cancer.[9] Importantly, nuclear enrichment of FBPs correlated with poor overall survival of liver cancer patients.[6] Besides c-MYC, other FBP downstream effectors have been identified, such as cell cycle regulators (p21, p15, and cyclin D2) as well as microtubule-destabilizing factors (stathmin and SCLIP).[10] This body of evidence strongly suggests that FBP enrichment in tumor cells may affect processes including cell cycle regulation and cytoskeletal reorganization. In accordance with the latter hypothesis,

functional analyses demonstrated a number of FBP-dependent protumorigenic cellular responses in tumor cells, including proliferation, migration, and invasiveness.[6,8] Interestingly, approximately 15% of oligodendrogliomas contain FBP mutations leading to truncated proteins that promote proliferation.[11] However, no such FBP mutations have been described for other neoplasms, indicating the existence of entity-specific oncogenic mechanisms. Concerning HCC, the mode of FBP dysregulation has not been completely understood.

In this study we show that activation of the phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K)/ AKT/mechanistic target of rapamycin (mTOR) signaling pathway stabilizes FBP1 and FBP2 in a caspase-3/-7-dependent manner. Phospho-AKT (pAKT) and nuclear FBP enrichment significantly correlate in human HCC tissues, and chemical perturbation of the PI3K/AKT/mTOR pathway by sorafenib or rapamycin reduces the amount of oncogenic FBP in two independent HCC mouse models. Thus, specific approaches targeting the PI3K/AKT/mTOR axis in a subgroup of HCC patients with high-level expression of FBPs may represent an interesting therapeutic strategy.

Materials and Methods

MOUSE TUMOR MODELS

Hydrodynamic injection of AKT, either alone or in combination with activated N-Ras (N-RasV12), was performed as described.[12] A group of AKT-injected mice was subjected, 4 weeks after hydrodynamic injection, to administration of either vehicle (n = 4) or rapamycin (5 mg/kg, n = 5) by oral administration for 5 days. Concerning AKT/Ras mice, 3 weeks after hydrodynamic injection, either vehicle (n = 8) or rapamycin (5 mg/kg, n = 8) was administered intraperitoneally for 3 weeks daily. In both mouse models, livers were harvested 5 hours after the last injection.

Male and female multidrug resistance 2-deficient ($Mdr2^{-/-}$) mice on an FVB/N background were held under pathogen-free conditions.[13] Wild-type controls were age-matched FVB/N mice. Mouse body weights during the experiments were 30–45 g. Sorafenib (Xing-cheng Chempharm, Taizhou, China) was administered daily (50 mg/kg) by oral gavage. Cremophor EL (Sigma)/95% ethanol/water in the ratio 1:1:6 was used as vehicle.

Mice were housed, fed, and treated in accordance with protocols approved by the Committee for Animal Research.

TISSUE MICROARRAY ANALYSIS AND IMMUNOHISTOCHEMISTRY

The tissue microarray contained representative areas (core diameter 0.6 mm) of seven normal human liver samples and 100 HCCs (grading: 10 × G1, 71 × G2, 15 × G3, and 4 × G4). Cases were surgically resected at the University Hospital of Heidelberg and histologically classified according to established criteria by two experienced pathologists (P.S., S.S.). The study was approved by the Institutional Ethics Committee of the Medical Faculty of Heidelberg University (application no. 206/05). Immunohistochemical stains on human and mouse liver tissue specimens were performed on 10% formalin-fixed, paraffin-

embedded sections as described.[6] Antibodies used in this study are listed in Supporting Table S4.

Detailed descriptions of all methods, protocols, sequences, and antibodies are listed in the Supporting Information.

Results

THE INTRACELLULAR PI3K/AKT AXIS STIMULATES FBP1/2 ENRICHMENT IN HCC CELLS

Human HCCs showed overexpression and strong nuclear enrichment of FBP1/2 in 70%–83% of all cases.[6,7] To define the underlying molecular mechanisms of FBP enrichment, we first tested if chromosomal alterations of the FBP1 gene (*fubp1*, chromosome 1p31.1) or the FBP2 gene (*khsrp*, chromosome 19p13.3) can explain the observed protein enrichment in HCCs. Only in 2.6% (2/76, *fubp1*) and 5.3% (4/76, *khsrp*) of all analyzed primary HCCs genomic gains were detectable at respective loci.[14] Moreover, genomic alterations did not correlate with FBP1/2 transcript level in a publicly available HCC data set,[15] illustrating that mechanisms different from genomic gains cause FBP1/2 overexpression in HCCs.

To identify regulatory upstream mechanisms for FBP1/2, different HCC cells were treated with a variety of stimuli or inhibitors. In particular, we focused on HLE cells with prominent FBP1 and FBP2 expression and Hep3B cells with high FBP2 concentrations. Most treatments did not affect FBP protein concentrations (e.g., hypoxia, PIM3 and transforming growth factor-β-, p38-, STAT3 pathway perturbation), while other stimuli moderately regulated FBP1/2 (e.g., cell density) (Supporting Fig. S1A–H). The strongest effects were observed after perturbation of receptor kinase activities, which typically activate the PI3K/AKT and Ras/RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathways (Fig. 1A).

Because silencing of ERK1/2 moderately regulated FBPs (Fig. 1B; Supporting Fig. S1I), we decided to further characterize the impact of the PI3K/AKT axis on FBP enrichment. Administration of different PI3K inhibitors (LY294002, wortmannin) resulted in significantly decreased FBP1/2 amounts, with strongest effects after 24 hours (Fig. 1C; Supporting Fig. S2A). Interestingly, the small interfering RNA (siRNA)-mediated inhibition of AKT2, but not AKT1, reduced FBP concentrations in both analyzed cell lines (Fig. 1D; Supporting Fig. S2B).

To confirm the AKT2 dependence of FBP expression, we used a novel approach for the specific induction of AKT1 or AKT2 in Hep3B cells. AKT activation was achieved by stable expression of the membranous FRB-ECFP fusion protein containing the binding domain for the chemically inert rapalog AP23102. The opposite rapalog-binding partner FK-BP was fused to the respective AKT isoform lacking the plextrin homology domain (ΔPH-AKT1/2; Fig. 2A). This strategy allowed the exclusive activation of AKT1 or AKT2 already 10–30 seconds after rapalog administration.[16] Treatment with AP23102 immediately led to an enrichment of the AKT isoforms near the plasma membrane and their phosphorylation (Fig. 2B,C). As indicated by the previous siRNA experiments, only AKT2, but not AKT1, led to a moderate but reproducible induction of FBP2 in this cell system (Fig. 2C). Together these

data illustrate that the PI3K/AKT pathway represents a physiological inducer of FBP1/2 enrichment in HCC cells.

FBP1/2 OVEREXPRESSION DEPENDS ON MTOR ACTIVITY

To test if the catalytic subunit of the mTOR complexes mTORC1 and mTORC2 are involved in the PI3K/AKT-dependent FBP1/2 expression, HCC cell lines were treated with different mTOR inhibitors such as PP242 (mTORC1/2 inhibitor) and rapamycin (mTORC1 inhibitor). Both compounds significantly reduced FBP1/2 concentrations (Fig. 3A,B; Supporting Fig. S3A,B). Equally, silencing of raptor and rictor by gene-specific siRNAs led to a reduction of FBP1/2 in HCC cells (Supporting Fig. S3C).

In order to examine if increased AKT activity was sufficient to induce FBP1/2 overexpression *in vivo* and if simultaneous mTORC1 silencing was able to reduce FBP expression, we performed injection of plasmids encoding for AKT using the sleeping beauty transposon system.[17] Because our first *in vitro* data also indicated moderate effects of the Ras/MAPK axis on FBP enrichment and because a close interplay between PI3K/AKT and Ras/MAPK pathways has been described, coinjections of AKT and an activated N-Ras isoform (N-RasV12) were equally performed. Since activation of the Ras/MAPK axis is not sufficient to induce HCC formation *in vivo*, N-RasV12 plasmids were not injected alone.[18] AKT expression induced hepatocyte proliferation and HCC development within 32 weeks, while AKT/N-RasV12 coexpression facilitated a massive formation of preneo-plastic and neoplastic lesions within 4 weeks.[12]

Immunohistochemical analyses revealed no FBP1/2 expression in normal liver tissues, while AKT and AKT/N-RasV12 expression led to an induction of FBPs in tumor tissues (Fig. 3C). Importantly, daily injection of rapamycin for 5 days in AKT-overexpressing mice resulted in FBP1/2 down-regulation. Similarly, administration of rapamycin 3 weeks after hydrodynamic vector delivery significantly reduced FBP1/2 levels in tumors developed after AKT/N-RasV12 injection (Fig. 3C). Western immunoblot analyses of total protein fractions isolated from these liver tissues confirmed these findings.

Because mTORC1 function is partly mediated by the eukaryotic translation initiation factor 4E-binding protein 1/eukaryotic translation initiation factor 4E (4EBP1/eIF4E) complex,[19] we next investigated the role of the 4EBP1/eIF4E axis on FBP1/2 regulation. For this purpose, HCC cells were subject to treatment with 4EGI-1, a soluble drug that mimics inhibitory 4EBP1 activity.[17] Quantitative analysis revealed that 4EGI-1 administration resulted in a moderate down-regulation of both FBP1 and FBP2 proteins, with FBP2 levels being more reduced (densitometric measurement: FBP1 −30%, FBP2 -43%; Fig. 3D). A similar FBP1/2 reduction occurred after transient transfection with 4EBP1 wild-type or the nonphosphorylatable form 4EBP1A4 (Fig. 3E). This suggests that protein synthesis affects FBP1/2 enrichment, though to a limited extent.

Together, these results demonstrate that mTOR is essential for PI3K/AKT-driven FBP overexpression in HCC cells.

EXPRESSION OF FBP1/2 IN HUMAN HCC TISSUES

Our *in vitro* and *in vivo* data illustrated that the PI3K/AKT/mTOR axis can efficiently diminish FBP enrichment in hepatic cells. To test whether this relation is also detectable in human HCC tissue samples, tissue microarrays containing normal liver tissues and HCCs (n = 107) were analyzed. Strong FBP1/2 accumulation was detectable in about 60% of all analyzed HCCs, which confirmed previous findings.[6,7] FBP1/ 2 levels were significantly elevated in HCC tissues compared to normal liver tissues ($P < 0.01$), and their expression was associated with nuclear KI67, which is a correlate for tumor cell proliferation ($r = 0.49$, $P < 0.001$; Fig. 4). Importantly, FBP1/2 expression was significantly associated with cytoplasmic pAKT amounts in the group of HCC samples ($r = 0.41$, $P < 0.001$) (Fig. 4). In addition, FBP1/2-positive and pAKT-positive tissues also showed increased phosphorylation of mTOR. These results supported our hypothesis that pAKT participates in the induction of FBPs in HCCs and that targeting upstream regulatory mechanism such as the PI3K/AKT/ mTOR signaling axis might be a suitable approach for blocking the production of oncogenic FBPs *in vivo*.

PERTURBATION OF REGULATORY UPSTREAM MECHANISMS OF ONCOGENIC FBP1/2

Because the clinically relevant multikinase inhibitor sorafenib blocks different kinases including vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), which in turn are potent regulators of the PI3K/AKT/ mTOR pathway, we tested if sorafenib affects FBP1/2 enrichment. In HCC cell lines, sorafenib administration reduced FBP1/2 levels 24 hours to 48 hours after administration (Fig. 5A; Supporting Fig. S4A). As expected, the phosphorylation of VEGFR and PDGFR was equally reduced by sorafenib (Supporting Fig. S4B).

To determine if this drug also regulates FBP1/2 amounts *in vivo*, $Mdr2^{-/-}$ mice were used, which developed an inflammatory cholangitis with portal inflammation and liver cancer by the age of 10–12 months.[13] Analysis of immunohistochemical stains for FBP1/2 revealed that tumors derived from $Mdr2^{-/-}$ mice exhibited prominent nuclear FBP immunolabeling (Fig. 5B). Quantitative analysis illustrated induction of FBP1/2 in tumor tissues compared to liver parenchyma.

Interestingly, sorafenib administration led to a strong FBP1/2 reduction in the tumor tissue but not in the adjacent liver tissue. These findings were con-firmed by western immunoblotting using protein extracts isolated from tumors and adjacent nontumorous liver tissues (Fig. 5C). The total mRNA amounts for FBP1/2 were not significantly affected in tumor tissues after sorafenib administration (Fig. 5D). Together, these results illustrate that sorafenib, which inhibits many upstream kinases regulating the PI3K/ AKT/mTOR pathway, may represent a suitable approach for the repression of FBPs in HCC cells.

HIGH STABILITY OF FBP1 AND FBP2 TRANSCRIPTS IN HCC CELL LINES

Because only minor effects of sorafenib on FBP1/2 mRNA levels were detected (Fig. 5D), we determined whether the PI3K/AKT/mTOR pathway regulates FBP1/2 transcription. For this purpose, the respective *fubp1* (−1182 to +70) and *khsrp* (−1273 to +92) promoters were cloned into Gaussia luciferase vectors (pEZX-PG04). After transfection of the reporter

constructs, luciferase activity was measured upon LY294002 administration. This treatment resulted in a strong reduction of *fubp1* and *khsrp* promoter-driven Gaussia luciferase expression (Fig. 6A; Supporting Fig. S5A). In contrast, LY294002-dependent PI3K inhibition only weakly affected the total amounts of FBP1/2 mRNAs (Fig. 6B; Supporting Fig. S5B).

One explanation for the absence of significant effects on FBP1/2 mRNA levels after PI3K inhibition or sorafenib administration could be high transcript stability. Indeed, blockade of transcription by actinomycin D revealed a high half-life of both mRNAs (Fig. 6C; Supporting Fig. S5C). Notably, FBP2 mRNA stability was higher than FBP1 stability (HLE cells: 17.3 hours for FBP1 and 67.4 hours for FBP2). The high stability of FBP1 and especially FBP2 transcripts suggested that transcriptional regulation by the PI3K/AKT/mTOR does not sufficiently explain the FBP1/2 decrease observed after pathway perturbation.

THE PI3K/AKT/MTOR PATHWAY REGULATES FBP1/2 PROTEIN STABILITY

Because FBP1/2 mRNAs are stable molecules, also the respective protein stability in HCC cells was defined. Treatment with the protein synthesis inhibitor cycloheximide (CHX) revealed that both proteins were also rather stable with a half-life of about 20.5 hours (FBP1) to 15.8 hours (FBP2) in HLE cells (Supporting Fig. S6).

Next we investigated if the PI3K/AKT/mTOR pathway affected FBP protein stability. For this, HCC cells were treated with CHX and/or LY294002 to block protein biosynthesis and/or PI3K signaling, respectively. Combined incubation with both substances led to a stronger protein reduction after 24 hours than with either CHX or LY294002 alone (LY294002 18.3 hours, CHX 16.0 hours, LY294002/CHX 10.0 hours for FBP1; LY294002 16.7 hours, CHX 11.6 hours, LY294002/ CHX 8.6 hours for FBP2) (Fig. 7A). Comparable results were generated using CHX and the PI3K inhibitor wortmannin or the mTORC1/2 inhibitors PP242 and rapamycin (Fig. 7B; Supporting Fig. S7). The results demonstrated that the PI3K/AKT/mTOR pathway stabilized FBPs in HCC cells.

Because the PI3K/AKT/mTOR pathway is known to counteract apoptosis[20] and caspase-3/-7-dependent FBP1 cleavage has been described,[21] we hypothesized that the PI3K/AKT/mTOR axis inhibited caspase activity and in this way elevated FBP levels. Indeed, the induction of apoptosis/caspases with doxorubicin efficiently reduced FBP levels in HCC cells (Fig. 8A; Supporting Fig. S9A). Identical effects were observed after using an independent apoptotic stimulus (Supporting Fig. S8A,B).[22] Importantly, inhibition of the PI3K/AKT/mTOR pathway by LY294002 led to a strong induction of caspase-3 activity after 24 hours, which was the time point when most prominent effects were detectable in most of our *in vitro* experiments (Fig. 8B; Supporting Fig. S9B).

Because the previous experiments showed that AKT2, but not AKT1, regulated FBP1/2 proteins (Figs. 1D and 2; Supporting Fig. S2B), we tested the impact of both AKTs on caspase-3 activity. Results from independent cell lines revealed that AKT2 inhibition more efficiently induced caspase activity than AKT1 (Supporting Fig. S9C).

We finally asked if caspases were involved in PI3K/ AKT/mTOR-dependent stabilization of FBPs and performed cotreatments using LY294002 and caspase-3/-7 inhibitor 1. Indeed, perturbation of caspase activity partly rescued FBP reduction after inhibition of PI3K activity (Fig. 8C; Supporting Fig. S9D). Identical results were obtained with another PI3K inhibitor (Supporting Fig. S9E). In summary, these data show that activity of the PI3K/AKT/mTOR pathway contributes to FBP stabilization through suppression of caspase activity in HCC cells.

Discussion

The bow tie structure of signaling pathways facilitates control and robustness with regard to perturbations and fluctuations under physiological conditions.[1,23] In this context, transcription factors (e.g., FBPs, c-MYC, p53) or transcriptional coactivators (e.g., YAP, β-catenin) represent structural pathway nodes that, if dysregulated, may cause or contribute to the development of liver cancer.[24] FBPs are highly expressed in different human malignancies, and especially for HCC it has been demonstrated that their overexpression induces tumor cell proliferation and is correlated with poor overall survival.[6,7] However, the mode of FBP dysregulation in tumor cells is only partly understood.

The findings presented in this study explain previous observations made in primary human HCC tissues, where only a moderate increase of FBP1 and FBP2 mRNAs in the vast majority of tumor samples compared to normal liver tissues was detected.[6] Indeed, perturbation of the PI3K/AKT/mTOR axis *in vitro* or administration of sorafenib *in vivo* caused moderate effects with regard to FBP1/2 mRNA levels. High transcript stability, especially of FBP2, suggested that mechanisms other than transcriptional regulation were responsible for the reduction of FBPs after PI3K/ AKT/mTOR pathway perturbation. Our data illustrate that next to the long-term regulation of FBP transcript abundance, PI3K/AKT/mTOR-dependent FBP enrichment is achieved through increased protein stability in the analyzed cell systems.

Different mechanisms for PI3K/AKT/mTOR-mediated protein abundance have been described. For example, frequently observed overexpression of the cell cycle regulator $p21^{Cip1}$ is partly due to its AKT/protein kinase B-dependent phosphorylation and subsequent protein stabilization.[25] Equally, recent data showed that AKT-mediated phosphorylation protected MDM4 from proteasomal degradation and increased its stability in HCC cells.[26] In addition, AKT indirectly facilitates protein stability in a glycogen synthase kinase-3-dependent manner as demonstrated for $p21^{Cip1}$ and cyclin D1.[27] Here we show that activation of especially the PI3K/AKT/mTOR pathway promotes FBP stability through inactivation of caspase-3/-7 activity. This is supported by previous data illustrating that perturbation of the PI3K/AKT/ mTOR axis efficiently induced apoptosis and caspase-3 activity in HCC cells.[28,29]

For both FBPs caspase cleavage sites have been described. Concerning FBP2, seven potential cleavage sites were identified, and two of them were confirmed experimentally,[30,31] while for FBP1 one caspase consensus site was defined, which allowed protein cleavage after induction of apoptosis in a caspase-3/-7-dependent

manner.[21] Indeed, our data show that caspase perturbation partly rescued FBP1 and FBP2 from degradation after PI3K inhibition. These results suggest the existence of a molecular mechanism which stabilized FBP1/ 2 upon stimulation of the antiapoptotic PI3K/AKT/ mTOR pathway in HCC cells. The presence of this mechanism is further supported by our data showing that different apoptotic stimuli such as doxorubicin, MG132,[22] and sorafenib[32] facilitated FBP1/2 depletion but weakly affected transcript concentrations. It is therefore tempting to speculate that PI3K/AKT/ mTOR-dependent FBP1/2 stabilization is part of an antiapoptotic and proproliferative cellular response in HCC cells.

Published data suggest additional modes of FBP regulation in nonneoplastic and malignantly transformed cells. For example, direct binding of FBP1 to p38/JTV-1, which is part of the aminoacyl-transfer RNA synthase complex, or the E3 ubiquitin ligase par-kin led to the proteasomal degradation of FBP1 in mouse embryonic fibroblasts.[33,34] Recently, we demonstrated that the functional FBP inhibitor FIR is highly expressed in HCC cells.[35] Nuclear FIR enrichment gains tumor-supporting functions partly through the induction of FBP expression. Therefore, different stimuli may affect FBP expression and stabilization under physiological and pathophysiological conditions. Our present results reveal an additional mode of FBP regulation, linking the activation of PI3K/ AKT/mTOR signaling with nuclear FBP enrichment. Both PI3K/AKT/mTOR pathway activation and nuclear FBP expression have been described as oncogenic events, which correlate with poor overall survival of HCC patients.[6,26,36]

HCC has long been an orphan tumor disease with regard to translational research efforts, clinical trial perspectives, and therapeutic options. So far, only sorafenib represents an effective and approved systemic treatment for progressed HCC; however, patients' responses have been modest and its administration has been associated with side effects.[37] For this reason, recent efforts focus on the identification of patient subgroups which could especially benefit from sorafenib treatment. For example, the VEGF/VEGFR signaling axis not only supports tumor vessel recruitment but promotes tumor cell growth in a cell-autonomous and autocrine manner.[38] VEGFR efficiently activates the PI3K/AKT/mTOR as well as the Ras/RAF/MAPK/ ERK1/2 pathway, and recent data illustrated that HCC patients with amplifications of the *vegfa* gene locus and higher VEGF-A secretion in particular could benefit from sorafenib treatment.[39] This is of special interest because bevacizumab, an antibody targeting VEGF-A, showed only moderate effects in HCC patients,[40] which could be due to the fact that activation of other ligands/receptors (and respective PI3K/AKT/ mTOR activation) may bypass the specific VEGF-A/VEGFR perturbation. Therefore, it is tempting to speculate that the effects of sorafenib in patients with *vegf* amplifications are partly due to reduction of PI3K/AKT/mTOR signaling and FBP stability.

Our *in vivo* results illustrate that sorafenib selectively blocks FBP enrichment in the tumor tissue of Mdr2-deficient mice but not in the nontumorous liver tissue. One reason for this might be that PI3K/AKT/ mTOR pathway activity and FBP expression are higher in HCCs than in adjacent liver tissues.[26,35] Interestingly, the perturbation of higher-level regulatory mechanisms (e.g., by sorafenib or other receptor/ pathway inhibitors) is probably the most promising approach for the reduction of FBPs in tumor cells. This is illustrated by the fact that small compounds that selectively disturb the physical interaction between FBP and

DNA are not soluble in an aqueous solution and therefore are not applicable *in vivo*.[41] In summary, this study systematically revealed a direct connection between the cellular PI3K/AKT/ mTOR signaling pathway, which is frequently activated in human hepatocarcinogenesis, and the enrichment of oncogenic transcription factors of the FBP family. Further studies are necessary to evaluate if specific perturbation approaches in HCC patients with elevated PI3K/AKT/mTOR activation and/or nuclear FBP enrichment may represent promising therapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Tissue samples were provided by the tissue bank of the National Center of Tumor Diseases (Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the Ethics Committee of the Medical Faculty of Heidelberg University.

Abbreviations

CHX	cycloheximide
4EBP1/eIF4E	eukaryotic translation initiation factor 4E-binding protein 1/eukaryotic translation initiation factor 4E
DMSO	dimethyl sulfoxide
ERK1/2	extracellular signal-regulated kinases 1 and 2
HCC	hepatocellular carcinoma
EGFR	epidermal growth factor receptor
FBP	far-upstream element binding protein
FIR	FBP-interacting repressor
FUSE	far-upstream element
MAPK	mitogen-activated protein kinase
Mdr2	multidrug resistance 2
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
mTORC	mTOR complex
pAKT	phospho-AKT

PARP	poly (adenosin diphosphate ribose) polymerase
PDGFR	platelet-derived growth factor receptor
PH	plextrin homology
PI3K	phosphati-dylinositol-4,5-biphosphate 3-kinase
siRNA	small interfering RNA
VEGF-A	vascular endothelial growth factor-A
VEGFR	VEGF receptor

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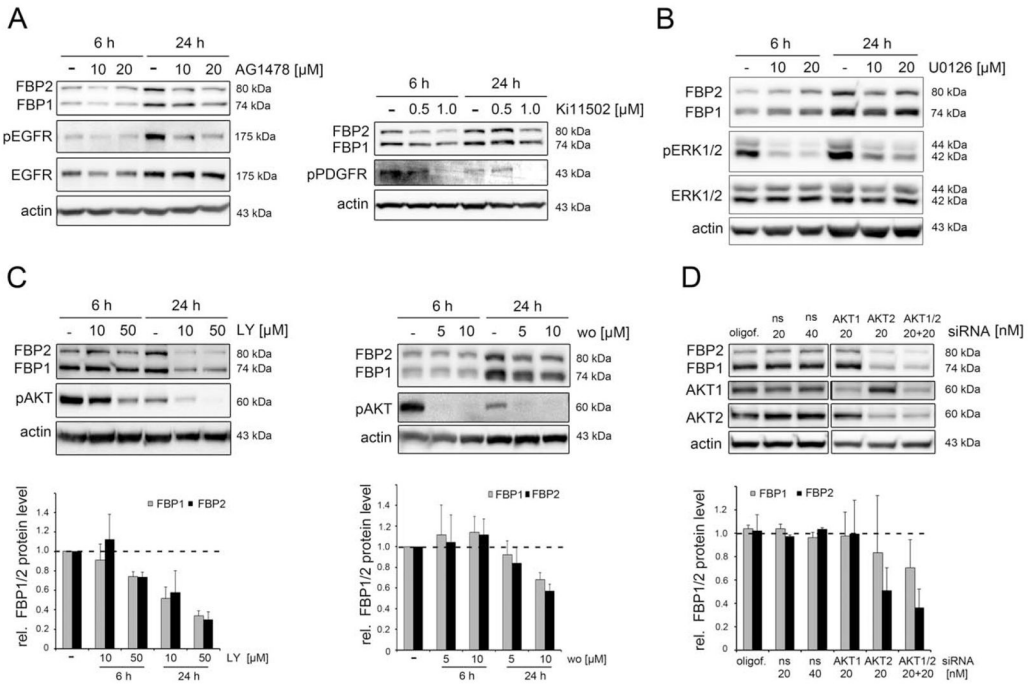

FIG. 1.

The PI3K/AKT pathway stimulates FBP expression in HCC cells. (A) HLE cells were treated with the EGFR inhibitor AG1478 (10 μM and 20 μM) or with the PDGFR inhibitor Ki11502 (0.5 μM and 1 μM) for the indicated time points. Protein levels of FBP1/2, EGFR/phospho-EGFR, and phospho-PDGFR were detected using total protein fractions by western immunoblotting. (B) Expression of FBP1/2, ERK1/2, and phospho-ERK1/2 was analyzed in HLE cells after administration of the MAPK 1/2 inhibitor U0126 (10 μM and 20 μM) in total cell lysates at the indicated time points. (C) FBP1/2 protein and pAKT levels in HLE cells 6 hours and 24 hours after treatment with the PI3K inhibitors LY294002 (10 μM and 50 μM) and wortmannin (5 μM and 10 μM). Untreated cells for each time point were used for normalization and set to 1. (D) Western immunoblot analysis of FBP1/2 and AKT1/2, 48 hours after siRNA-mediated knockdown of AKT1 and/or AKT2 in HLE cells (20 nM and 40 nM). Nonsense siRNA was used as control. Bar diagrams in (C) and (D) summarize the quantitative results from independent experiments (n = 2–3). Identical results were obtained with another HCC cell line (Supporting Fig. S2). Abbreviations: LY, LY294002; ns, nonsense; wo, wortmannin.

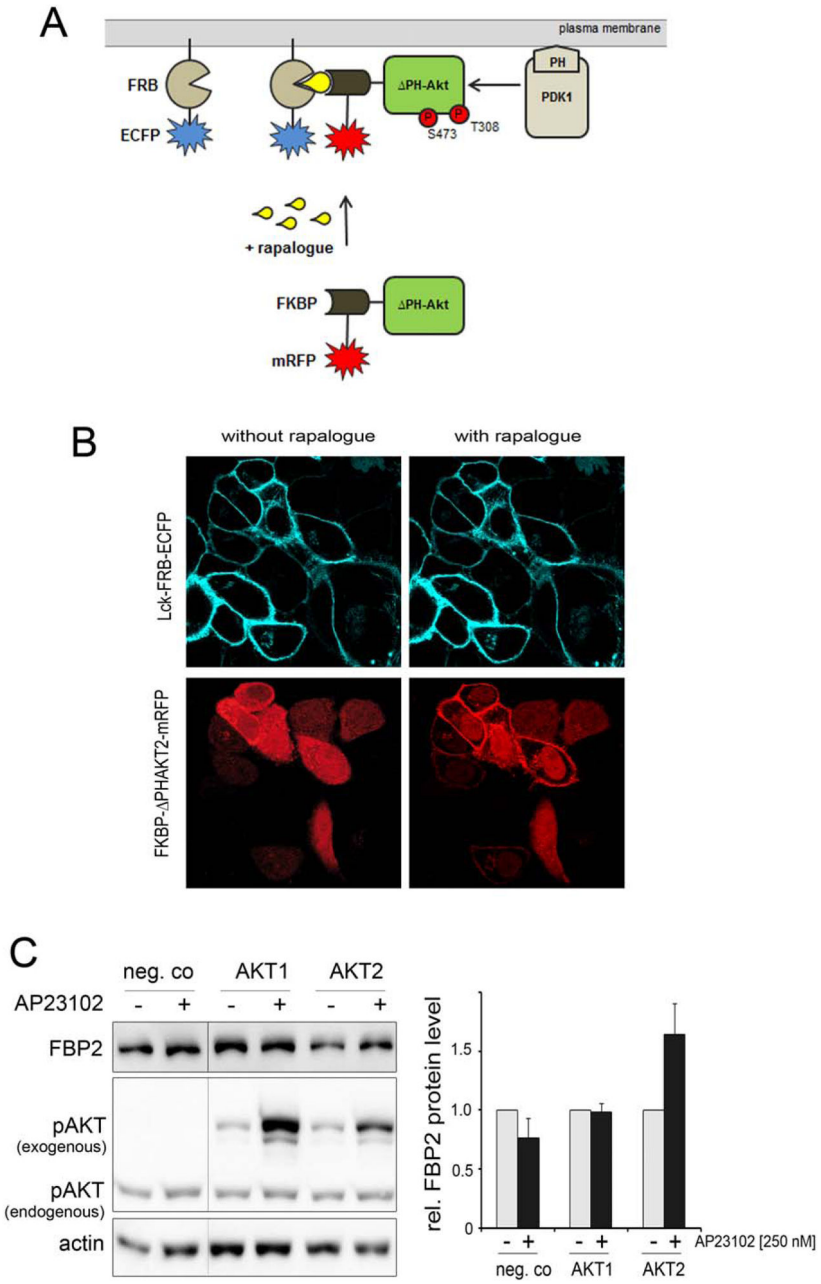

FIG. 2.

Rapalog-dependent dimerization system for AKT induces FBP expression in HCC cells. (A) Cells were transiently transfected with Lck-FRB-ECFP and one of the following constructs: FKBP-ΔPHAKT1-mRFP, FKBP-ΔPHAKT2-mRFP, or FKBP-mRFP (as negative control). Administration of the inert rapalog AP23102 (250 nM) induces membrane translocation and eventually activation of ΔPH-AKT isoforms. (B) Representative immunofluorescence pictures illustrating membranous localization of Lck-FRB-ECFP as well as cytoplasmic and membranous localization of FKBP-ΔPHAKT2-mRFP before AP23102 administration and after 15 minutes, respectively. (C) Hep3B cells were stably transfected with Lck-FRB-ECFP,

while the mRFP vectors were transiently transfected. AP23102 was administered 24 hours after transfection of the mRFP vectors. FBP1/2, AKT/pAKT, and actin protein levels were detected 24 hours after AKT activation. FBP amounts were normalized using the respective actin amounts and the corresponding control without AP23102. Densitometric quantification of protein amounts was performed using the Quantity One software. The graph summarizes the data of two independent Hep3B cell clones stably expressing the Lck-FRB-ECFP fusion protein. Abbreviations: ECFP, enhanced cyan fluorescent protein; FKBP, FK-506 binding protein; FRB, FK-506 binding protein rapamycin binding domain; mRFP, monomeric red fluorescent protein.

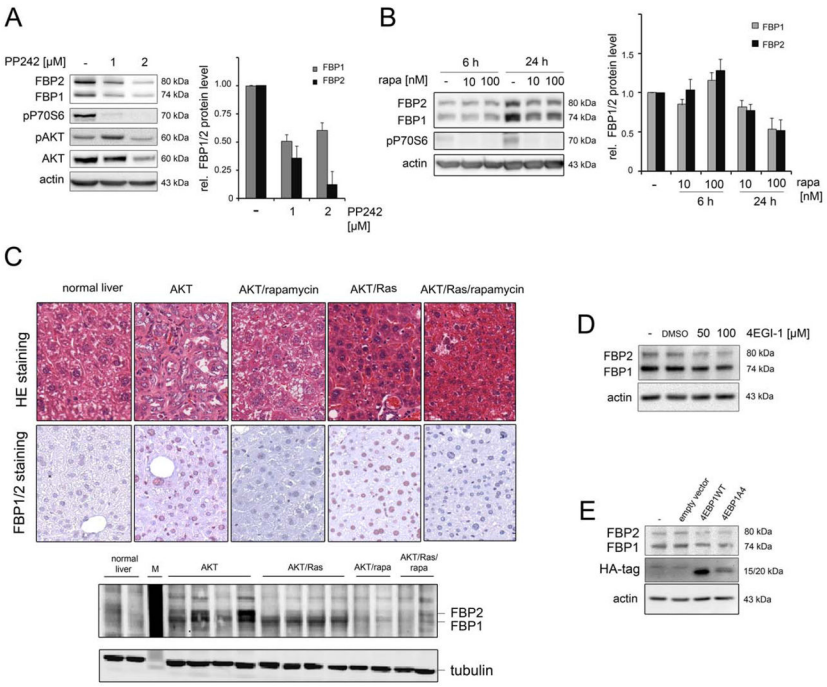

FIG. 3.

mTORC-dependent up-regulation of FBP proteins in HCC cells. (A) HLE cells were treated with the mTORC1/2 inhibitor PP242 for 24 hours (1 μM and 2 μM). Protein levels for FBP1/2, pP70S6, pAKT, and AKT were detected. (B) HLE cells were incubated with the mTORC1 inhibitor rapamycin for 6 hours and 24 hours (10 nM and 100 nM). Expression of FBP1/2 and pP70S6 in HLE total cell lysates was quantitatively analyzed. Untreated cells for each time point were used for normalization and set to 1. (C) Hydrodynamic injection of myr-AKT and/or N-RasV12 along with sleeping beauty transposase induced FBP expression. mTORC1 activity was blocked by rapamycin injections. Representative FBP1/2 immunohistochemical stains and hematoxylin and eosin overview stains are shown. Magnification ×20. In addition, protein lysates derived from the respective liver samples were analyzed by western immunoblotting. (D) Treatment of HLF cells with the eIF4E/eIF4G disruptor 4EGI-1 (50 μM and 100 μM) for 24 hours. HLF cells were chosen because they were resistant to 4GI-1-induced apoptosis. (E) Transfection of HA-tagged wild-type 4EBP1 or its unphosphorylatable isoform 4EBP1A4 in HLF cells. Bar diagrams in (A) and (B) summarize the quantitative results from independent experiments (n = 4). Identical results were obtained with another HCC cell line (Supporting Fig. S3). Abbreviations: HA, hemagglutinin; HE, hematoxylin and eosin; rapa, rapamycin; WT, wild type.

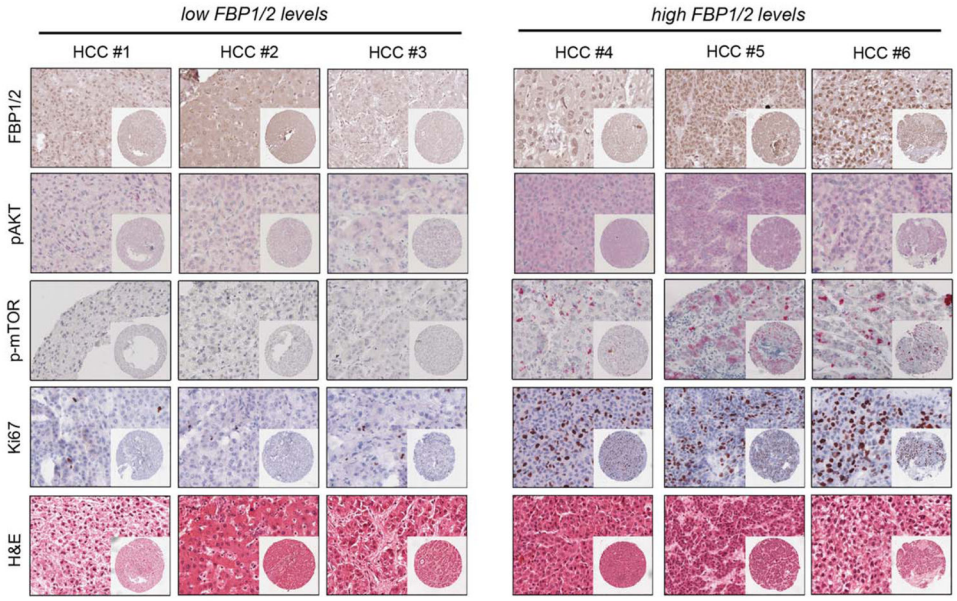

FIG. 4.

Association between FBP1/2, activated AKT, and KI67 in human HCC tissues. Tissue microarray containing seven normal human liver tissues and 100 HCCs (grading: 10 × G1, 71 × G2, 15 × G3, and 4 × G4) was prepared, and sections were stained for FBP1/2, pAKT, phospho-mTOR, and the proliferation marker KI67. Respective H&E stains and the tissue cores are shown. High magnification ×40, low magnification ×10. Three exemplary tissues with high nuclear FBP1/2, cytoplasmic pAKT, and phospho-mTOR levels as well as low nuclear FBP1/2, cytoplasmic pAKT, and phospho-mTOR levels are shown. Abbreviation: H&E, hematoxylin and eosin.

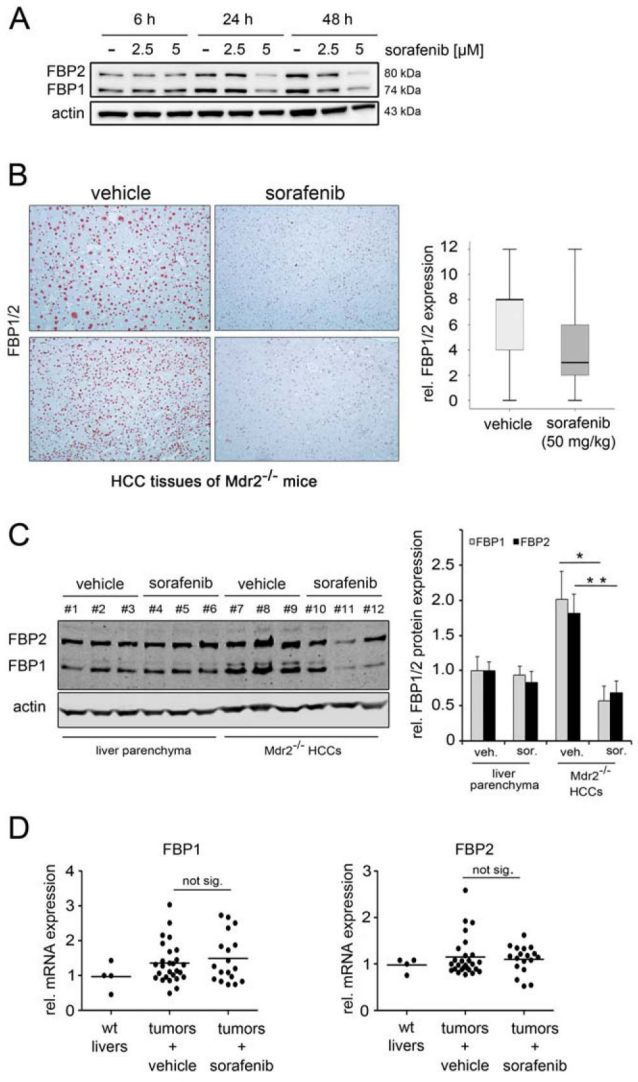

FIG. 5.

Sorafenib reduces FBP1/2 expression in HCCs. (A) Measurement of FBP1/2 protein levels in HLE cells after sorafenib administration (2.5 μM and 5 μM) at the indicated time points. Identical results were obtained with another HCC cell line (Supporting Fig. S4). (B) Tumor-bearing $Mdr2^{-/-}$ mice were treated with sorafenib (50 mg/kg, n = 17) or Cremophor EL/ethanol/water vehicle (n = 21) for 3 days. Expression of FBPs in tissues was analyzed by immunohistochemistry. Exemplary stains for two tumors with and without sorafenib treatment are shown (magnification ×10). Box plot summarizes the data of all analyzed specimens. (C) Representative FBP1/2 protein expression in total protein extracts derived from HCC and adjacent liver tissue of $Mdr2^{-/-}$ mice with and without sorafenib administration. The graph summarizes the data of five (parenchyma with or without sorafenib) and seven (HCC with or without sorafenib) tumors per group. For (C) the Mann-Whitney U test was used. (D) FBP1/2 transcript levels were analyzed in normal liver tissue (n = 4) and HCC with (n = 18) and without (n = 27) sorafenib treatment. Abbreviations: not sig., not significant; wt, wild type.

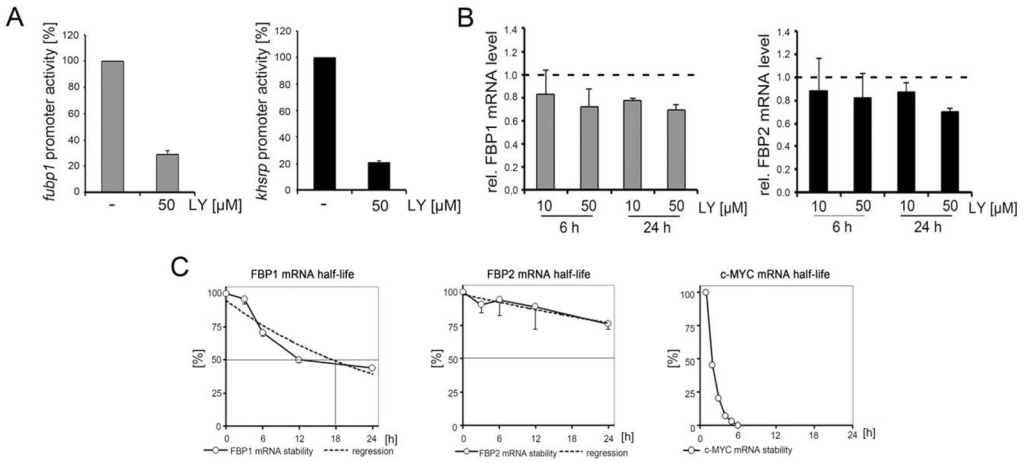

FIG. 6.

High mRNA stability of FBP1 and FBP2. (A) HLE cells were transfected with *fubp1* (n = 6) or *khsrp* (n = 2) promoter-luciferase constructs. Four hours after transfection, cells were treated with DMSO or LY294002 (50 μM). Gaussia luciferase activity was analyzed 48 hours after reporter construct transfection. (B) After incubation of HLE cells with LY294002 for 6 hours and 24 hours (10 μM and 50 μM), FBP1/2 mRNA levels were quantitatively analyzed by real-time polymerase chain reaction. Bars represent ratios between untreated and LY294002-treated cells (n = 3). The housekeeping gene RPL41 was used for normalization. (C) HLE cells were treated with DMSO or actinomycin D (1 μg/mL) for the indicated time points. The mRNA levels of FBP1/2 and c-MYC (positive control) were detected by real-time polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used for normalization. The graph summarizes the data of three experiments. For (C) exponential regression was used. Abbreviation: LY, LY294002.

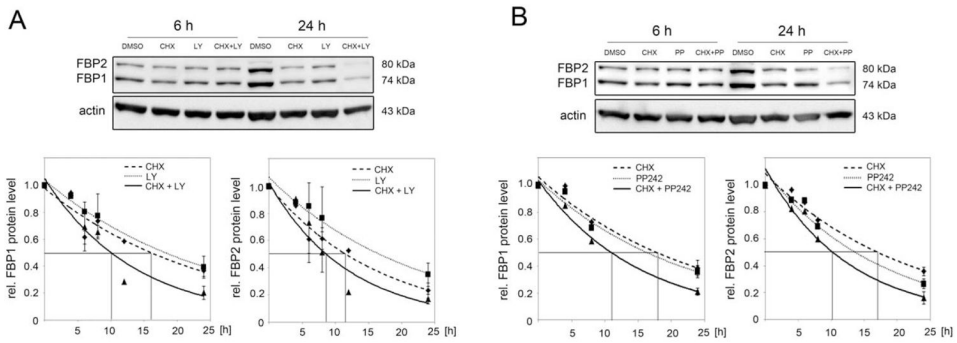

FIG. 7.

The PI3K/AKT/mTOR pathway prolongs FBP protein half-life. (A) FBP1/2 protein level in HLE cell lysates after treatment with DMSO, CHX (450 μM), and the PI3K inhibitor LY294002 (LY, 50 μM) alone or in combination were detected by western immunoblotting. (B) FBP1/2 protein level in HLE cell lysates after treatment with DMSO, CHX (450 μM), and mTORC1/2 inhibitor PP242 (2 μM) alone or in combination. Exemplary blots are shown. Graphs summarize the data of at least three experiments. Untreated cells for each time point were used for normalization and set to 1. Identical results were obtained with another HCC cell line (Supporting Fig. S7). For (A) and (B) exponential regression was used. Abbreviations: LY, LY294002; PP, PP242.

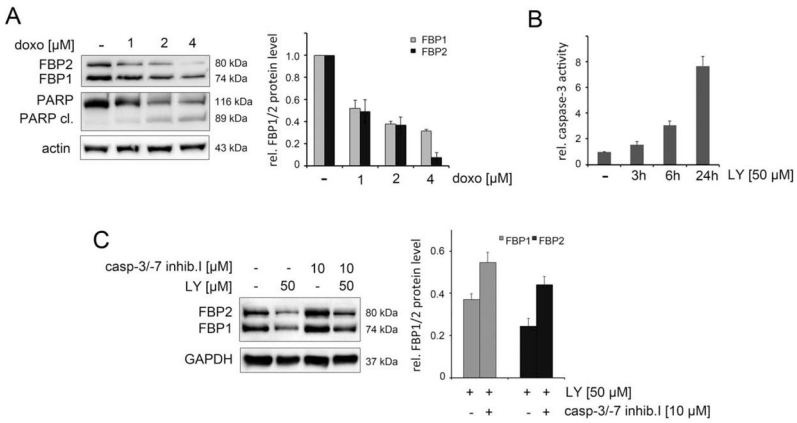

FIG. 8.

PI3K signaling prevents FBP1/2 from caspase-3-mediated degradation. (A) Apoptosis was induced in HLE cells using doxorubicin (1 μM, 2 μM, and 4 μM) for 24 hours. FBP1/2 and PARP were detected by western immunoblotting. PARP cleavage served as positive control for the efficient induction of apoptosis. (B) HLE cells were treated with LY294002 (50 μM) or DMSO for the indicated time points. The activity of caspase-3 was analyzed using the fluorogenic substrate Ac-DEVD-AFC. (C) HLE cells were cultured with and without caspase-3/-7 inhibitor 1 (10 μM) for 30 minutes and then incubated with or without LY294002 (50 μM) for 24 hours. Protein levels of FBP1/2 and GAPDH were analyzed by western immunoblotting. Bar diagrams in (A–C) summarize the quantitative data from independent experiments (n = 2). Identical results were obtained with another HCC cell line (Supporting Fig. S8). Abbreviations: casp-3/-7 inhib. I, caspase-3/-7 inhibitor 1; doxo, doxorubicin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LY, LY294002; PARP cl., PARP cleavage.