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PIK3IP1, a negative regulator of PI3K, suppresses the development of hepatocellular carcinoma

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Abstract

Phosphatidylinositol-3-kinase (PI3K) is a well-known regulator of cell division, motility and survival in most cell types. Recently we characterized a novel protein that we call PI3K Interacting Protein 1 (PIK3IP1) which binds to the p110 catalytic subunit of PI3K and reduces its activity in vitro. Little is known about PIK3IP1's role in normal and neoplastic growth in vivo. Proper liver function and development depend on intact PI3K signal transduction; when dysregulated, the PI3K pathway is linked to the development of liver cancer. To begin to dissect the contribution of PIK3IP1 to hepatic PI3K signaling in vivo and to liver tumorigensis in particular, we formulated the following hypothesis: since PIK3IP1 downregulates PI3K signaling and uncontrolled PI3K signaling is associated with liver cancer, then PIK3IP1-mediated downregulation of the PI3K pathway should inhibit hepatocellular carcinoma (HCC) development. To test this idea, we generated transgenic mice overexpressing PIK3IP1 in hepatocytes in a mouse strain prone to develop HCC. Isolated PIK3IP1 transgenic mouse hepatocytes showed blunted PI3K signaling, DNA synthetic activity, motility and survival as compared to controls. In vivo, spontaneous liver tumorigenesis was significantly dampened in the transgenic animals. This was accompanied by decreased hepatic PI3K activity and reduced hepatocyte proliferation in the transgenics as compared to controls. We also observed that human HCC expressed less PIK3IP1 protein than adjacent matched liver tissue. Our data show that PIK3IP1 is an important regulator of PI3K in vivo, and its dysregulation can contribute to liver carcinogenesis.

Keywords

PIK3IP1; PI3K; liver; hepatocytes; hepatocellular carcinoma

Introduction

The class IA phosphoinositol-3-kinases (PI3Ks) regulate important cellular processes such as proliferation, growth, survival, motility and metabolism. PI3K is stimulated by association of the p85 regulatory subunit with tyrosine phosphorylated proteins at the plasma membrane which leads to activation of the p110 catalytic subunit and generation of the second messenger phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] from phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$]. The appearance of $PI(3,4,5)P_3$ attracts Akt (also known as Protein

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Kinase B [PKB]) and other signal transduction molecules to the membrane, the association of which triggers typical PI3K dependent cellular responses (1). We recently identified and characterized a new negative regulator of PI3K we named Phosphatidylinositol-3-kinase Interacting Protein 1 (PIK3IP1) that binds to the p110 catalytic PI3K subunit by virtue of a region sharing homology with the p85 regulatory subunit. When overexpressed in cultured cells, PIK3IP1 suppresses PI3K activity, while PI3K activity rises when PIK3IP1 levels are experimentally reduced (2). However, the consequences of PIK3IP1 on PI3K signaling in vivo remain unknown.

A variety of normal tissues such as the liver utilize the PI3K cascade to convey extracellular signals inside the cell leading to changes in gene and protein expression. Abnormal activation of the PI3K pathway, though, can cause unchecked growth and increased cell survival (1). Several human malignancies including ovarian, breast, and liver cancer display dysregulation of the PI3K signal transduction pathway (1,3,4). In human hepatocellular carcinoma (HCC), for example, Lee et al. (5) detected mutations in *pik3ca* (the gene for p110 alpha). Of the various tumor types tested, liver cancer shows the highest percentage of cases with *pik3ca* mutations (36%) (1). Another PI3K pathway constituent that is targeted in liver cancer is PTEN (phosphatase and tensin homologue). PTEN is the product of a well-known tumor suppressor gene and acts as a lipid and protein phosphatase that regulates the relative cellular concentration of $PI(3,4,5)P_3$ (6). PTEN protein abundance is downregulated in human HCC (7,8). Mutations and loss of heterozygosity of the *PTEN* gene (9) have been identified as well. Experimentally, liver tumors developed in mice lacking functional PTEN in hepatocytes (10). Given the emerging importance of PI3K to tumorigenesis in tissues like liver, we chose to explore the possibility that PIK3IP1 plays a role in normal and cancerous hepatic cell growth by regulating PI3K signaling. To do so, we utilized a mouse model of PIK3IP1 gain-of-function in hepatocytes.

Materials and Methods

Chemicals and Reagents

The following reagents were described previously: polyclonal antibody against PIK3IP1, anti-p110 antibodies, anti-phospho-Akt and anti-Akt antibodies as well as Protein-A agarose and IgG control antibody (2). Anti-phospho-p85 antibody was obtained from Cell Signaling (Beverly, MA). Mouse epidermal growth factor (EGF) was purchased from BD Biosciences (Mountain View, CA). Hepatocyte growth factor (HGF—five amino acid deleted isoform) was a kind gift from Snow Brand Milk Products, Ltd. (Japan). Ly294002 was purchased from LC Laboratories (Woburn, MA). All other chemical reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Assurances

Appropriate University of Pittsburgh Institutional Review Board (IRB), Institutional Animal Care and Use Committee (IACUC) and Recombinant DNA (rDNA) approvals were obtained prior to commencing these studies.

Human Tumor Tissues

Archived frozen human hepatocellular carcinoma and adjacent liver tissues were obtained through the Health Systems Tissue Bank at UPMC.

Generation and Characterization of Liver Specific PIK3IP1 Transgenic Mice

A 0.8 kb mouse *pik3ip1* cDNA containing the entire coding region of PIK3IP1 was derived from a mouse mammary gland est (GenBank I.D.: AA754893). PCR primers (forward primer:

5′-TTAGGATCCCATTTGGACACTGGCTG-3′; reverse primer: 5′-GATCCTAGGCCTGTACCAGTGTTTAC-3′) were prepared that incorporated BamHI sites in the 5′ and 3′ non-coding regions of the cDNA near the translation stop and start codons, respectively. The amplified region was inserted into the BamHI site of an albumin promoter-driven expression vector which was kindly provided by Dr. Richard Palmiter (University of Washington, Seattle, Washington) as described by Bell et al. (11). This transgene was used to generate transgenic (TG) mice on a B6SJL background at the Duke University Medical Center Transgenic Mouse Facility (Durham, NC). TG mice were identified by Southern blot analysis (using standard procedures) of BamHI digested genomic tail DNA using a ^{32}P-labeled 0.8 kb mouse PIK3IP1 cDNA probe. To confirm the results, PCR analysis of blood DNA was performed using oligonucleotide primers designed from the PIK3IP1 gene as follows: forward primer: 5′-GACGTGAGTTGCCCAGAGACC-3′; reverse primer: 5′-TTACCTGCAGCCATTGCCGCTAGTGAG-3′. TG mice were propagated by breeding with wildtype C3H mice for greater than four generations. Livers of young mice (≤6 mo.) were harvested, and liver and body weights were recorded to calculate the liver:body weight ratio. Grossly, the livers appeared to be normal in the TG and non-transgenic (NTG) animals, and the liver:body weight ratios were similar between the two groups. Histologically, major architectural changes attributable to the presence of the PIK3IP1 transgene were not observed.

For tumor studies, eight male TG and eight male NTG littermates (average age 14 mo.) were sacrificed by cervical dislocation and necropsied. Gross examination of the liver for tumors was performed. The presence of grossly visible tumor was documented. A portion of each liver lobe was placed in formalin, embedded, sectioned and stained with H&E. Tissue sections were examined histologically to confirm the presence of hepatocellular carcinoma. The remaining liver tissue was snap frozen for additional experimentation.

Assessment of Proliferation and Apoptosis

Ki67 or PCNA immunostaining and TUNEL staining of formalin fixed liver using standard procedures was performed to assess hepatocyte proliferation and apoptosis, respectively. The Ki67 antibody was purchased from Abcam (Cambridge, MA) and used at a concentrationof 1:50. The PCNA antibody was purchased from Dako (Glostrup, Denmark) and used at a concentration of 1:4000. The DeadEnd™ Colorimetric TUNEL System (Promega Corporation, Madison, WI) was used to evaluate apoptosis. Three to five thousand hepatocyte nuclei in non-tumorous tissues were examined per mouse following Ki67 or TUNEL staining. About 600 hepatocytic nuclei were assessed in tumorous and non-tumorous human tissues following PCNA. The formula used to determine the proliferation or apoptotis indices was: number of Ki67, PCNA or TUNEL positive hepatocyte nuclei, respectively, divided by the total number of hepatocyte nuclei counted.

Extraction of Total RNA and qRT-PCR

Total RNA was extracted according to the TRIzol manufacturer’s protocol (Invitrogen, Carlsbad, CA) from TG (n = 8) and NTG (n = 11) mouse liver tissue. Animals ranged in age from 1–6 mo. The RNA was reverse transcribed by standard procedures and then subjected to qRT-PCR analysis for 40 cycles using the ABI Prism 7000 Sequence Detection System with TaqMan® Gene Expression Assays reagents as well as primers specific for mouse PIK3IP1 purchased from Applied Biosystems (Foster City, CA, Assay ID: Mm01191492_m1). 18S rRNA primer (Applied Biosystems, Assay ID: HS99999901_S1) was used as an internal control for sample quality, and DEPC water was used as a negative control for product contamination.

Protein Isolation, SDS-PAGE and Western Blot Analyses

Human and mouse liver tissues as well as isolated mouse hepatocytes were subjected to western blot (WB) analysis as described previously (2). Commercially available antibodies were utilized at the dilutions recommended by their manufacturers. Western Lightning Chemiluminescence Reagent PLUS (PerkinElmer Inc., Boston, MA, #NEL102) and Biomax film (Eastman Kodak Company, New Haven, CT) were used to detect the WB signals, the intensities of which were measured using Scion Image 1.63 software (Scion Corporation, Frederick, MD).

In Vitro PI3K Activity Assay

PI3K activity assays were performed as previously described (2). Briefly, protein lysates (1 mg) were immunoprecipitatedwith anti-p110 antibodies, washed 3X with RIPA buffer, followed by three washes with PI3K reaction buffer (PI3K-RB) (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.5 mM EGTA). Immunoprecipitates were suspended in 50 μl of PI3K-RB containing 0.2 mg/ml phosphatidylinositol (PI, Sigma) followed by incubation for 10 min. at RT. After incubation at RT, 440 ul of PI3K-RB containing 30 μCi of ^{32}P-gammaATP, 0.88mM ATP and 20 mM $MgCl_2$ was added to the resuspended immunoprecipitates which were then incubated for 10 min. at RT. 100 ul of chloroform:methanol:HCl (200:100:2) was used to halt the reaction. Thin layer chromatography was used to separate the products in chloroform:methanol:ammonium hydroxide:water (86:76:10:14) running buffer. Lysates were routinely assessed for p110 abundance by IP and WB analysis.

Isolation and Culture of Primary Mouse Hepatocytes

Mouse hepatocytes were isolated from male PIK3IP1 TG and NTG littermates by the in situ two-step collagenase perfusion technique described previously (11). Hepatocytes were pelleted by centrifugation, assessed for viability by Trypan Dye exclusion which typically ranged from 70–90%, and cultured in Eagle's Minimal Essential Medium (EMEM—Cellgro, Herndon, VA) containing 10% fetal bovine serum. The medium was then changed to serum free after 3 hr. For some experiments, hepatocytes were plated in hepatocyte growth medium (HGM (12)) with slight modification as follows: 1) EMEM was used in place of Dulbecco's Modified Essential Medium; 2) nicotinamide was omitted; and 3) dexamethasone was added at a final concentration of 10^{-8}M.

^{3}H-Thymidine Incorporation Assay

Mouse hepatocytes from either TG or NTG littermates were seeded at 250,000 cells per well in 6 well plates in triplicate in HGM. Following 18 hr. under serum free conditions, the cells were changed to HGM medium containing 2.5 μCi ^{3}H-thymidine with or without a combination of HGF (30 ng/ml) and EGF (25 ng/ml) and with either Ly294002 (10 μM in DMSO) or DMSO alone as a vehicle control. After 48 hr., radioactivity was measured by scintillation counting as described (13).

Transwell Migration Assay

Transwell migration assays were performed on hepatocytes as described previously (14). Briefly, TG or NTG hepatocytes were seeded onto the upper part of a 12 μm pore Transwell chamber (collagen I coated) at a density of 50,000/cm^2 and allowed to attach for 3 hr. Unattached cells were washed; adherent cells were maintained in serum-free EMEM for 12 hr. prior to transfer of the Transwell to a fresh 12-well plate containing EGF (20 ng/ml) or control media with either Ly294002 (10 μM in DMSO) or DMSO alone as a vehicle control. Cells were then allowed to migrate for 24 hr. prior to fixing with 4% paraformaldehyde in PBS for 30 min. Cells were then stained with 0.1% Coomassie blue in 10% methanol/10% acetic acid for 1 hr. Transwells were washed; stationary cells were removed from the filter top with

a cotton-tipped applicator; cell debris was washed away; then, cells that had migrated to the bottom of the Transwell filter were enumerated by counting ten fields for each Transwell at 200× magnification.

In Vitro Scratch Assay

One million TG or NTG hepatocytes were seeded in 6-well plates to obtain confluent monolayers. After 12 hr., a scratch to dislodge cells was made in the monolayers using a blunted Pasteur pipette, and hepatocytes were washed several times to remove floating cells and debris. Cultures were treated with or without EGF (20 ng/ml) and with either Ly294002 (10 μM in DMSO) or DMSO alone as a vehicle control for up to 5 days. Daily, the medium was changed and reconstitution of the scratched surface was monitored and photographed.

UV-induced Apoptosis Assay

One hundred thousand TG or NTG hepatocytes were cultured for 12 hr. on single well chamber slides (9.4 cm^2/well—Lab-Tek, Germany). Some slides were treated with a dose of 50 J/m^2 UV irradiation without medium. Mock treatment consisted of medium removal for an equivalent time period but no UV exposure. Serum free medium was replaced, and after 24 hr., slides were fixed with 4% paraformaldehyde and TUNEL stained for detection of apoptotic cells. Cleaved caspase 3 was assessed by WB using cell lysates harvested from some cultures.

Statistical Analysis

Statistical analysis was carried out using a two-tailed Student's t-Test unless otherwise specified. Results were considered to be statistically significant when *p* values were determined to be less than 0.05.

Results

Generation and characterization of hepatocyte-specific PIK3IP1 overexpressing mice

We subcloned the full-length mouse PIK3IP1 cDNA into a construct containing the human growth hormone gene under the transcriptional control of the mouse albumin promoter/enhancer (Fig. 1A) as we described previously for human HGF (11) and generated transgenic mice. We utilized this particular promoter/enhancer cassette because albumin promoter activity is specific for hepatocytes, rises after birth and plateaus within approximately one month (15).

PIK3IP1 expression reduces PI3K activity and phospho-Akt levels in mouse liver

We first ascertained that TG mice overexpress PIK3IP1 in the liver by qRT-PCR (Fig. 1B) and western blot (Fig. 1C) as compared to controls. We noted no differences in p110 alpha or beta protein levels between the livers of TG and NTG mice (data not shown). However, we did observe a significant diminution in PI3K activity by about 50% (Figs. 2A&B) and a marked decrease in phospho-Akt levels by western blot (Fig. 2C) in the TG livers as compared to controls suggesting that PIK3IP1 regulates p110 activity and signaling in liver.

PIK3IP1 downregulates Akt in isolated mouse hepatocytes

To further investigate PIK3IP1's effects on liver cell biology and the PI3K pathway, we isolated hepatocytes from TG and NTG animals. We confirmed that PIK3IP1 is overexpressed in the TG hepatocytes as compared controls by qRT-PCR (data not shown). Next, we examined the hepatocytes for basal Akt activation by WB and detected decreased levels of endogenous phospho-Akt in cultured TG hepatocytes as compared to control cells (Fig. 2D). This indicates that PIK3IP1 expression does indeed downregulate the PI3K pathway in TG hepatocytes.

PIK3IP1 suppresses mouse hepatocyte DNA synthesis and motility in culture

Since we determined that PIK3IP1 reduces signaling through the PI3K pathway in TG hepatocytes and since PI3K activity is linked to increased cell motility and proliferation in a wide variety of cell types, we next explored the functional consequences of PIK3IP1 expression and PI3K downregulation on DNA synthesis and motility in the TG and NTG hepatocytes. When cultured hepatocytes were induced to undergo DNA synthesis by addition of known hepatic mitogens (HGF and EGF) (16), the DNA synthetic response in TG hepatocytes measured by fold increase in ^{3}H-thymidine incorporation over control cultures was strongly impaired (Fig. 3A). We also added Ly294002 (LY), a known PI3K inhibitor (17), to some cultures to determine the effect of PI3K inhibition on hepatocyte DNA synthesis; LY treatment also blunted growth factor-induced hepatocyte DNA synthesis (data not shown). Overall, our results demonstrate that PIK3IP1 expression diminishes mouse hepatocyte DNA synthetic activity likely through inhibition of PI3K signaling.

To examine the effect of PIK3IP1 on cell motility, another cellular function ascribed to PI3K (18), we subjected TG and NTG hepatocytes to a transwell migration assay. In response to EGF, a well-characterized hepatocyte motogen (14), three fold fewer TG hepatocytes than NTG hepatocytes migrated across the transwell insert (mean number of migrated hepatocytes per hpf: 23.8 ± 13.8 vs. 70.3 ± 22.5, respectively [Figs. 3B&C]). This difference was found to be statistically significant (p=0.00003). As a control for PI3K inhibition, LY was added to some EGF-treated TG and NTG cultures; LY reduced migration of both cell types by over two fold (Figs. 3B&C).

In parallel, we performed a second type of motility assay (i.e., an in vitro scratch assay (19)) which compares the ability of TG and NTG hepatocytes to reepithelialize a scratched surface. As shown in Fig. 3D, reepithelialization was significantly delayed in TG hepatocyte cultures as compared to controls which typically reconstituted the void in 5 days in our assay. We added LY to some EGF-treated cultures; it halted reepithelialization by both TG and NTG hepatocytes. Taken together, our data indicate that PIK3IP1 impedes mouse hepatocyte motility, a response that is likely to be at least in part PI3K dependent.

PIK3IP1 promotes apoptosis in isolated mouse hepatocytes

Considering the key role of PI3K in cell survival (20), we next assessed if PIK3IP1 spurs hepatocyte cell death. To do so, we induced apoptosis in TG and NTG hepatocytes with UV exposure and examined the cells for apoptosis by TUNEL staining and by western blot (WB) for cleaved caspase 3. Normal cultured hepatocytes are known to undergo apoptosis upon UV treatment (21). In our experiments, we observed that 47.4 ± 6.3% of TG hepatocytes and only 28.5 ± 3.3% of control cells became TUNEL positive 48 hr. following UV irradiation (Figs. 4A&B) which was determined to be a statistically significant difference (p=0.00005). TG hepatocytes also showed significantly more cleaved caspase 3 by WB at this time point when compared to NTG cells (Fig. 4C). Taken together, these data support the idea that PIK3IP1 promotes apoptosis in isolated hepatocytes, an observation that is in agreement with our previous finding that PIK3IP1 enhanced apoptotic cell death induced by staurosporine addition to C33A human endometrial cancer cells (2).

PIK3IP1 suppresses hepatocyte proliferation and spontaneous HCC development in vivo

Once we established that PIK3IP1 overexpression alters PI3K activity in mouse liver and in isolated hepatocytes, we wanted to determine whether its presence affects the appearance of liver tumors in vivo. Our transgenics were crossed onto a C3H background. Male mice of the C3H strain spontaneously develop hepatocellular carcinoma (HCC) without carcinogen treatment: the incidence of liver tumors is estimated to be 72–91% in males at 14 months of age (http://jaxmice.jax.org/strain/000659.html). On necropsy, we found that 62.5% of NTG

male mice (avg. age 14 mo.) had histologically confirmed HCC in their livers while only 12.5% of PIK3IP1 matched male TG littermates developed HCC (Figs. 5A&B). This comparison was statistically different (p=0.0387) between the groups using Chi-squared analysis.

To begin to decipher how PIK3IP1 impacts liver tumorigenesis in our animals, we examined the hepatocyte proliferation rate in the adjacent normal liver tissue of TG and NTG mice utilized in the tumor study. We immunostained their livers for Ki67, a nuclear antigen which appears in G_1-G_M. We observed that about 1.00 ± 0.52% of NTG hepatocyte nuclei stained for Ki67 while only 0.36 ± 0.21% of TG hepatocyte nuclei were Ki67 positive (Fig. 5C&D). This difference was found to be statistically significant between the two groups using the Mann-Whitney test (p<0.0039). The percentage of apoptotic hepatocytes as determined by TUNEL staining was not statistically different between the NTG and TG groups (0.27 ± 0.15% vs. 0.44 ± 0.32%, respectively) using the Mann-Whitney test. Collectively, these data indicate that the presence of PIK3IP1 in hepatocytes suppresses hepatocyte proliferation in vivo and spontaneous liver tumorigenesis.

PIK3IP1 protein expression is reduced in human HCC

Lastly, to gain insight as to whether PIK3IP1 plays a role in human liver tumorigenesis, we examined PIK3IP1 expression in human hepatocelluar carcinoma (HCC) and adjacent non-tumor tissue by western blot and found that PIK3IP1 protein abundance was diminished in the majority of HCC cases (9/12) as compared to matched adjacent liver (Figs. 6A&B). On average, tumors expressed 22% less PIK3IP1 protein than control tissues (p=0.0025). However, three of the twelve cases expressed only half the amount of PIK3IP1 protein as compared to adjacent tissue (Cases 2, 6 and 9—Figs. 6A&B). We then carried out analysis of human HCC and adjacent liver tissues for PI3K activity as measured by the abundance of tyrosine phosphorylated p85 (which is a surrogate of PI3K activation (22)) relative to total p85 in WB and for proliferation activity (as determined by PCNA nuclear labeling). We next compared these findings to PIK3IP1 abundance in the same tissues. As expected, we observed a high proliferative index (average labeling index: 30% in tumors vs. 2.3% in the corresponding adjacent livers) and readily detectable PI3K activity in tumors which were associated with reduced PIK3IP1 abundance in several HCC cases tested (data not shown).

Discussion

Unregulated signaling through the PI3K pathway is linked to the development of certain types of cancer including hepatocellular carcinoma (1,5). While others have demonstrated that hepatocyte specific gene deletion of the PI3K signaling regulator *PTEN* results in the appearance of liver tumors in a mouse model (10), no studies of which we are aware have examined the effects of directly suppressing PI3K activity on liver tumor development. Our laboratory discovered and characterized a novel negative regulator of Class Ia PI3Ks called Phosphatidyl inositol-3-kinase interacting protein 1 (PIK3IP1). PIK3IP1 is a transmembrane protein that possesses a region in its intracellular domain that shares homology with the p85 regulatory subunit of PI3K. Previously, we showed through a variety of in vitro experiments that PIK3IP1 binds to the p110 catalytic subunit of PI3K through PIK3IP1's intracellular p85-like domain to downregulate PI3K activity (2). However, we had not elucidated the role of PIK3IP1 in controlling PI3K activity in vivo. The liver requires sufficient PI3K signaling to function appropriately. For example, mice lacking all isoforms of p85 alpha (thus showing substantially reduced PI3K activity because of a destabilizing effect on p110) die perinatally partly due to liver necrosis (23). As mentioned, unfettered PI3K signaling also alters liver homeostasis. Aberrant PI3K signal transduction is implicated in liver tumorigenesis in both humans and rodents (5,7,8,10). Given all of this information as well as our own published observations that PIK3IP1 negatively regulates the PI3K pathway in vitro, we wanted to

ascertain whether PIK3IP1 is involved in normal liver growth and hepatic tumorigenesis. We hypothesized that PIK3IP1 expression in hepatocytes suppresses PI3K activity in these cells and thereby inhibits hepatocyte growth, motility and liver tumorigenesis.

To test this hypothesis, we took a gain-of-function approach and generated transgenic mice in which PIK3IP1 expression is directed to the hepatocytes. Our decision to target PIK3IP1 expression to hepatocytes is supported by physiologically relevant evidence summarized as follows: 1) the liver has high endogenous PI3K activity as compared to most other tissues (i.e., relative mouse tissue PI3K activity measured by phospho-/total AKT WB: brain—0.4; heart —1.0, testis—1.2; spleen—1.5; kidney—1.6; lung—1.8; and liver—2.9), 2) liver survival is dependent on a functioning PI3K pathway (23), 3) PIK3IP1 mRNA and protein are expressed by liver and hepatocytes ((2) and data not shown, respectively); and 4) the expression levels of PI3K pathway constituents (5,7,8,10) are altered in human liver tumors as compared to adjacent liver.

Analysis of our PIK3IP1 expressing mice revealed that hepatic PI3K activity is reduced as compared to control animals supporting an in vivo role for PIK3IP1 as a PI3K regulatory molecule. Hepatocytes isolated from these animals showed a blunted response to growth factor induced DNA synthesis. In addition, we found that LY294002 (LY), a known PI3K inhibitor, staunched DNA synthesis in isolated mouse hepatocytes, a finding that is in agreement with studies by others showing that PI3K blockade by wortmannin (another well-studied PI3K inhibitor (24)) cuts HGF-induced DNA synthesis in cultures of rat hepatocytes by about half as compared to HGF treatment alone (25). Given the fact that the hepatocyte DNA synthetic response stimulated by growth factors is sensitive to PI3K inhibition, it is reasonable to postulate that PIK3IP1's suppressive effect on this process is mediated by its ability to downregulate PI3K activity.

Growth factors such as EGF or HGF stimulate hepatocyte motility in culture. Nakanishi et al. (26) reported that blocking PI3K signaling with wortmannin mitigated HGF-induced motility of human liver cancer cell lines in culture; however, it is unknown whether the motility of *normal* hepatocytes depends on signal transduction through the PI3K pathway. Our experiments are the first to demonstrate that normal mouse hepatocytes treated with LY are significantly less motile than controls in transwell and scratch assays suggesting that hepatocyte motility does indeed rely at least in part on intact PI3K signaling. In the same types of studies, we found that, akin to our observations in LY treated hepatocytes, motility in PIK3IP1 overexpressing TG hepatocytes was reduced compared to controls which may well be a consequence of its ability to suppress PI3K.

Tumor development requires the activation of a set of cellular functions such as division, proliferation, migration, survival and tissue remodeling, many of which are influenced by PI3K. In our transgenic animal model, we observed a significant reduction in spontaneous liver tumorigenesis in PIK3IP1 overexpressing TG male mice as compared to controls. Because our studies indicated that PIK3IP1 expression in hepatocytes suppressed hepatic PI3K activity as well as hepatocyte DNA synthesis in culture, we chose to examine hepatocyte replication in situ in the livers of the TG and NTG animals. Typically, the vast majority of hepatocytes in the adult are in a state of quiescence (i.e., in G_0) in vivo. For example, Counts et al. (27) pulsed mice of two different strains (B6C3F1 and C57B/6) with BrdU for one week, sacrificed the animals, and determined the hepatocyte BrdU labeling index as a measure of proliferation. They noted that only 2–4% of hepatocytes labeled with BrdU indicating that a minor proportion of normal hepatoyctes were engaged in the cell cycle. We observed that hepatocyte replication in our TG animals was reduced: the percentage of hepatocytes staining with the proliferation marker Ki67 was about two-thirds less in TG mice than that observed in control animals. Thus, one mechanism by which PIK3IP1 suppresses liver tumorigenesis in mice may be through

inhibition of innate hepatocyte replication, a process that we show is likely to be at least partially dependent on PI3K signaling.

As noted, we observed PIK3IP1-induced inhibition of hepatocyte proliferation as well as motility, two functions crucial not only to hepatic tumor development and growth but to governing the liver's ability to physiologically replenish itself or regenerate following insult. Liver regeneration is a remarkable process whereby the liver parenchyma regrows in an orderly fashion following limited hepatic damage (by exposure to toxins or anoxia, for example) or surgical excision (i.e., two-thirds partial hepatectomy). Proper liver regrowth requires a combination of controlled cell division, migration and tissue remodeling and is stimulated and regulated by a variety of endocrine and paracrine growth factors, cytokines and hormones, some of which are known to signal through PI3K such as HGF (16). It seems likely then that the PI3K signaling network plays an instrumental part of the normal regenerative response in hepatic tissue. Despite the obvious link between PI3K and liver regeneration, little has been published elucidating the role of PI3K in regrowth of the liver. A recent report by Jackson et al. (28) demonstrates that treatment of mice with wortmannin slowed liver regeneration at 2d. following partial hepatectomy as measured by the percent liver remnant to body mass as well as by BrdU incorporation into hepatocyte nuclei. When the mice were treated with siRNA to either p85 alpha or p110 alpha, a more robust inhibition of liver regeneration was observed. We are currently assessing the role of PIK3IP1 in liver regeneration following surgical resection in our PIK3IP1-overexpressing mice.

Altogether, our in vivo and in vitro data demonstrating that hepatic PIK3IP1 expression negatively regulates PI3K activity in this tissue and suppresses the development of HCC coupled with our findings that PIK3IP1 protein expression is reduced in most cases of human HCC point to a tumor suppressor-like function for PIK3IP1 and suggest that downregulating PI3K may well have an inhibitory effect on liver tumorigenesis, a notion that deserves further attention.

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List of Abbreviations

EGF
: epidermal growth factor

HCC
: hepatocellular carcinoma

HGF
: hepatocyte growth factor

hpf
: high power field

LY
: LY294002

NTG
: non-transgenic

PI3K
phosphatidylinositol-3-kinase

PIK3IP1
phosphatidylinositol-3-kinase interacting protein 1

PTEN
phosphatase and tensin homologue

TG
transgenic

TUNEL
terminal dUTP nick-end labeling

UV
ultraviolet

WB
western blot

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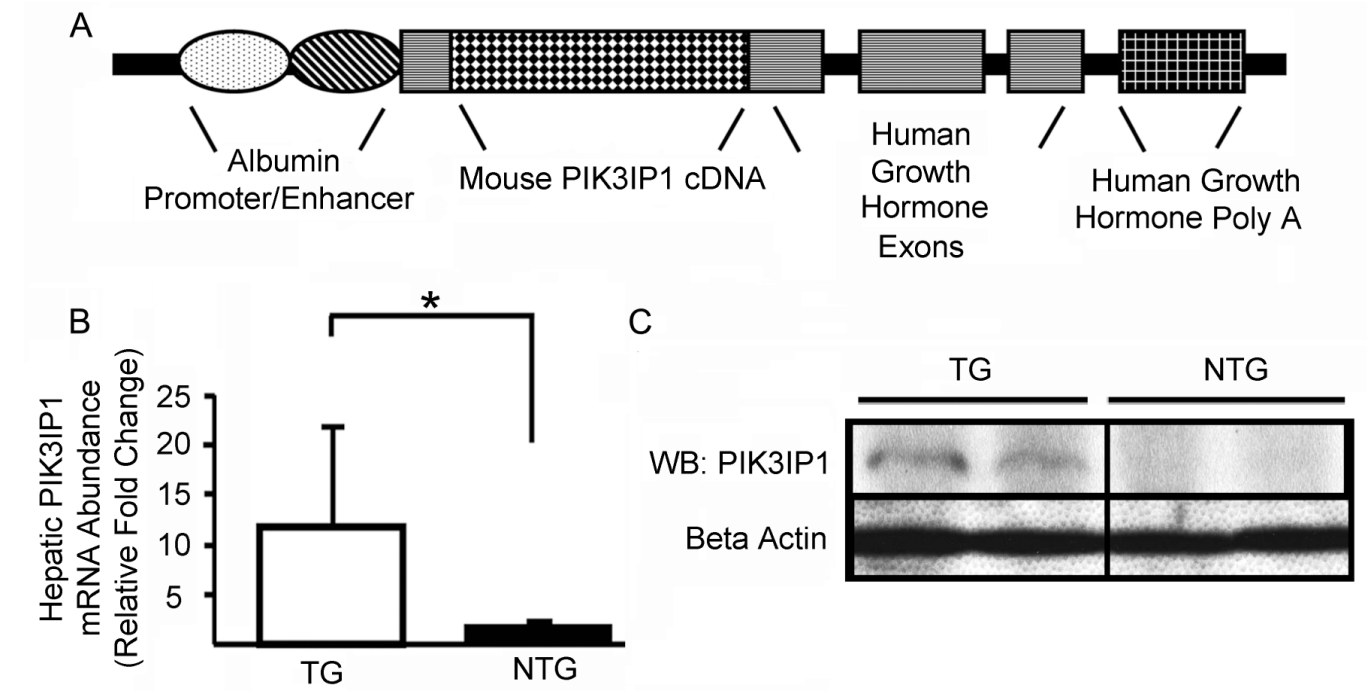

Figure 1. Generation and characterization of transgenic mice expressing PIK3IP1 in hepatocytes

A) PIK3IP1 transgene construct. Full-length mouse PIK3IP1 cDNA was subcloned into an albumin promoter/enhancer growth hormone expression construct. **B) PIK3IP1 mRNA is overexpressed in PIK3IP1 transgenic mouse liver.** Total liver RNA from transgenic (TG—n = 8) and non-transgenic (NTG—n = 11) mice was subjected to qRT-PCR. Data were normalized to 18S rRNA; the relative fold change in PIK3IP1 mRNA abundance was calculated and graphed. *p=0.0006 **C) PIK3IP1 protein is overexpressed in PIK3IP1 transgenic mouse liver.** Liver protein lysate from transgenic (TG—n = 2) and non-transgenic (NTG—n = 2) mice was subjected to western blot (WB) for PIK3IP1 protein. Beta Actin was used as a loading control.

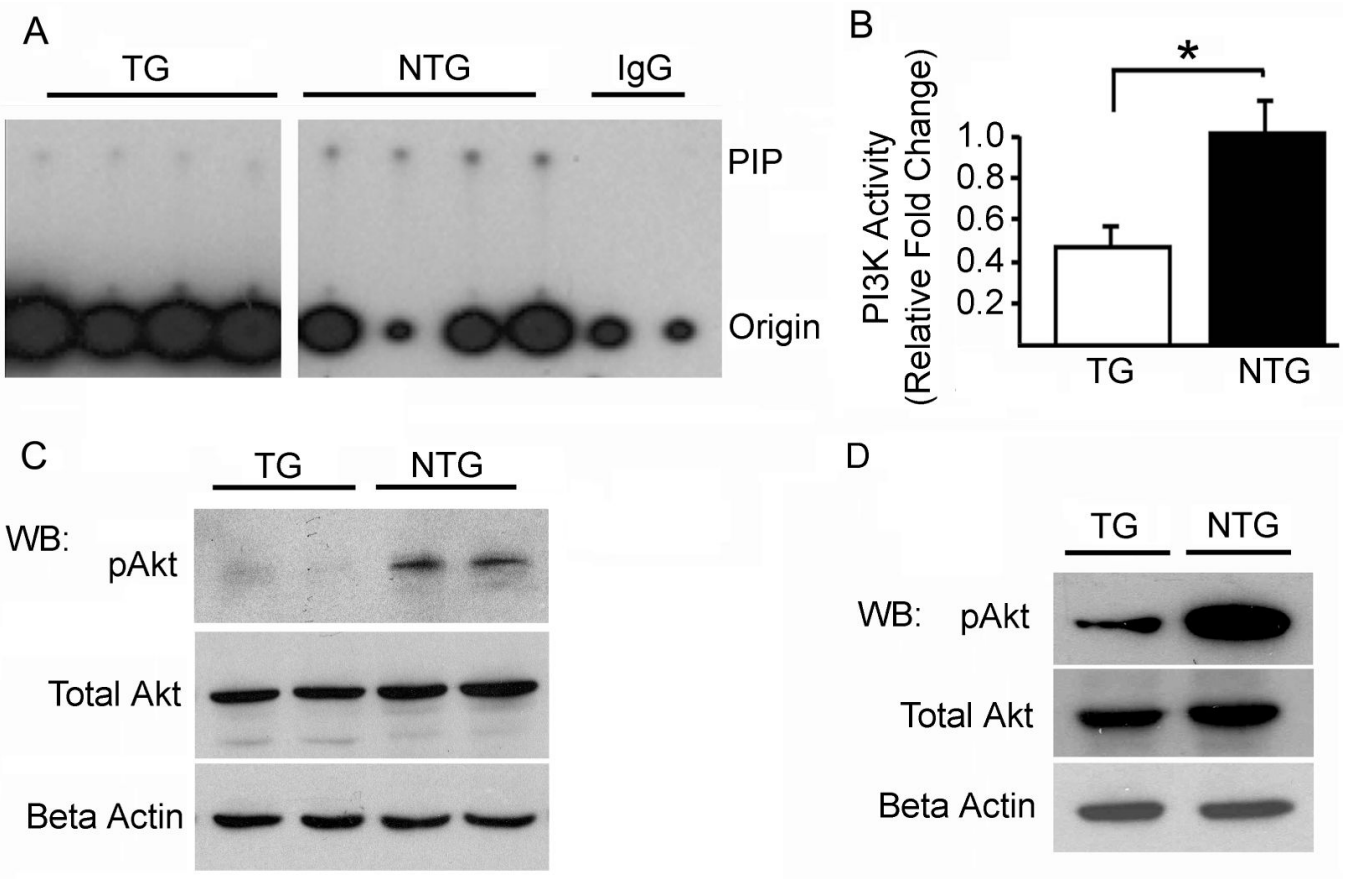

Figure 2. PIK3IP1 expression suppresses PI3K activity and phospho-Akt abundance in transgenic mouse liver and hepatocytes

A&B) PI3K activity is reduced in PIK3IP1 transgenic mouse liver. PI3K activity in the livers of transgenic (TG—n = 2) and non-transgenic (NTG—n = 2) littermates was assessed in duplicate by standard PI3K assay. Production of phosphatidylinositol-3-phosphate (PIP) is shown. The relative fold change in PIP production was calculated following densitometric analysis of the PIP signals and is graphed in B. **p*<0.003 **C) Phospho-Akt is downregulated in PIK3IP1 transgenic mouse liver.** Western blot (WB) analysis of liver protein lysates from transgenic (TG—n = 2) and non-transgenic (NTG—n = 2) littermates was carried out using antibodies to phospho-Akt and total Akt. Beta Actin was used as a loading control. **D) Phospho-Akt is dampened in PIK3IP1 transgenic mouse hepatocytes.** Transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes were subjected to western blot (WB) analysis using anti-phospho-Akt and total Akt antibodies. Beta Actin was used as a loading control.

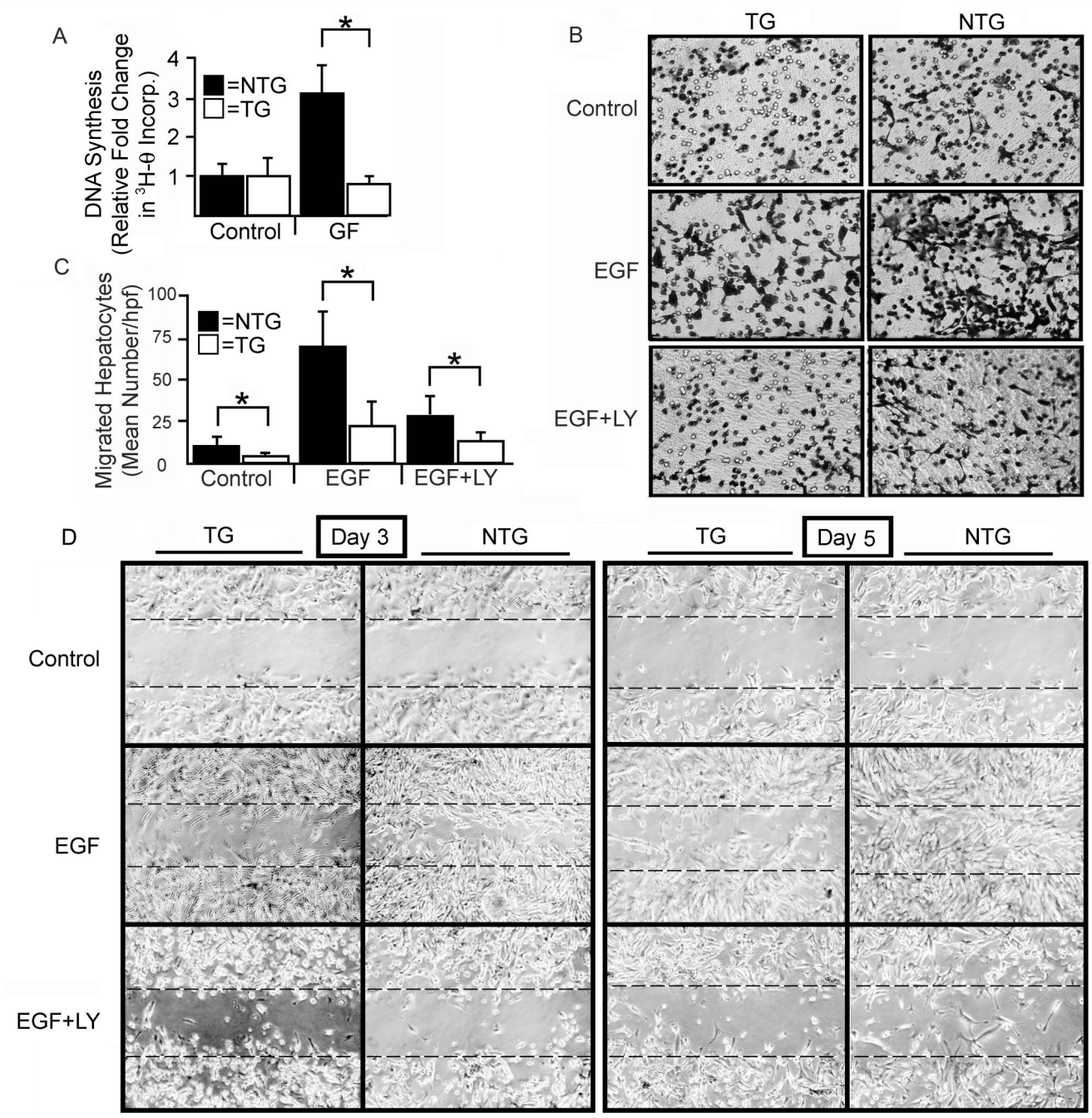

Figure 3. PIK3IP1 reduces hepatocyte DNA synthesis and migration

A) DNA synthesis is blunted in PIK3IP1 transgenic mouse hepatocytes. Transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes were stimulated to undergo DNA synthesis by addition of HGF and EGF (GF; Control = no growth factor added). A standard ^{3}H-thymidine (^{3}H-θ) incorporation assay was performed in triplicate. The relative fold change in average ^{3}H-thymidine incorporation over control cultures was assessed. *p=0.01 This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. **B&C) Hepatocyte migration is reduced by PIK3IP1: transwell migration assay.** A transwell migration assay was performed using transgenic (TG) and non-transgenic (NTG) plated at a density of 50,000/cm^2. Migration was stimulated by addition of EGF. Some

cultures were also treated with Ly294002 (LY—a PI3K inhibitor). Hepatocytes that migrated to the underside of the transwell insert were stained with Coomassie blue, enumerated and photographed; the images are shown in B. The mean number of migrated hepatocytes per high power field (hpf) is graphed in C. *p<0.01 Control = no growth factor added. This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. **D) Hepatocyte migration is reduced by PIK3IP1: in vitro scratch assay.** An in vitro scratch assay was carried out using transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes. Migration was stimulated by addition of EGF. Some cultures were also treated with Ly294002 (LY—a PI3K inhibitor). Restitution of the scratched surface was observed over five days. The photographs shown were taken at days 3 and 5 post-scratch. Control = no growth factor added. This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome.

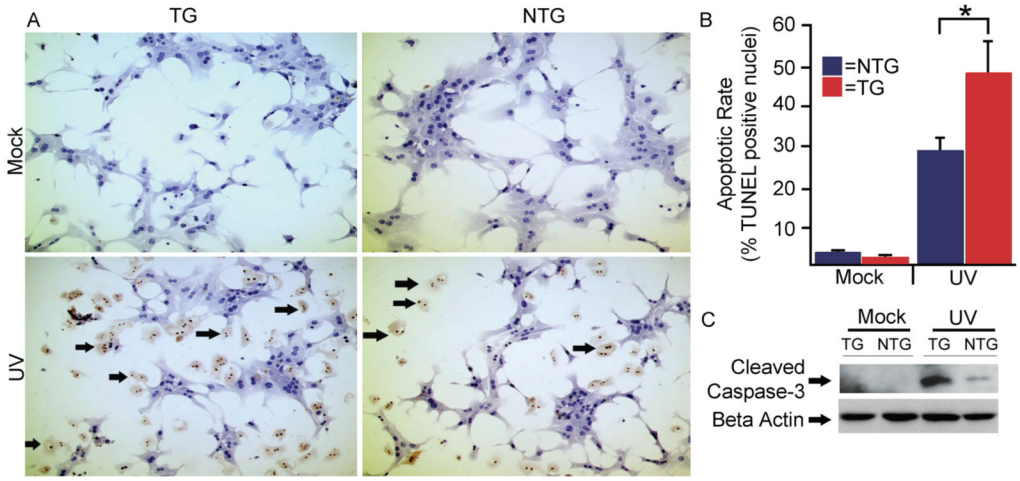

Figure 4. PIK3IP1 augments hepatocyte apoptosis

Transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes were induced to undergo apoptosis with ultraviolet light (UV–50 J/m^2) or mock treatment. **A) TUNEL assay.** The cells were fixed, immunostained for TUNEL and photographed. TUNEL positive hepatocyte nuclei are indicated with arrows in A. The apoptotic rate was calculated and graphed (B). This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. **C) Measurement of cleaved caspase-3 by Western Blot.** Transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes were induced to undergo UV-induced apoptosis as above. Protein lysates were prepared and subjected to western blot (WB) for cleaved caspase 3. Beta actin was used as a loading control.

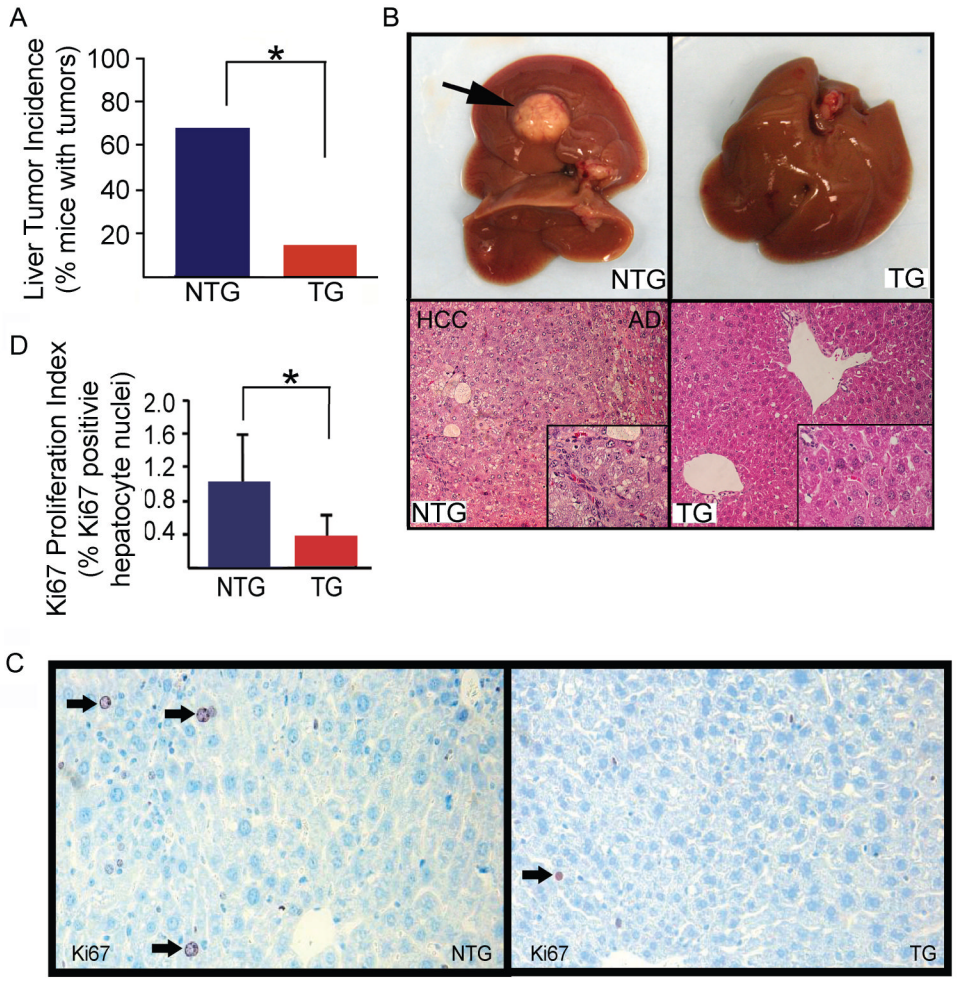

Figure 5. PIK3IP1 expression suppresses spontaneous tumorigenesis and hepatocyte proliferation in mouse liver

A&B) Spontaneous liver tumor development is inhibited in PIK3IP1 transgenic mice. Graphic representation (A) showing the percentage of PIK3IP1 transgenic (TG—n = 8) and non-transgenic (NTG—n = 8) control mice with liver tumors (avg. age = 14 mo.). *p = 0.0387. Gross (top) & histologic (bottom—200×) images of representative livers and liver tumors are shown in B. For the NTG animal, the liver tumor is indicated by an arrow in the gross image and by 'HCC' in the histologic image. 'AD'–adjacent liver tissue. Bottom insets—400× magnification of tumor or normal liver for NTG and TG, respectively. **C&D) Hepatocyte proliferation is reduced in PIK3IP1 transgenic mouse liver.** Adjacent non-tumorous liver tissue from the transgenic (TG) and non-transgenic (NTG) mice described in A&B was subjected to immunostaining for the Ki67 proliferation marker. Representative histologic images are shown in C. The Ki67 positive (indicated by arrows) and negative hepatocyte nuclei were enumerated, and the Ki67 proliferation index was calculated and graphed (D). *p = 0.0039.

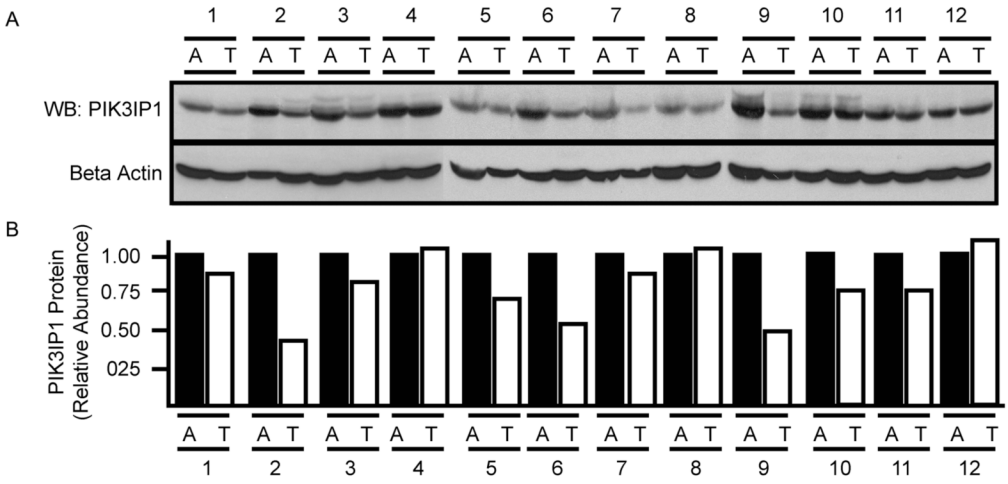

Figure 6. PIK3IP1 protein expression is downregulated in human HCC

A&B) Twelve cases of human HCC (T) and matched adjacent (A) liver tissue were probed for PIK3IP1 protein expression by western blot (WB—panel A). Beta Actin was used as a loading control. The average relative PIK3IP1 abundance in each tumor vs. its matched adjacent tissue was calculated following densitometric analysis and graphed (B).