

**Role of PIK3IP1, a negative PI3K regulator, in hepatic
tumorigenesis and metabolism**

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tumorigenesis and metabolism**

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University of Pittsburgh, 2008

Phosphatidylinositol-3-kinase (PI3K) is a well-known regulator of cell division, motility, metabolism and survival in most cell types. Proper liver function and development highly depend on intact PI3K signal transduction. Aberrant PI3K pathway signaling in the liver is associated with hepatocellular carcinoma (HCC). In addition, PI3K signaling is involved in the homeostasis of lipid and glucose metabolism. Activation of the PI3K pathway induces lipogenesis and glycogenesis in the liver, since both Akt overexpressing transgenic mice and PTEN knockout mice develop fatty liver and hypoglycemia.

Our laboratory 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 tumorigenesis and metabolism in vivo. Therefore we constructed PIK3IP1 transgenic mice (TG) which overexpress PIK3IP1 in hepatocytes under an albumin promoter in the C3H mouse strain to investigate the effect of PIK3IP1 on hepatocyte growth and metabolism, as well as HCC tumorigenesis.

We detected a high expression level of PIK3IP1 in the livers from TG animals. The PI3K pathway was successfully suppressed both in liver tissues and isolated hepatocytes, which was confirmed by Western blots and phospho-protein array studies.

Given the fact that PI3K signaling is associated with liver tumorigenesis, our next objective was to determine whether PIK3IP1 inhibits HCC development through PIK3IP1-mediated downregulation of the PI3K pathway. 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. Isolated PIK3IP1 transgenic mouse hepatocytes showed blunted PI3K signaling, DNA synthetic activity, motility and survival as compared to controls.

We then investigated the effect of PIK3IP1 on the maintenance of whole-body glucose and fat homeostasis. We observed that mice overexpressing PIK3IP1 have increased body weight, hyperglycemia, as well as increased visceral fat deposition. This suggests PIK3IP1 is an important regulator of metabolism.

In conclusion, we successfully generated a transgenic mouse model with PIK3IP1 overexpression in hepatocytes to assess the biological functions of PIK3IP1, an important negative regulator of PI3K, in liver tumorigenesis and insulin signaling. A high level expression of PIK3IP1 suppressed PI3K signaling pathway *in vivo* and *in vitro*, which curbs hepatic tumorigenesis. Furthermore, we show that PIK3IP1 overexpression can contribute to glucose homeostasis and fatty deposition.

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PREFACE

Publications during Ph.D. study in the University of Pittsburgh:

1. Zhu, Z., He, X., Johnson, C., Stoops, J., Eaker, AE., Stoffer, D.S., Bell, A., Zarnegar, R., DeFrances, M.C. (2007). PI3K is negatively regulated by PIK3IP1, a novel p110 interacting protein. *Biochemical and Biophysical Research Communications* 358:66-72.
2. He, X., Zhu, ZQ., Johnson, C., Stoops, J., Eaker, AE., Bowen, W., DeFrances, M.C. (2008). PIK3IP1, a negative regulator of PI3K, suppresses the development of hepatocellular carcinoma. *Cancer Research* 68: (14) 1-8
3. He, X., Zhu, ZQ., Johnson, C., Stoops, J., Eaker, AE., DeFrances, M.C. (2008) Post-transcriptional and -translational modification of PIK3IP1, a novel regulator of PI3K. (In Preparation)

1.0 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)P₃] from phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂]. The appearance of PI(3,4,5)P₃ attracts Akt (also known as Protein Kinase B [PKB]) and other signal transduction molecules to the membrane, the association of which triggers typical PI3K dependent cellular responses (Engelman, et al., 2006).

Growth factor→Receptor tyrosine kinase→PI3K (GF→RTK→PI3K) pathways control cell proliferation, differentiation and apoptosis and are key regulators of liver development, tumorigenesis, regeneration and metabolism. For example, among the growth factors studied, HGF is now recognized as one of the most critical to maintaining proper liver homeostasis. It can also be considered a multifunctional cytokine with effects on many tissues and organs besides liver. HGF and Met, the HGF receptor, are essential for hepatocyte DNA replication and mitosis during liver regeneration, and deletion of the HGF or Met genes during development causes an embryonic lethal phenotype (Schmidt et al., 1995). Additionally, HGF overexpression accelerates DEN-induced hepatocarcinogenesis (Horiguchi, et al., 2002) and ameliorates high-fat diet-induced fatty liver (Kosone et al., 2007).

1.1 PIK3IP1: A NOVEL NEGATIVE REGULATOR OF PI3K

PIK3IP1 (mRNA ID: NM_052880; protein ID: NP_443112) was first identified as a novel kringle containing transmembrane protein in our laboratory (Zhu et al., 2007). The human PIK3IP1 gene structure contains 6 exons and 5 introns spanning about 12,000 bp, and it is located on human chromosome 22q12.2. In addition, we identified its ortholog in several species such as mouse (chromosome 11A1; GenBank I.D.:NP_835362), rat (chromosome 14q1; GenBank I.D.:XP_223593), chicken (GenBank I.D.: XP_415257) and zebrafish (chromosome 5; GenBank I.D.: CAM14081). They are highly homologous at the mRNA and protein levels.

Human p85 beta shares homology with PIK3IP1 (Figure 1). Overall similarity is 70% and 65% between PIK3IP1 (AA 197–219) and p85 beta (AA 502–524) or alpha (AA 505–527), respectively. An amino acid stretch (AA 210–218: NH₂-EREMQRITL-COOH) within the p85-like domain of PIK3IP1 is 78% identical to p85 beta (AA 515–523).



Figure 1: Amino acid comparison of human PIK3IP1's p85-like domain to the homologous region of human p85 beta.

Alignment of the homologous regions of hPIK3IP1 and hp85 beta is shown. Solid lines indicate identical AA; dotted lines indicate similar AA.

Functional assays show that PIK3IP1 binds to the p110 subunit of PI3K, and the interaction between PIK3IP1 and p110 leads to downregulation of PI3K activity (Zhu et al., 2007). Interestingly, PIK3IP1 overexpressing cells also show enhanced sensitivity to staurosporine induced apoptosis.

Altogether, our published data confirm that PIK3IP1 is the only adaptor protein identified so far that can directly bind to p110 and suppresses its activity. Furthermore, PIK3IP1 can also suppress pMEK1/2, pERK(p44/42), pp38MAPK and pSAPK/JNK, probably through inhibiting the PI3K pathway.

1.2 PI3K: ROLES IN THE LIVER

Class IA PI3K is activated by associating with RTKs by binding through one or two SH2 domains in the regulatory PI3K subunit to phosphotyrosine consensus motifs in receptors and adaptor molecules. This leads to allosteric activation of the catalytic subunit to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) from the substrate phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2). PIP3 recruits a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane, including PDK1 and Akt. (Figures 2 and 3) (Hunter, 2000; Fruman, et al., 1998; Fresno Vara et al., 2004).

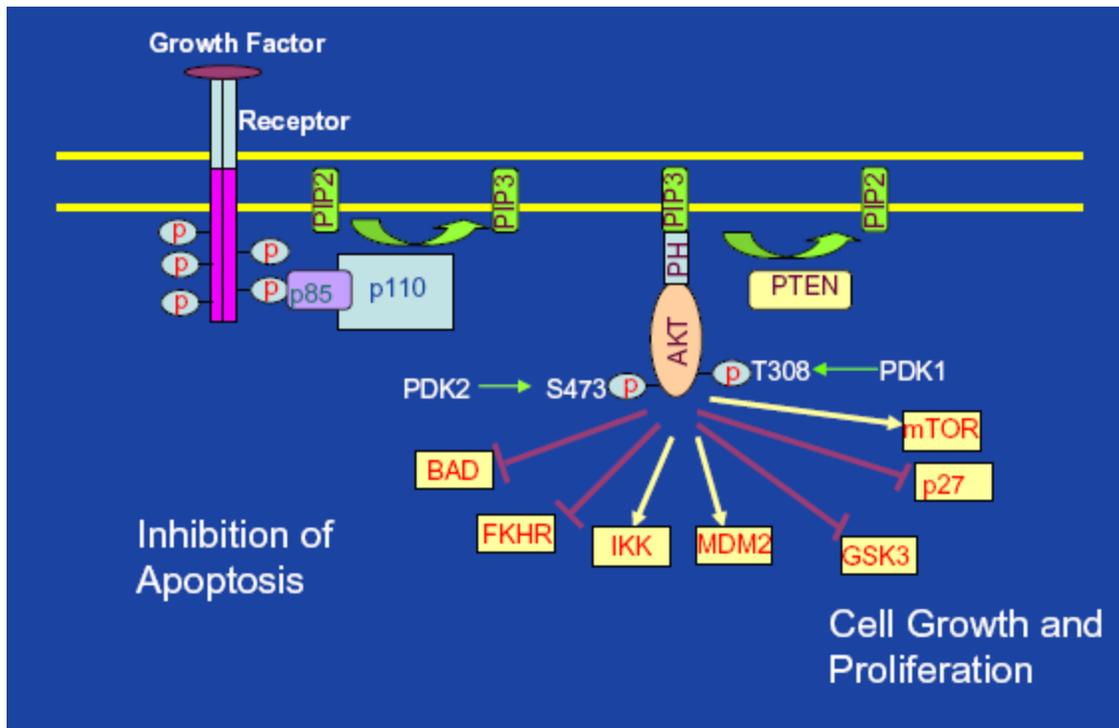


Figure 2: Class Ia PI3K signal cascades activated by RTKs

PI3K directly binds to phosphotyrosine consensus residues of growth factor receptors or adaptors and the p110 catalytic subunit of PI3K gets activated through allosteric activation. This will produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), an important second messenger. PIP3 recruits a subset of signalling proteins with PH domains to the membrane, including PDK1 and Akt. The activation of Akt by PDK1 will mediate the activation and inhibition of several targets resulting in survival, growth and proliferation through various mechanisms. PTEN, a PI-3,4,5-P3 phosphatase, negatively regulates the PI3K/Akt pathway by dephosphorylation of PIP3. (HFresno Vara JAH, et al., 2004)

In general, cell growth and survival are controlled by PI3K and other cellular kinases (e.g.,MAPK) in response to growth factors. Furthermore, activating PI3K/Akt dependent antiapoptotic pathways protects cells from apoptosis (Xiao et al., 2001; Franke et al., 2003; Kozman et al., 2002).

Although the effects of HGF on liver regeneration, development and tumorigenesis have been extensively studied, the role of PI3K activity and function with regards to hepatocyte proliferation and apoptosis has not yet been completely clarified (Cho et al., 2003). Published studies suggest an important role for the PI3K pathway in hepatocyte metabolism, growth, survival and tumorigenic transformation. For example, hypoxic preconditioning can reduce hepatocyte killing by hypoxia by about 35% (Carini et al., 2001). Hypoxic preconditioning stimulated phosphatidylinositol-3,4,5-triphosphate production and the phosphorylation of Akt, suggesting that PI3K can play a protective role in the development of hepatic tolerance to hypoxia/reperfusion (Carini et al., 2004). Interestingly, both p110 and p85 gene knock-out mice showed prenatal lethality (Bi L et al., 1999; Bi L et al., 2002; Fruman et al., 2000), and hepatocyte necrosis was reported in p85 knock-out mice (Bi et al., 1999).

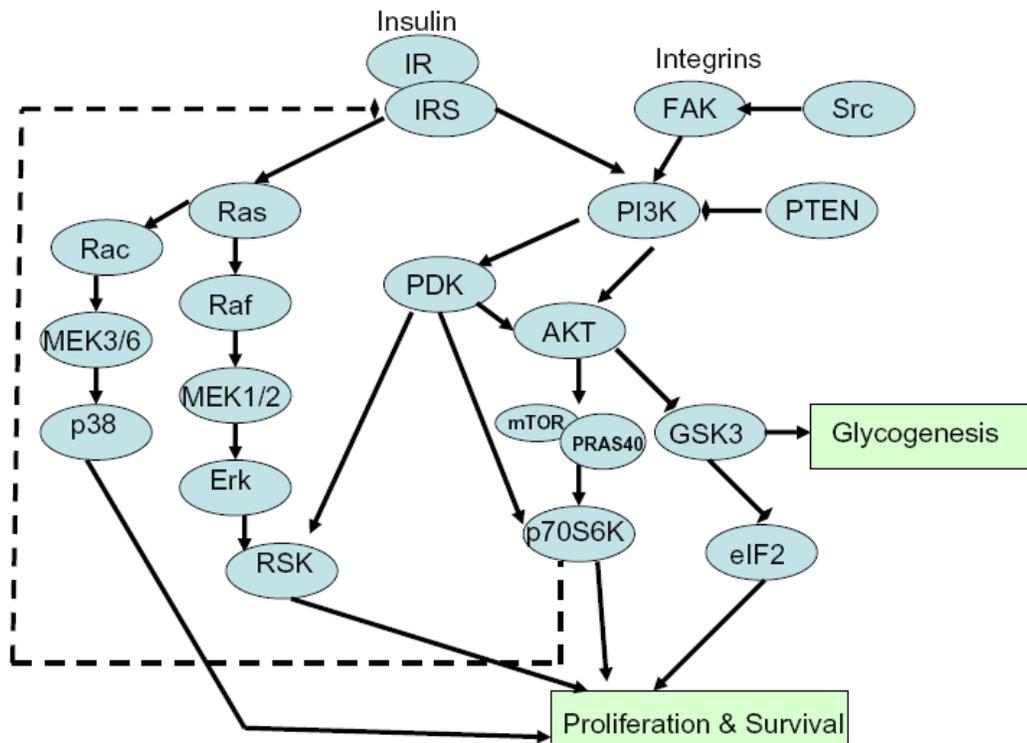


Figure 3: Scheme displaying the involvement of PI3K in the control of intracellular signaling pathways.

PI3K is activated via interaction with growth factor receptors (such as insulin receptor/IRS) or non-receptor (Src) tyrosine kinases. The main downstream effectors of PI3K are: Akt/PKB (responsible for anti-apoptotic signal transduction), Ras/Raf/ERK (the main mitogen-induced signal transduction pathway), and Rac/JNK/JNK (partially controls the mitogen signal transduction but is involved mainly in other cell functions such as motility)

1.3 RAS AND MAPK: ROLES IN LIVER DEVELOPMENT AND REGENERATION

The Ras-MAPK pathway is one of the most important cell signaling families in controlling liver function. Activation of p21Ras allows the recruitment and phosphorylation/activation of the cytosolic protein Raf (Marshall 1995; Madhani 2001; Plyte et al., 2000). Activation of Raf initiates sequential phosphorylation of MEK and ERK (MAPK) cascades (Seger et al., 1995). In spite of the fact that Ras induces MAPK, Ras can also activate PI3K and vice versa (Rodriguez-Viciana et al., 1994; Downward J 1998). These kinases have been demonstrated to play essential roles in cell growth, transformation differentiation and apoptosis.

Ras increases DNA synthesis through C/EBP during partial hepatectomy (Ludde et al., 2001). During liver development, K-Ras specifically mediates cytokine signaling for formation of the E-cadherin-based adherens junction, since fetal hepatocytes derived from K-Ras knockout (K-Ras^{-/-}) mice failed to form adherens junctions in response to Oncostatin M (OSM), whereas adherens junction formation was induced normally by OSM in mutant hepatocytes lacking both H-Ras and N-Ras (Matsui et al., 2002).

Inhibition of p42/44 MAPK does not significantly alter proliferation of hepatocytes isolated from regenerating liver, although inhibition of p38 MAPK results in decreased DNA synthesis in the same cells (Spector et al., 1997). Prominent, sustained ERK activation was seen in the liver during early postimplantation mouse development (5-10.5 days postcoitum) suggesting that the Erk-MAPK cascade participates in liver development (Corson et al.,)

2.0 POST-TRANSLATIONAL MODIFICATION OF PIK3IP1

2.1 INTRODUCTION

We identified and characterized a novel negative regulator of Class Ia phosphatidylinositol-3-kinases (PI3Ks) that we named phosphatidylinositol-3-kinase interacting protein 1 (PIK3IP1) (Zhu et al., 2007). Our initial intention was to identify new kringle containing proteins by mining GenBank databases using the kringle signature amino acid sequence (NYCRNPD) and the cDNA of a well known kringle containing protein, HGF. Kringles are three disulfide-linked triple-looped motifs found in proteins with various functions such as coagulation, fibrinolysis, cell growth, and organogenesis. They mediate protein-protein interactions. Our search led us to the discovery of human and mouse expressed sequence tags (Ests) encoding for a putative transmembrane protein of about 263 amino acids bearing a signal peptide, a kringle domain extracellularly and a region intracellularly with homology to the p85 regulatory subunit of PI3K (Figure 4).

```

1 atgetgttggcctgggtacaagcattcctegtcagcaacatgctc
M L L A W V Q A F L V S N M L
46 ctagcagaagcctatggatctggaggctgttctctgggacaacggc
L A E A Y G S G G C F W D N G
91 cacctgtaccgggaggaccagacctcccccgcgccgggctccgc
H L Y R E D Q T S P A P G L R
136 tgcctcaactggctggacggcagagcgggctggcctcggccccc
C L N W L D A Q S G L A S A P
181 gtgtcgggggcccggcaatcacagt tactgccgaaaccgggacgag
V S G A G N H S Y C R N P D E
226 gacccgcgcgggccctgggtgctacgtcagtggcgaggccggcgctc
D P R G P W C Y V S G E A G V
271 cctgagaaacggccttgcgaggacctgcgctgtccagagaccacc
P E K R P C E D L R C P E T T
316 tcccaggccctgccagccttcacgacagaaatccaggaagcgtctc
S Q A L P A F T T E I Q E A S
361 gaagggccaggtgcagatgaggtgcaggtgttcgctcctgccaac
E G P G A D E V Q V F A P A N
406 gcctgcccgcctcggagtgaggcggcagctgtgcagccagtgatt
A L P A R S E A A A V Q P V I
451 gggatcagccagcgggtgccgatgaactccaaggagaaaaaggac
G I S Q R V R M N S K E K K D
496 ctgggaactctgggctacgtgctgggcattaccatgatggtgatc
L G T L G Y V L G I T M M V I
541 atcattgccatcggagctggcatcatcttgggctactcctacaag
I I A I G A G I I L G Y S Y K
586 agggggaaggat ttgaaagaacagcatgatcagaaagtatgtgag
R G K D L K E Q H D Q K V C E
631 agggagatgcagcgaatcactctgcccttgtctgccttcaccaac
R E M Q R I T L P L S A F T N
676 ccacctgtgagattgtggatgagaagactgtcgtggteccacacc
P T C E I V D E K T V V V H T
721 agccagactccagttgaccctcaggagggcaccaccccccttatg
S Q T P V D P Q E G T T P L M
766 ggccaggccgggactcctggggcctga 792
G Q A G T P G A *

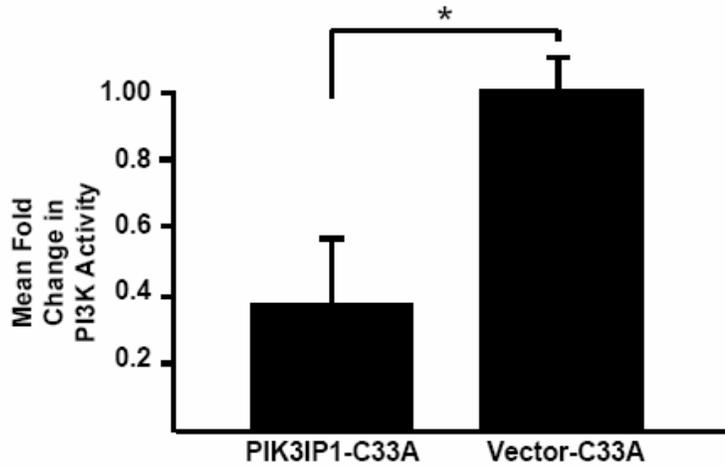
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Figure 4: Schematic of human PIK3IP1 cDNA and amino acid sequences.

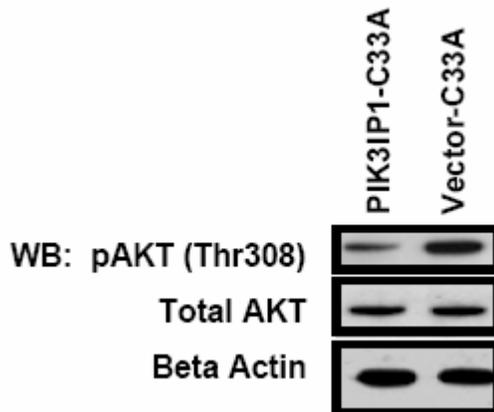
Green box–signal peptide; grey box–kringle domain; yellow box–transmembrane domain; aqua box–p85-like domain; pink stealth arrow–putative N-glycosylation site; black solid arrow–putative O-glycosylation site; green open arrow–last amino acid encoded by exon 5.

We conducted several in vitro and in vivo functional assays which revealed that PIK3IP1 binds to and negatively regulates the p110 catalytic subunit of PI3K. PI3Ks are key transducers of intracellular signals from tyrosine phosphorylated proteins such as growth factor receptors and

adapter proteins ultimately leading to cellular responses like changes in gene expression and modifications to metabolic pathways. PI3Ks do so by converting the phospholipid PI(4,5)P2 to PI(3,4,5)P3 at the plasma membrane which in turn recruits and leads to the activation of Akt/PKB serine/threonine kinase, a well known PI3K transduction target (Cantley 2002). When PIK3IP1 binds to p110, PI3K activity and subsequently the activation of Akt/PKB are reduced (Zhu et al., 2007).



A



B

Figure 5: PIK3IP1 negatively regulates PI3K activity and activated Akt abundance.

*PI3K activity was assessed in PIK3IP1-C33A (two independent clones) and Vector-C33A (two independent clones) cells by conventional PI3K assay using anti-p110 antibody or Ig antibody (negative control) for immunoprecipitation (Figure 5A). The average PIP level was calculated, normalized and graphed following densitometric analysis for each cell type. * $p=0.04$. The results are representative of three independent trials*

carried out in triplicate. Western blot analysis of the PI3K downstream effector Akt (phosphorylated and total) was assessed in PIK3IP1- and Vector-C33A cells. pAKT (Thr308) – antibody recognizing the activated form of AKT phosphorylated on Thr308 (a site which is phosphorylated specifically in response to PI3K signaling); Total AKT – antibody recognizing activated and unactivated forms of AKT; and Beta-actin – antibody used to determine loading. Densitometric readings of the western signals were normalized for the PIK3IP1-C33A or Vector-C33A cells; the results are shown graphically. (Figure 5B) (Zhu et al., 2007)

In our initial biochemical analyses of PIK3IP1, we noted that PIK3IP1 protein migrated variably in SDS-PAGE/Western blot analysis. We were puzzled as to why this may be so. We observed that in vitro transcribed/translated ³⁵S-labeled PIK3IP1 protein measured 37 kDa in SDS-PAGE, but the predominant forms of endogenously expressed PIK3IP1 protein in human tissues and cell lines had apparent molecular masses of about 43 kDa, 37 kDa, and 22-25 kDa in western blot analysis (Zhu et al., 2007).

To address these issues, we performed several experiments. We determined that PIK3IP1 is a bone fide membrane bound protein and that PIK3IP1 is a target of serine protease cleavage following growth factor- or serum treatment of cells. This latter attribute accounts for the appearance of the PIK3IP1 species of about 22-25 kDa. Next, we found that PIK3IP1 undergoes N- and O-linked amino acid glycosylation. The major glycosylated form of PIK3IP1 migrates at about 43 kDa in SDS-PAGE/western blot analysis, while the unglycosylated form migrates at 37 kDa. Thus, our analyses of PIK3IP1 protein have uncovered modifications that may affect its ability to act as an inhibitor of PI3K.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and reagents.

A polyclonal antibody was made in a rabbit against a PIK3IP1 antigenic peptide as described previously (Zhu et al., 2007). Secondary antibodies were obtained from Chemicon International, Inc. (Temecula, CA). G418 was obtained from Invitrogen Corporation (Carlsbad,

CA). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma-Aldrich (St. Louis, MO) and stored in dimethyl sulfoxide (DMSO) at -20°C.

2.2.2 Cell culture

C33A, human uterine cervical epithelial carcinoma cells, and HepG2, human hepatocellular carcinoma cells, were purchased from ATCC (Manassas, VA). PIK3IP1-C33A and Vector-C33A cells were produced by transfecting in an either an empty expression vector (pCR3.1) or one bearing the full length PIK3IP1 human cDNA as described by Zhu et al. (Zhu et al., 2007). All C33A cells were grown in EMEM containing 10% fetal bovine serum; HepG2 cells were cultured in DMEM containing 10% fetal bovine serum. Both were typically grown to 80% confluence. For some studies, cells were serum starved overnight (O.N) before treatment.

2.2.3 Protein isolation, SDS-PAGE, immunoprecipitation and Western blot analysis

Cells such as C33A or HepG2 were washed twice with cold-PBS (pH 7.5) and lysed with RIPA lysis buffer (1X PBS, 1% Igepal CA630 [Sigma Aldrich Corp.], 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml PMSF, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml, 0.5 µg of antipain/ml, and 0.5 µg of pepstatin/ml). Lysates were sonicated, and cellular debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C. Generally, 40 µg of protein were analyzed. 2X SDS sample buffer was added to protein lysates which were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with the appropriate primary antibodies, followed by secondary goat anti-mouse, or donkey or goat anti-rabbit antibodies as indicated. Commercial antibodies were used at the concentrations recommended by their manufacturers. Signals were illuminated by the Western Lightning Chemiluminescence Reagent PLUS (PerkinElmer Inc., Boston, MA, #NEL102) followed by exposure to Biomax film (Eastman Kodak Company, New Haven, CT).

2.2.4 Non-detergent cell fractionation

Both cytosol and plasma membrane enriched fractions were prepared under non-detergent conditions. Briefly, HepG2 cell lysates were prepared by the standard freeze-thaw method in 10mM Tris-HCl (pH 7.5) in the presence of protease inhibitor cocktail (1:100, Sigma-Aldrich Corp., #p8340). After centrifugation at 17,000 rpm for 3 hr. at 4 °C, the supernatant (soluble) containing cytosolic proteins was decanted. The plasma membrane containing pellet (insoluble) was resuspended in 1% SDS in 10 mM Tris-HCl (pH 7.5) in the presence of protease inhibitor cocktail. The cytosolic and particulate fractions were then subjected to SDS-PAGE and Western blot analysis.

2.2.5 Deglycosylation assay

Deglycosylation of PIK3IP1 protein was carried out using the Enzymatic Deglycosylation Kit (Glyko/ProZyme, San Leandro, CA, #GK80110) according to the manufacturer's instructions. Briefly, PIK3IP1-C33A cell lysate (100 µg) was placed in an Eppendorf tube containing 30 µl dH₂O. Ten (10) µl 5X Incubation Buffer and 2.5 µl of Denaturation Solution were added, and the contents were gently mixed. The lysate was heated to 100°C for 5 min. after which it was cooled to RT. Detergent Solution (2.5 µl) was then added followed by 1 µl each of N-Glycanase, Sialidase A or O-Glycanase. The lysate was then incubated for 3 hr. at 37°C and analyzed for mobility shift of PIK3IP1 protein by SDS-PAGE/Western blot analysis. Densitometric analysis of specific signal was carried out using Scion Image 1.63 software (Scion Corporation, Frederick, MD).

2.2.6 Serum-induced cleavage assays

To induce PIK3IP1 cleavage, PIK3IP1-C33A cells were serum starved ON; cells were harvested in RIPA buffer after exposing them to either medium containing 10% fetal bovine

serum or serum free medium from 0 to 30 min. The reaction was stopped by the addition of SDS sample buffer; then, samples were subjected to SDS-PAGE and Western blot analysis.

2.2.7 Serine protease inhibition assay

To inhibit serine proteases, PIK3IP1-C33A cells were incubated at 37 °C in medium containing either 1 mM PMSF in DMSO or DMSO alone as a control for 4 hr. The cells were lysed with RIPA buffer, and samples were subjected to SDS-PAGE and Western blot analysis.

2.2.8 Ras activity assay

Ras activity was compared between C33A-PIK3IP1 and C33A vector cells by using Raf-RBD domain protein agarose beads (Upstate Biotechnology, Lake Placid, NY), which measure the GDP/GTP ratio of Ras in vitro.

2.2.9 Statistical analysis

Stastical analysis was carried out using the two tailed Student's t-test. Values were considered to be statistically different when the p-value was less than 0.05, and such values are indicated in the figures with asterisks.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization of the PIK3IP1 protein

We examined HepG2 cell lysates by western blot for endogenous PIK3IP1 protein. We found that HepG2 cells express PIK3IP1 proteins measuring approximately 65 kDa, 43 kDa, and 22-25 kDa and that these variants of PIK3IP1 are all associated with the insoluble particulate fraction upon cell fractionation (Figure 6). Similar results were obtained with PIK3IP1-C33A cells, human cervical carcinoma cells engineered to overexpress PIK3IP1, although a 37 kDa variant was more prominent and the 65 kDa variant was undetectable in these cells as compared to HepG2 cells (data not shown). These findings suggested to us that PIK3IP1 is indeed membrane bound and that these variants arise from alternative splicing and/or post-translational processing events such as enzymatic proteolysis and/or glycosylation.

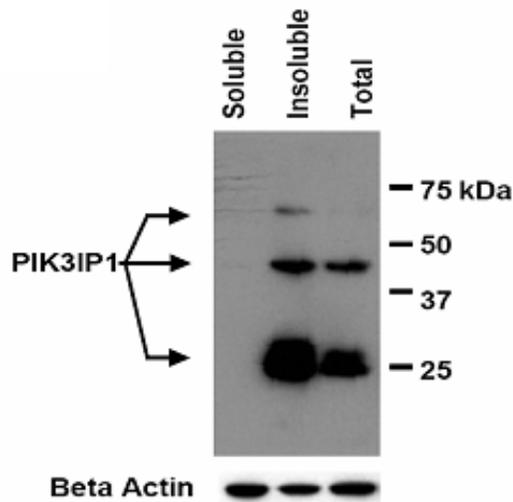


Figure 6: PIK3IP1 is a membrane protein.

HepG2 cells were fractionated into soluble and insoluble components under non-detergent conditions. PIK3IP1 was found to associate with the insoluble membrane-containing fraction by Western blot analysis indicating that it is membrane-bound. Specific PIK3IP1 protein bands of 65, 43, 22-25 kDa were identified in the insoluble fraction. Beta actin was used as a loading control. Total – unfractionated input lysate

2.3.2 PIK3IP1 suppresses Ras activity

Cross-talk between the PI3K/Akt and Ras/Raf/Erk pathways has been reported. Since PIK3IP1 negatively regulates PI3K (Zhu et al., 2007), as well as Erk MAPK and p38 MAPK (data not shown), we were curious as to whether PIK3IP1 could also negatively regulate Ras. To confirm our hypothesis, Raf-RBD domain protein agarose beads were used to measure the GTP/GDP ratio of Ras in vitro. We observed that C33A cells with high expression of PIK3IP1 showed an obvious reduction in Ras kinase activity in pull-down assay with Raf-RBD domain protein (Figure 7). This result, together with our observations that PIK3IP1 inhibits Erk/p38 MAPK suggests that PIK3IP1 inhibits the Ras pathway too. Whether the effect of PIK3IP1 on Ras is direct or indirect through PI3K is unknown. The goal of future experiments is to fully characterize this relationship.

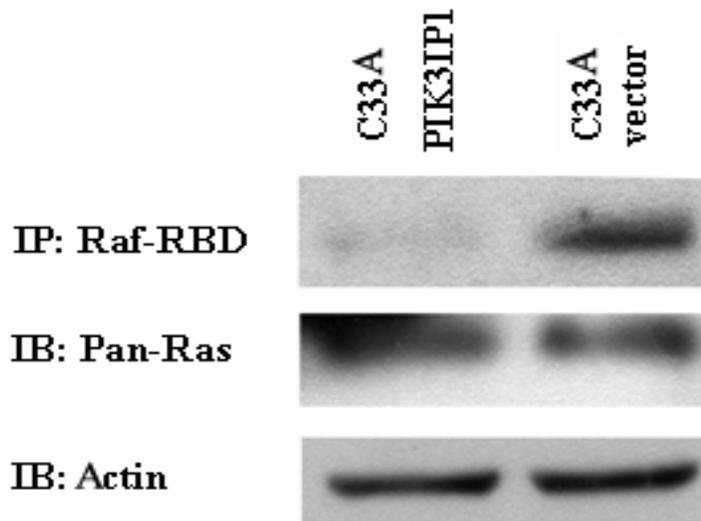


Figure 7: PIK3IP1 suppresses Ras activity.

Ras activity was compared between C33A-PIK3IP1 and C33A vector cells.

2.3.3 PIK3IP1 protein is cleaved by a serine protease induced by serum or growth factor treatment

Some kringle containing proteins, such as hepatocyte growth factor (HGF), are targets of serine proteases (Mars et al., 1993). We entertained the possibility that PIK3IP1 protein which has a kringle in its extracellular domain is likewise targeted for enzymatic cleavage given the identification of both higher (43 and 37 kDa) and lower (22-25 kDa) molecular weight PIK3IP1 species (Figure 6) in Western blot analysis.

To evaluate the possibility that PIK3IP1 protein is a target for enzymatic cleavage, we determined whether addition of serum (10%) which is known to contain a variety of proteases, some of which are involved in enzymatic activation of kringle bearing proteins, promotes the appearance of the lower molecular PIK3IP1 species in PIK3IP1-overexpressing C33A cells. In Figure 8, we observed that addition of serum to quiescent cells in a time course analysis resulted in the appearance of the lower molecular weight PIK3IP1 species within 5 min. consistent with the notion that PIK3IP1 protein cleavage is stimulated by addition of serum.

As mentioned above, we postulated that the cleavage of PIK3IP1, like other kringle containing proteins, is carried out by a serine protease. If this were to be true, the serine protease inhibitor, PMSF, should inhibit PIK3IP1 cleavage. In the presence of PMSF, PIK3IP1 cleavage induced by serum addition to PIK3IP1-C33A cells was significantly suppressed as compared to control conditions (Figure 9) suggesting that a serine protease is responsible for PIK3IP1 protein processing. We are currently attempting to determine the exact cleavage site in PIK3IP1's extracellular domain and to identify the responsible cleavage enzyme.

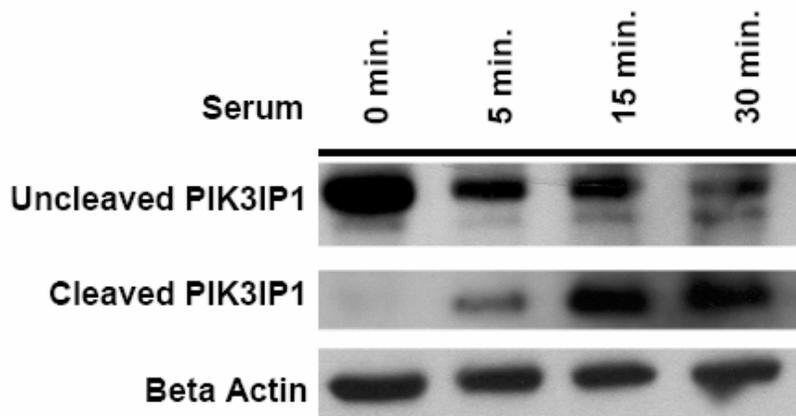


Figure 8: Serum addition induces PIK3IP1 cleavage in PIK3IP1-C33A cells.

Addition of serum (10%) to quiescent PIK3IP1-C33A cells for the indicated times resulted in the appearance of the lower molecular weight species of PIK3IP1 of about 22 kDa as early as 5 min. post-induction.

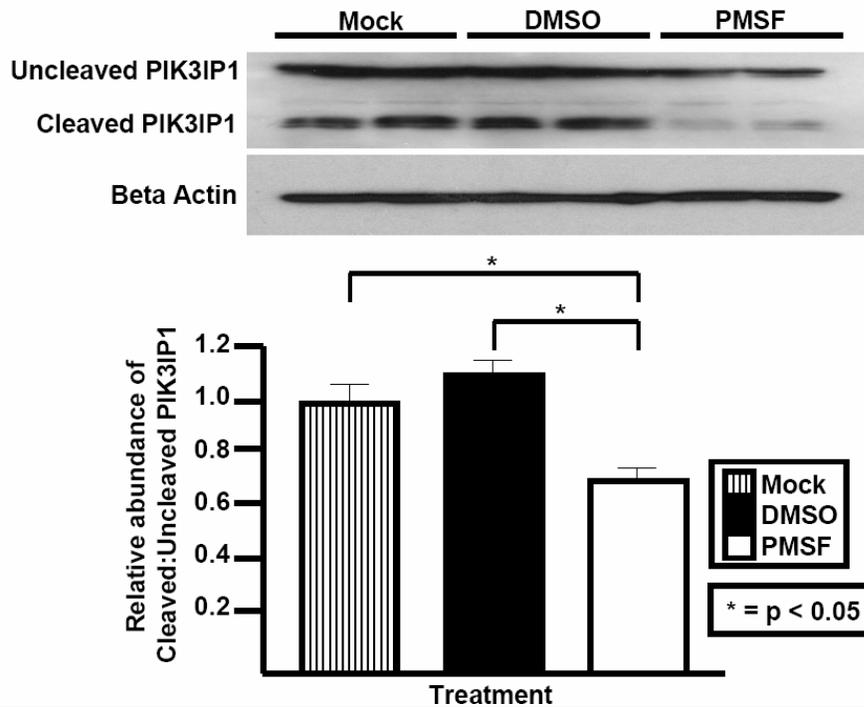


Figure 9: PIK3IP1 cleavage is PMSF sensitive.

PIK3IP1-C33A cells were incubated O.N. at 37°C with the serine protease inhibitor PMSF (1 mM final concentration) or its solvent DMSO. The cells were lysed with RIPA lysis buffer and analyzed by Western blot for

PIK3IP1 and Beta actin (as a loading control). The data were analyzed by densitometry and normalized. The relative abundance of cleaved PIK3IP1 to uncleaved PIK3IP1 is shown in the graph. Values of statistical significance are shown with an asterisk.

2.3.4 PIK3IP1 protein is glycosylated on O- and N-linked amino acid residues

To explore whether PIK3IP1 is glycosylated, we examined the human PIK3IP1 amino acid sequence with NetNGly 1.0 search engine (<http://www.cbs.dtu.dk/services/NetNGly/>) (Blom et al., 2004). It predicted the existence of a single putative N-glycosylation site in the extracellular portion of PIK3IP1 at residue 66 (Asn) with an N-glycosylation potential of about 65% (pink stealth arrow—Figure 4). This Asn residue is conserved in human, mouse and rat. To search for O-glycosylation sites in PIK3IP1 protein, we used the YinOYang 1.2 search engine (<http://www.cbs.dtu.dk/services/YinOYang/>). Several putative residues that have O-glycosylation potential were identified in the extracellular portions of the human, mouse and rat PIK3IP1 proteins; however, the residue showing the highest average potential (62%) among the species was residue 106 (Ser) (black solid arrow—Figure 4).

Next, we carried out enzymatic deglycosylation of PIK3IP1 protein in lysate from overexpressing human PIK3IP1-C33A cancer cells using the Enzymatic Deglycosylation Kit (Glyko/Prozyme) according to the manufacturer's instructions. We found that the 43 kDa species of PIK3IP1 underwent deglycosylation following treatment with either N- or O-glycanase to an apparent molecular mass of about 40 kDa (Figure 10). However, when N- and O-glycanase were added in combination, the apparent molecular mass of PIK3IP1 shifted to about 37 kDa suggesting that PIK3IP1 protein undergoes both N- and O-glycosylation in vivo which accounts for the major high molecular weight (i.e. 43 kDa) PIK3IP1 form we observe in various human tissues and cell lines.

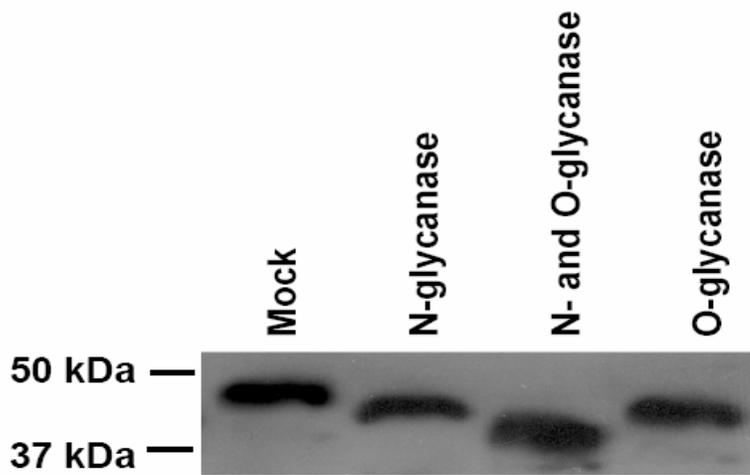


Figure 10: PIK3IP1 protein is glycosylated on N- and O-linked amino acid residues.

Enzymatic deglycosylation of PIK3IP1 protein in lysate from PIK3IP1-overexpressing human cancer cells (PIK3IP1-C33A) was performed. The 43 kDa species of human PIK3IP1 protein underwent deglycosylation following treatment with either N- or O-glycanase alone to an apparent molecular mass of about 40 kDa. However, when N- and O-glycanases were added in combination, the apparent molecular mass of PIK3IP1 shifted to about 37 kDa. Mock – input protein treated identically except no enzyme was added.

In conclusion, we confirmed that PIK3IP1 is a membrane protein which undergoes post-translation modifications including glycosylation and cleavage. Furthermore, in addition to curbing PI3K pathway by direct binding, PIK3IP1 can also suppress Ras activity. This may occur indirectly through its effect on PI3K and/or MAPK.

3.0 PIK3IP1 SUPPRESSES THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

3.1 INTRODUCTION

Abnormalities in PI3K-AKT signaling contribute to the pathogenesis of several human cancers. For example, gain-of-function mutations in the PI3K catalytic subunit (p110 alpha/PIK3CA) were frequently identified in cases of human ovarian carcinomas and breast carcinomas (Lee JW et al., 2005), whereas loss-of-function mutations in PTEN, a negative regulator of the PI3K signaling pathway, were reported in a variety of human cancers such as melanoma (Celebi JT et al., 2000; Zhou X et al., 2000) and endometrial carcinomas (Salvesen H et al., 2001), suggesting an important role of aberrant PI3K signaling in tumorigenesis of the liver and other tissues.

Abnormalities in the PI3K pathway were also detected in human HCC. For example, of the various tumor types tested, liver cancer shows the highest percentage of cases with *pik3ca* mutations (36%) (Engelman et al., 2006). 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)P₃ (Maehama et al., 1999). PTEN protein abundance is downregulated in human HCC (Hu et al., 2003; Wan et al., 2003). Mutations and loss of heterozygosity of the *PTEN* gene (Fujiwara et al., 2000) have been identified as well. Experimentally, liver tumors developed in mice lacking functional PTEN in hepatocytes (Horie et al., 2004).

Considering the high level of PI3K activity in HCC, we hypothesized that overexpression of PIK3IP1 in hepatocytes could alter the natural activity of the PI3K pathway as well as hepatocellular carcinoma development in this tissue. To test this hypothesis, we developed a PIK3IP1 TG mouse model directing expression of PIK3IP1 to hepatocytes under the regulatory control of the albumin promoter. The genetic background of our TG mice is C3H, a strain which spontaneously develops HCC at a high incidence (reportedly 72-91% in males by 14 months). Therefore, in the present study, we investigated the role of PIK3IP1 as a tumor suppressor in mouse hepatocarcinogenesis by using our PIK3IP1-C3H TG mouse model. First, we show that PIK3IP1 overexpression suppresses HCC tumorigenesis in C3H mice. Second, we found that Ki67 labeling index is reduced by 3 fold in TG mouse hepatocytes in vivo. Finally, we demonstrate that in isolated hepatocytes, PI3K activity and signaling, proliferation, survival and motility/migration are all significantly reduced in TG mice as compared to control animals. Our results indicate that PIK3IP1 is a novel tumor suppressor and may play an important role in hepatocarcinogenesis.

3.2 MATERIALS AND METHODS

3.2.1 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 (Zhu et al., 2007). 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.

3.2.2 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.

3.2.3 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'-TTAGGATCCCATTGGACTGGCTG-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. (Bell et al., 1999). This transgene was used to generate 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 ³²P-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'-GACGTGAGTTGCCAGAGACC-3'; reverse primer: 5'-TTACCTGCAGCCATTGCCGCTAGTGAG-3'. TG mice were propagated by breeding with wildtype C3H mice for greater than four generations.

3.2.4 Mouse tumor tissues

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 mouse 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. Ki67 and TUNEL immunostaining 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 concentration of 1:50. 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 animal following Ki67 or TUNEL staining. The formula used to determine the Ki67 or apoptosis indices was: number of Ki67 or TUNEL positive hepatocyte nuclei, respectively, divided by the total number of hepatocyte nuclei counted.

3.2.5 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 (Zhu et al., 2007). 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).

3.2.6 In vitro PI3K activity assay

PI3K activity assays were performed as previously described (Zhu et al, 2007). Briefly, protein lysates (1 mg) were immunoprecipitated with 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 μ l of PI3K-RB containing 0.2 mg/ml phosphatidylinositol (PI, Sigma) followed by incubation for 10 min. at RT. After incubation at RT, 440 μ l of PI3K-RB containing 30 μ Ci 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 μ l 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.

3.2.7 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 (Bell et al., 1999). 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.

3.2.8 ³H-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 μ Ci ³H-thymidine with or without a combination of HGF (30 ng/ml) and EGF (25 ng/ml) and with either Ly294002 (10 μ M in DMSO) or DMSO alone as a vehicle control. After 48 hr., radioactivity was measured by scintillation counting as described (DeFrances et al., 1992).

3.2.9 Transwell migration assay

Transwell migration assays were performed on hepatocytes as described previously (Stolz et al., 1994). Briefly, TG or NTG hepatocytes were seeded onto the upper part of a 12 μ m pore Transwell chamber (collagen I coated) at a density of 50,000/cm² 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 μ M 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 200X magnification.

3.2.10 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 μ M in DMSO) or DMSO alone as a vehicle control for up to 5 days. The medium was changed daily.

3.2.11 UV-induced apoptosis assay

1x10⁵ TG or NTG hepatocytes were cultured for 12 hr. on single well chamber slides (9.4 cm²/well—Lab-Tek, Germany). Some slides were treated with a dose of 50 J/m² 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.

3.2.12 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.

3.3 RESULTS

3.3.1 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 (Figure 11) as we described previously for human HGF (Bell et al., 1999) 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 (Tilghman et al., 1982).

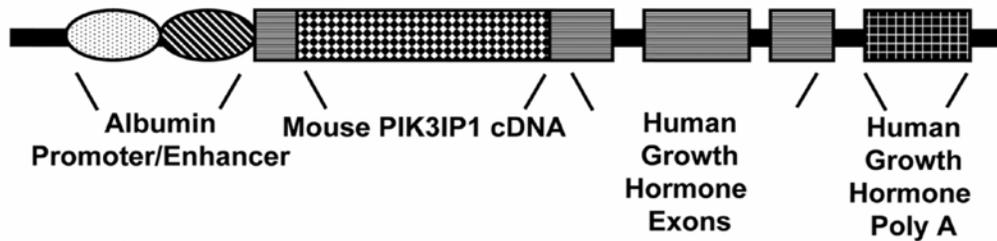


Figure 11: PIK3IP1 transgene construct.

Full-length mouse PIK3IP1 cDNA was subcloned into an albumin promoter/enhancer growth hormone expression construct.

3.3.2 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 (Figure 12) 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% (data not shown) and a marked decrease in phospho-Akt levels by western blot (Figure 13) in the TG livers as compared to controls suggesting that PIK3IP1 regulates p110 activity and signaling in liver.

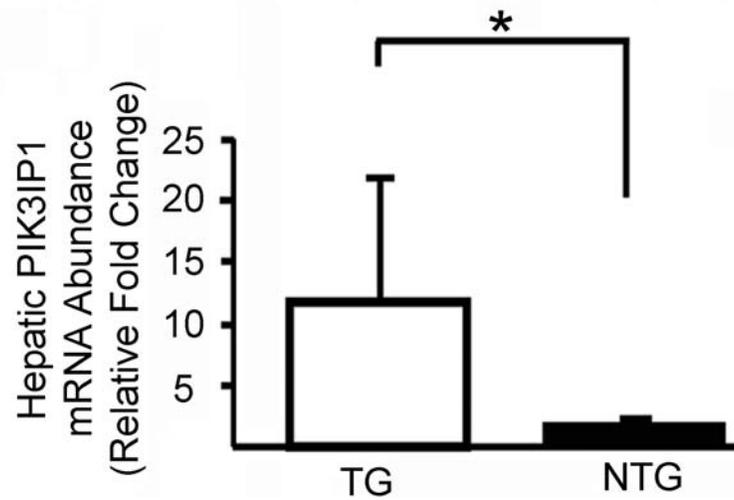


Figure 12: PIK3IP1 mRNA expression is increased in PIK3IP1 TG mouse livers.

Total liver RNA from transgenic (TG— $n = 13$) and non-transgenic (NTG— $n = 16$) mice was subjected to qRT-PCR. Data were normalized to 18S rRNA; the relative fold change in PIK3IP1 mRNA abundance was calculated and graphed. * $p=0.000023$

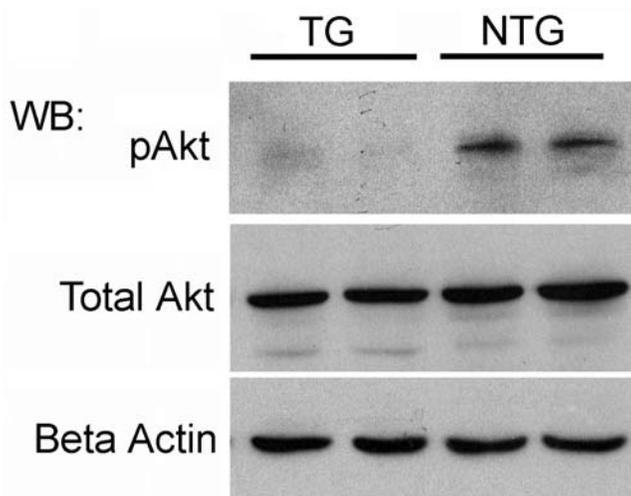


Figure 13: Phospho-Akt is downregulated in PIK3IP1 transgenic mouse liver.

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.

3.3.3 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 (Figure 14). This indicates that PIK3IP1 expression does indeed downregulate the PI3K pathway in TG hepatocytes.

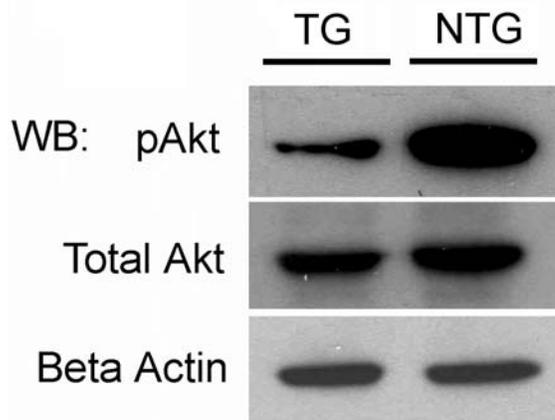


Figure 14: Phospho-Akt is dampened in PIK3IP1 transgenic mouse hepatocytes.

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.

3.3.4 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) (Michalopoulos et al., 2005), the DNA synthetic response in

TG hepatocytes measured by fold increase in ^3H -thymidine incorporation over control cultures was strongly impaired (Figure 15). We also added Ly294002 (LY), a known PI3K inhibitor (Vlahos et al., 1994), 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.

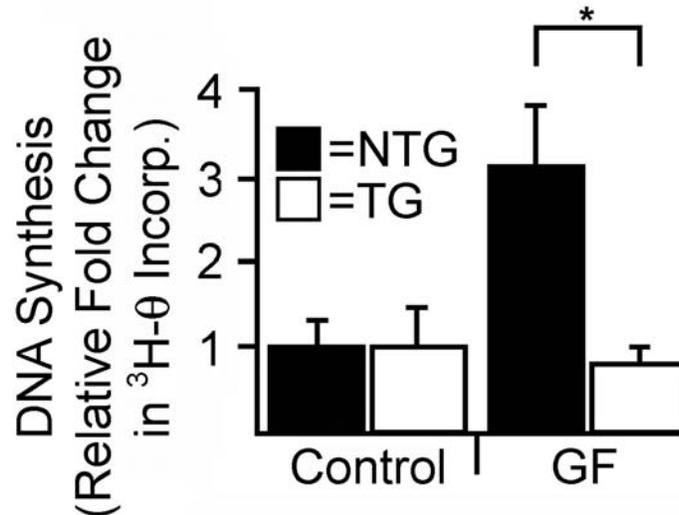


Figure 15: DNA synthesis is blunted in PIK3IP1 transgenic mouse hepatocytes.

*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 ^3H -thymidine (^3H - θ) incorporation assay was performed in triplicate. The relative fold change in average ^3H -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.*

To examine the effect of PIK3IP1 on cell motility, another cellular function ascribed to PI3K (Keely et al., 1997), we subjected TG and NTG hepatocytes to a transwell migration assay. In response to EGF, a well-characterized hepatocyte motogen (Stolz et al., 1994), 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 (Figures 16A&B). 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 (Figures 16A&B).

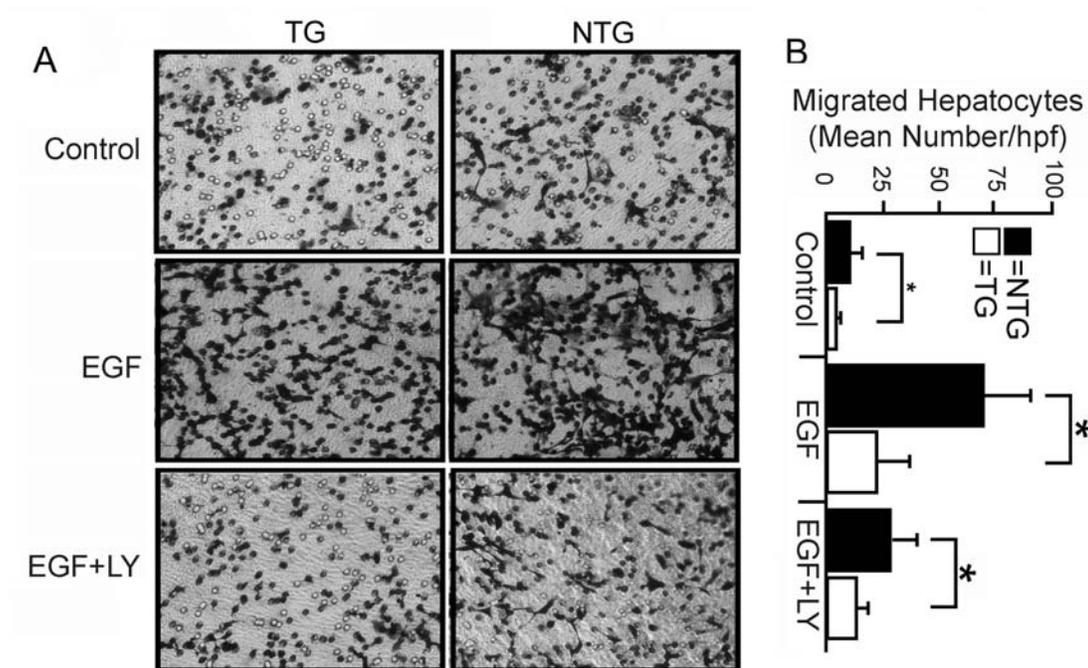


Figure 16: Hepatocyte migration is reduced by PIK3IP1: transwell migration assay.

A transwell migration assay was performed using transgenic (TG) and non-transgenic (NTG) plated at a density of 50,000/cm². 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 A. The mean number of migrated hepatocytes per high power field (hpf) is graphed in B. **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.

In parallel, we performed a second type of motility assay (i.e., an in vitro scratch assay (Liang et al., 2007)) which compares the ability of TG and NTG hepatocytes to reepithelialize a scratched surface. As shown in Figure 17, 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.

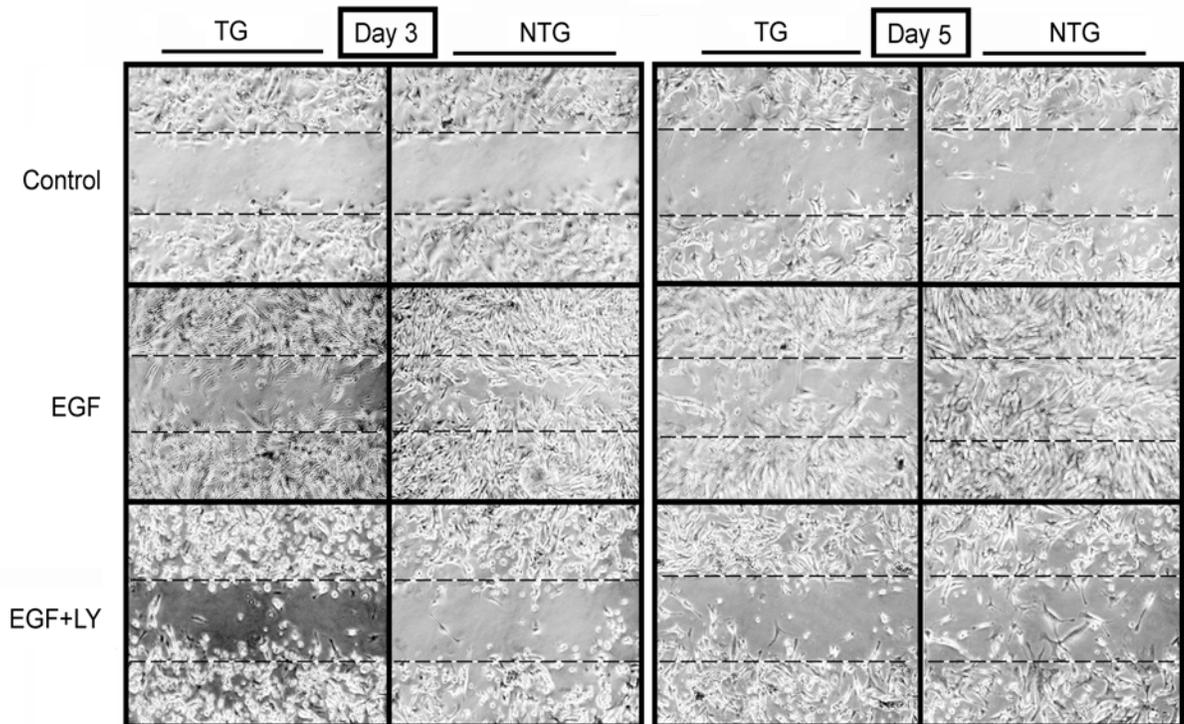


Figure 17: Hepatocyte migration is reduced by PIK3IP1: in vitro scratch assay.

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.

3.3.5 PIK3IP1 promotes apoptosis in isolated mouse hepatocytes.

Considering the key role of PI3K in cell survival (Downward et al., 2004), 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 (Prost et al., 1998). In our experiments, we observed that $47.4 \pm 6.3\%$ of TG hepatocytes and only $28.5 \pm 3.3\%$ of control cells became TUNEL positive 48 hr. following UV irradiation (Figures 18A&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 (Figure 18 C). 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 (Zhu et al., 2007).

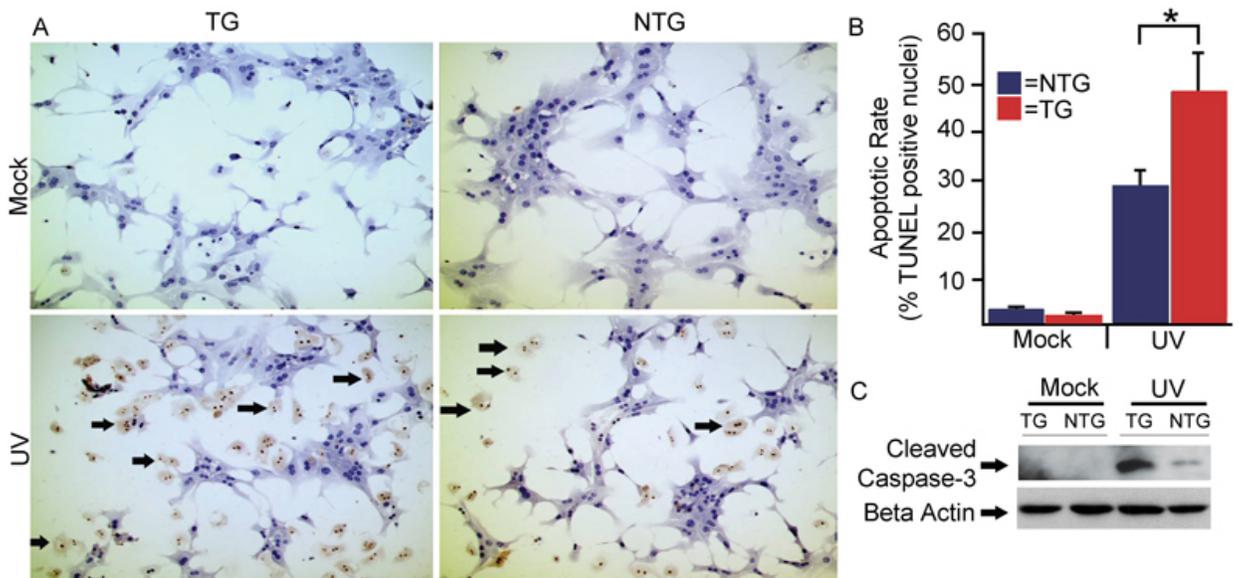


Figure 18: PIK3IP1 augments hepatocyte apoptosis.

Transgenic (TG) and non-transgenic (NTG) cultured mouse hepatocytes were induced to undergo apoptosis with ultraviolet light (UV – 50 J/m²) 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.

3.3.6 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 (Figures 19A&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 livers of TG and NTG mice utilized in the tumor study. We immunostained their livers for Ki67, a nuclear antigen which appears in G₁-G_M. We observed that about $1.00 \pm 0.52\%$ of NTG hepatocyte nuclei stained for Ki67 while only $0.36 \pm 0.21\%$ of TG hepatocyte nuclei were Ki67 positive (Figures 19C&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 ($0.27 \pm 0.15\%$ vs. $0.44 \pm 0.32\%$, 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.

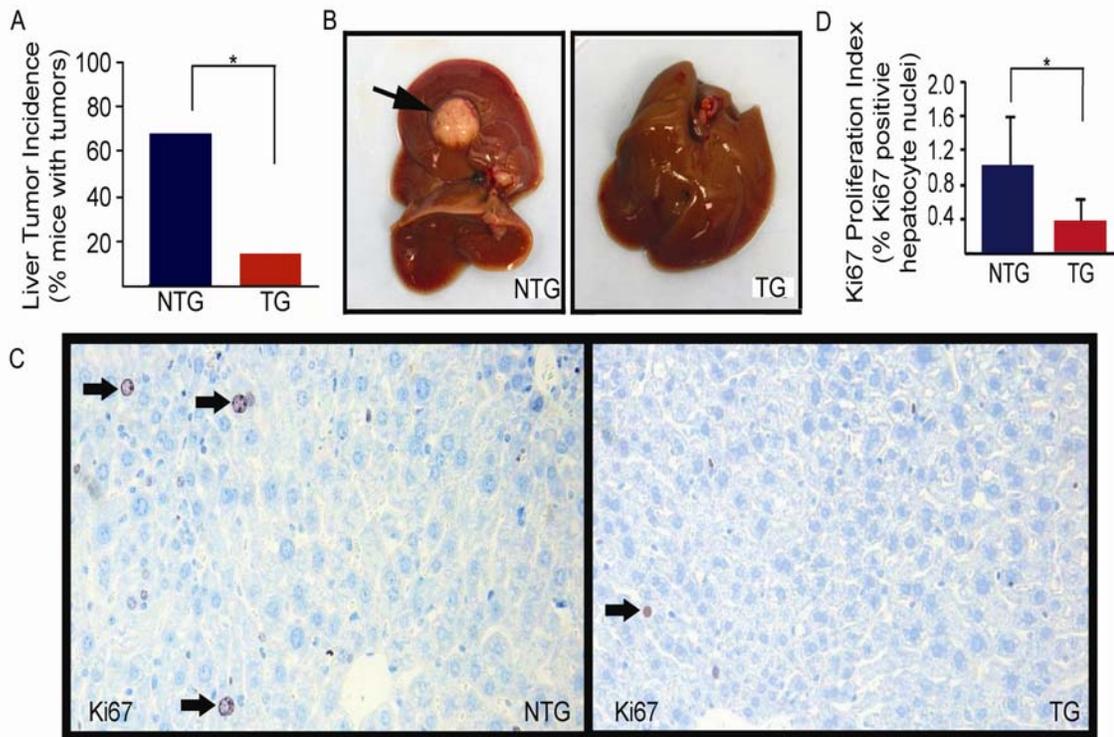


Figure 19: PIK3IP1 expression suppresses spontaneous tumorigenesis and hepatocyte proliferation in mouse liver.

*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 images of representative livers are shown in B. A liver tumor is indicated by the arrow. 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). $*p = 0.0039$.*

3.4 DISCUSSION

Unregulated signaling through the PI3K pathway is linked to the development of certain types of cancer including hepatocellular carcinoma (Engelman et al., 2006; Lee JW et al., 2005). 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 (Horie et al., 2005), 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 (Zhu et al., 2007). 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 (Fruman et al., 2000). As mentioned, unfettered PI3K signaling also alters liver homeostasis. Aberrant PI3K signal transduction is implicated in liver tumorigenesis in both humans and rodents (Lee et al., 2005; Hu et al., 2003; Wan et al., 2003; Horie et al., 2005). 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 other tissues (data not shown), 2) liver survival is dependent on a functioning PI3K pathway (Fruman et al., 2000), 3) PIK3IP1 mRNA and protein are expressed by liver and hepatocytes ((Zhu et al., 2007) and data not shown, respectively); and 4) the expression levels of PI3K pathway constituents (Lee et al.,

2005; Hu et al., 2003; Wan et al., 2003; Horie et al., 2005) 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, stifled 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 (Kanai et al., 1993)) cuts HGF-induced DNA synthesis in cultures of rat hepatocytes by about half as compared to HGF treatment alone (Skouteris et al., 1996). 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. (Nakanishi et al., 1999) 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.

In our 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 are in a state of quiescence (i.e., in G_0) *in vivo*. For example, Counts et al. (Counts et al., 1996) 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 hepatocytes 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.

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.

4.0 PIK3IP1 ALTERS THE LIVER PHOSPHOPROTEOME IN VIVO AND IN VITRO

4.1 INTRODUCTION

Given the multiple functions of PIK3IP1 in cell signaling, especially in relationship to PI3K, MAPK and Ras, we were prompted to investigate its role on modifying the phosphoproteome in the liver. We determined that endogenous hepatic PIK3IP1 expression is low in liver tissue, while levels of phospho-Akt (pAkt), a marker of PI3K activity, are upregulated. We next wanted to investigate the role of PIK3IP1 on gene and protein expression in liver tissues and hepatocytes. To do so, we utilized the PIK3IP1 overexpressing transgenic mice discussed in chapter 3. We then interrogated protein expression changes between PIK3IP1 transgenic (TG) and nontransgenic (NTG) livers and hepatocytes.

4.2 MATERIALS AND METHODS

4.2.1 Phospho-protein analysis in mouse livers

Liver tissues were collected from 6 male littermates (3 TGs and 3 NTGs) aged 6 months. For each animal, 0.1g of liver tissue was subjected to protein extraction and each group of samples were pooled equally. The liver lysates were sent to Kinexus Bioinformatics Corporation

(Kinexus) where Kinexus Kinetworks™ Biosource Phospho-Site Screening (KPSS-7.0) was performed for detection of 38 phosphorylation sites (Figure 20).

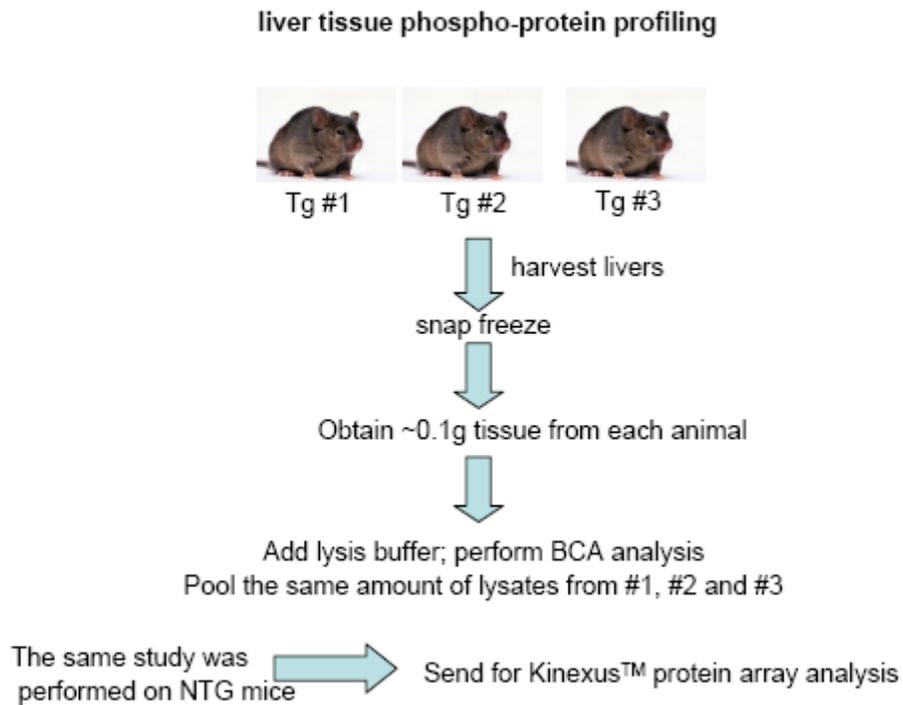


Figure 20: Flow chart for phospho-protein profiling of liver tissues harvested from PIK3IP1 TG and NTG littermates.

4.2.2 Phospho-protein analysis in mouse hepatocytes

Six male littermates (3 TGs and 3 NTGs), aged 6 months, were selected to collect the hepatocytes by liver perfusion. For each animal, 9 million hepatocytes were seeded in each of two 100 mm² dishes. In total, 6 dishes of hepatocytes from either 3 TGs or 3 NTGs were harvested after 12 hrs in culture under serum-free condition. Cell pellets were washed three times with PBS and were dissolved in protein lysis buffer. Five hundred microliter of protein lysate was taken from each animal and animal samples were pooled, mixed with 2x sample buffer, and sent to Kinexus where Kinetworks™ Biosource Phospho-Site Screening (KPSS-7.0) was

performed for hepatocytes phosphoprotein profiling, which can detect 38 phosphorylation sites at the same time (Figure 21 and Table 1).

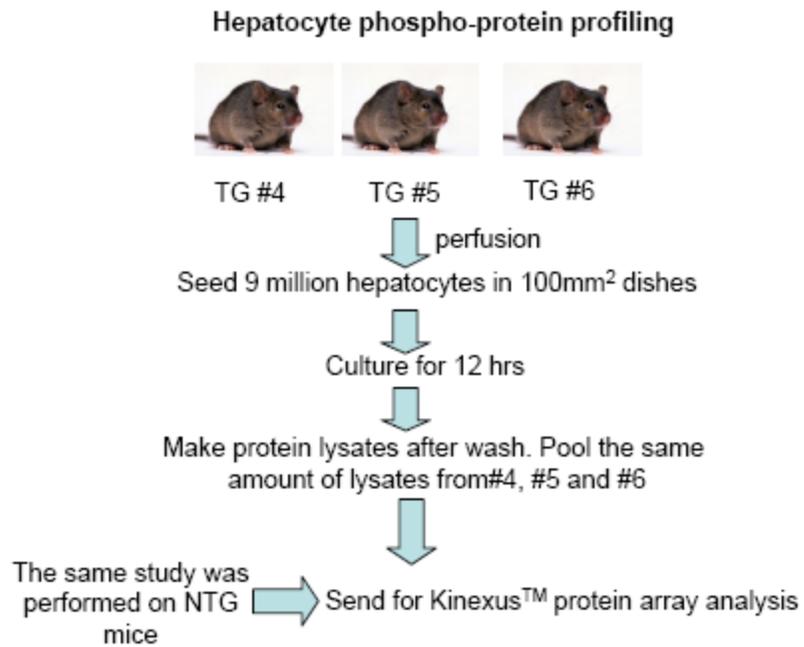


Figure 21: Flow chart for phospho-protein profiling of hepatocytes isolated from PIK3IP1 TG and NTG littermates.

Table 1. Phospho-protein targets interrogated by Kinetworks™ KPSS 7.0 phosphoprotein analysis.

The cognate targets of 38 different phospho-specific antibodies applied in the present study are listed. Y = tyrosine S = serine T = threonine

No.	Abbreviation	Full Name of Protein	Epitope(s)
1	CDK1/2	Cyclin-dependent protein-serine kinase 1/2	T14+Y15
2	Dok-2	Docking Protein 2 (mouse)	Y142
3	EGFR	Epidermal growth factor receptor-tyrosine kinase	Y1148
4	eIF-2 a	Eukaryotic translation initiation factor 2 alpha	S51
5	eIF4G	Eukaryotic translation initiation factor 4 gamma 1	S1107
6	Erk1	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	T202+Y204
7	Erk2	Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)	T185+Y187
8	FAK	Focal adhesion protein-tyrosine kinase	Y576
9	FAK	Focal adhesion protein-tyrosine kinase	S722
10	FAK	Focal adhesion protein-tyrosine kinase	S843
11	FAK	Focal adhesion protein-tyrosine kinase	Y861
12	FAK	Focal adhesion protein-tyrosine kinase	S910
13	GSK3 a	Glycogen synthase-serine kinase 3 alpha	Y279
14	GSK3 b	Glycogen synthase-serine kinase 3 beta	Y216
15	Integrin a4	Integrin, alpha 4 (VLA4)	S988
16	Integrin b1	Integrin, beta 1 (fibronectin receptor beta subunit, CD29 antigen)	S785
17	IR	Insulin receptor	Y999
18	IRS1	Insulin receptor substrate 1	Y612
19	IRS1	Insulin receptor substrate 1	Y1179
20	JNK	Jun N-terminus protein-serine kinases	T183+Y185
21	MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	T334
22	MEK1	MAPK/ERK protein-serine kinase 1(MKK1)	T291
23	MEK1	MAPK/ERK protein-serine kinase 1(MKK1)	S297
24	MEK1	MAPK/ERK protein-serine kinase 1(MKK1)	T385
25	MEK2	MAPK/ERK protein-serine kinase 2 (MKK2) human	T394
26	MEK2	MAPK/ERK protein-serine kinase 2 (MKK2) mouse	T394
27	p38 a	MAPK Mitogen-activated protein-serine kinase p38 alpha	T180+Y182
28	PKBa (Akt1)	Protein-serine kinase B alpha (Akt1)	S473
29	PRAS40	Proline-rich Akt substrate 40 kDa (Akt1S1)	T246
30	PTEN	Phosphatidylinositol-3,4,5-trisphosphate3-phosphatase and protein	S380+S382+S385
31	RSK1/2	Ribosomal S6 protein-serine kinase 1/2	S221/S227
32	RSK1/2	Ribosomal S6 protein-serine kinase 1/2	S363/S369
33	RSK1/2	Ribosomal S6 protein-serine kinase 1/2	S380/S386
34	S6Ka	p70 ribosomal protein-serine S6 kinase alpha	T229
35	Shc1	SH2 domain-containing transforming protein 1	Y349+Y350
36	Src	Src proto-oncogene-encoded protein-tyrosine kinase	Y418
37	Src	Src proto-oncogene-encoded protein-tyrosine kinase	Y529
38	Vinculin	Vinculin	Y821

4.2.3 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 as we described in chapter 3 (Bell et al., 1999). 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.

4.2.4 Protein isolation, SDS-PAGE and Western blot analysis

For Kinexus phospho-protein analysis, liver tissues were homogenized in lysis buffer (20 mM Tris, 20 mM β -glycerophosphate, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na_3VO_4 , 0.5% Nonidet P-40, and 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Sigma aldrich, Cat# P8340) and phosphatase inhibitor cocktail 2 (Sigma Aldrich, Cat# P5726). For hepatocytes, cells were washed with PBS before scraped in lysis buffer. By centrifugation at 100,000 rpm for 30 min at 4 °C, tissue or cell debris was removed. Protein concentration was determined by the BCA protein assay.

4.2.5 Extraction of total RNA and Taqman qRT-PCR

To detect hepatic PIK3IP1 mRNA expression, 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. 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.

4.2.6 PIK3IP1 abundance and PI3K activity among different mouse tissues

Two C3H wild type mice aged 6 months were sacrificed and the spleen, liver, lung, kidney and heart tissues were collected for protein extraction and total RNA extraction.

For comparison of PI3K activity among different tissues, protein was isolated by RIPA buffer and Western blot was performed against phospho-Akt antibody and total Akt antibody (as described in Chapter 2.2.5.) Phospho-Akt level in each tissue was first normalized by total Akt level and then was converted to relative fold of that in the heart tissue.

For comparison of PIK3IP1 abundance among different tissues, total RNA was extracted and Taqman qRT-PCR was carried out to detect the endogenous PIK3IP1 mRNA levels in these tissues..

4.2.7 Kinexus protein kinase profiling assay

The protein lysates were sent to Kinexus for the KinetworksTM phosphor-protein profiling assay, which was performed according to the handbook of KinetworksTM. Briefly, 300 µg of total protein were loaded on a 13% single lane SDS-polyacrylamide gel (SDS-PAGE). Proteins were transfer from SDS-PAGE to nitrocellulose membrane and the latter was incubated with different mixtures of antibodies with distinct molecular masses. After further incubation with secondary antibodies (Santa Cruz Biotechnology), the blots were developed using ECL Plus reagent (Amersham Biosciences), and signals were quantified using Quantity One software (Bio-Rad). The panel of target phosphoproteins detected by the KinetworksTM KPSS 7.0 screen is listed in Table 1. Detailed information and protocols of the KinetworksTM analysis have been reported (Pelech et al., 2003) and can also be found at the Kinexus website (www.kinexus.ca)

4.2.8 Statistics and data analysis

For the Taqman qRT-PCR data, a $2^{-\Delta\Delta Ct}$ method was adopted to compare the relative fold change between TG and NTG PIK3IP1 mRNA expression levels (Livak et al., 2001). PIK3IP1 expression level was normalized to 18S rRNA expression by calculating ΔCt (Average PIK3IP1 Ct - Average 18S Ct). Next, we compared normalized PIK3IP1 expression between TGs and NTGs, adjusting the PIK3IP1 expression value by calculating $\Delta\Delta Ct$ (Average ΔCt_{TG} - Average ΔCt_{NTG}). Then to obtain the relative fold, $2^{-\Delta\Delta Ct}$ was calculated, and $2^{-\Delta\Delta Ct}$ is the normalized PIK3IP1 amount relative to NTGs (relative fold). Student's t-test was used for the comparison of relative fold, and $p < 0.05$ was considered statistically significant.

For the PIK3IP1 abundance among different tissues, relative abundance of PIK3IP1 mRNA from each tissue was normalized according to that in the heart. Then Pearson correlation coefficient was used to predict the possible correlation between pAkt levels and PIK3IP1 levels in different tissues.

4.3 RESULTS

4.3.1 Endogenous PIK3IP1 abundance is negatively correlated with PI3K activity among different tissues

We examined PIK3IP1 mRNA expression and pAkt protein levels in 5 normal tissues including heart, spleen, lung, kidney and liver isolated from two wild type male C3H mice. Each tissue harvested from two animals was pooled and homogenized for protein extraction. We performed western blots for pAkt (normalized by total Akt) and qRT-PCR for PIK3IP1 mRNA

(normalized by 18S ribosome RNA). Relative fold expression was normalized to that of heart tissue. We observed that tissues with the lowest PIK3IP1 mRNA expression, such as the liver and kidney, have the highest pAkt level, whereas heart tissue with the highest PIK3IP1 mRNA expression has the lowest pAkt level. After a correlation study, we found a negative correlation (Pearson's $r = -0.92813$) between the Akt phosphorylation level and PIK3IP1 mRNA level among the tissues (Figure 22). This suggests that high amount of PIK3IP1 may contribute to low PI3K activity in tissues.

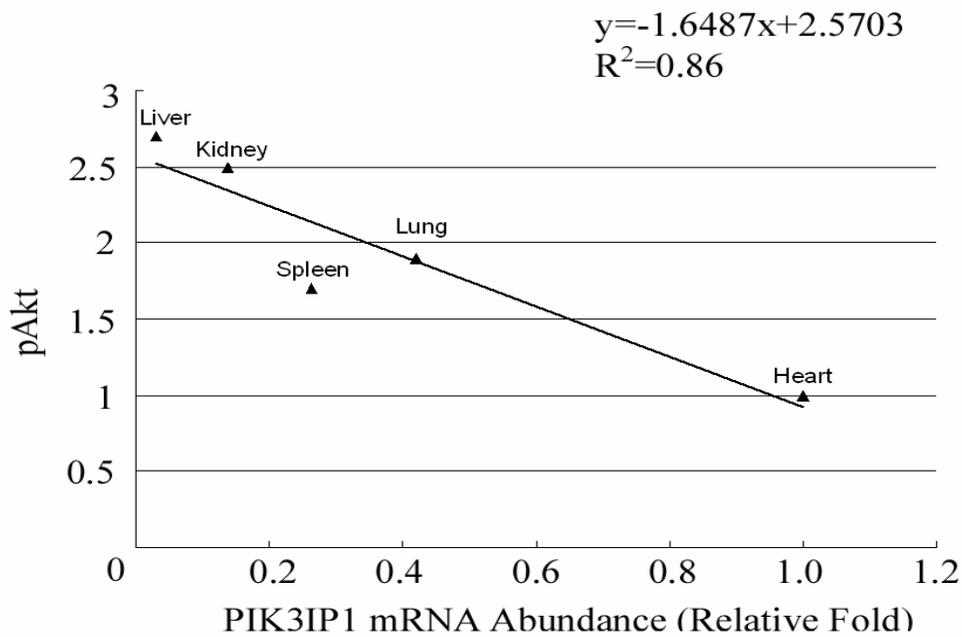


Figure 22: PIK3IP1 abundance is negatively correlated with PI3K activity in different tissues.

Normalized pAkt and PIK3IP1 mRNA levels from different tissues were compared, relative fold to heart was determined, and a correlation study was performed.

4.3.2 Time-course expression of PIK3IP1 mRNA in liver tissues

As shown in Figure 23, in the NTGs, PIK3IP1 mRNA expression remained stable at a low level following birth. However, for TGs, the high hepatic PIK3IP1 mRNA expression was noted as early as 1 month and remained elevated at 16 months. (*= $p < 0.01$)

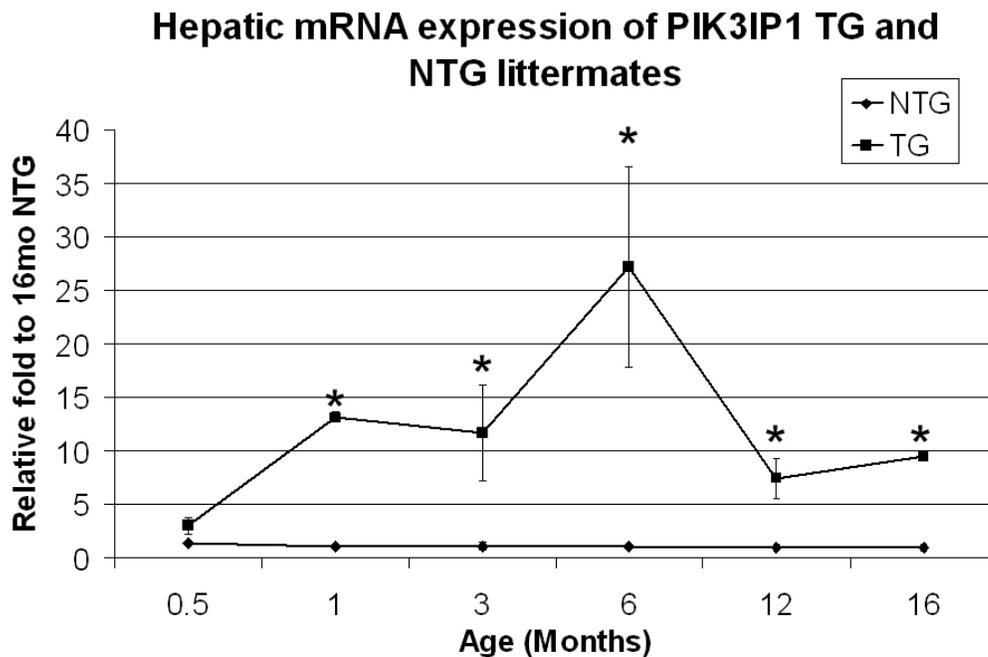


Figure 23: PIK3IP1 mRNA is overexpressed in PIK3IP1 TG mouse livers.

Time-course comparison of hepatic PIK3IP1 mRNA levels between TG and NTG littermates. Mice littermates aged from 0.5 to 16 months were sacrificed and the liver tissues were harvested for mRNA extraction. Two to three animals in each group were compared for the relative fold of PIK3IP1 mRNA abundance.

4.3.3 Comparison of protein phosphorylation between TG and NTG mouse livers

Because PI3K/Akt pathway is involved in protein phosphorylation, and because PIK3IP1 negatively regulates PI3K, we suspected that PIK3IP1 overexpression would change the protein

phosphorylation pattern in hepatocytes and liver tissue. Therefore, we used the Kinexus phospho-antibody screening system to compare the protein phosphorylation profile of TG and NTG livers. This is an efficient tool for discovery and definition of kinase signaling pathways (Sayed et al., 2000). The Kinetworks™ KPSS 7.0 array interrogates 38 protein phosphorylation sites involved in cell proliferation at the same time (Table 1).

We found that phosphorylation of RSK1/2 [S363/S369], GSK3 beta [Y216], Src [Y529], MEK1[S297], Erk2 [T185/T187], FAK [S910] and Akt [S473] were reduced in TG mouse livers as compared to controls. At the same time, we observed that phosphorylation of Dok2 [Y142], eIF2a [S51], PTEN [S380+T382+S385] and IR [Y999] were increased in TG mouse livers (Table 2 and Figure 24). Most of these proteins are downstream targets of the PI3K (Figure 3). Therefore, these results are consistent with our previous findings that PIK3IP1 suppresses the PI3K signaling pathway.

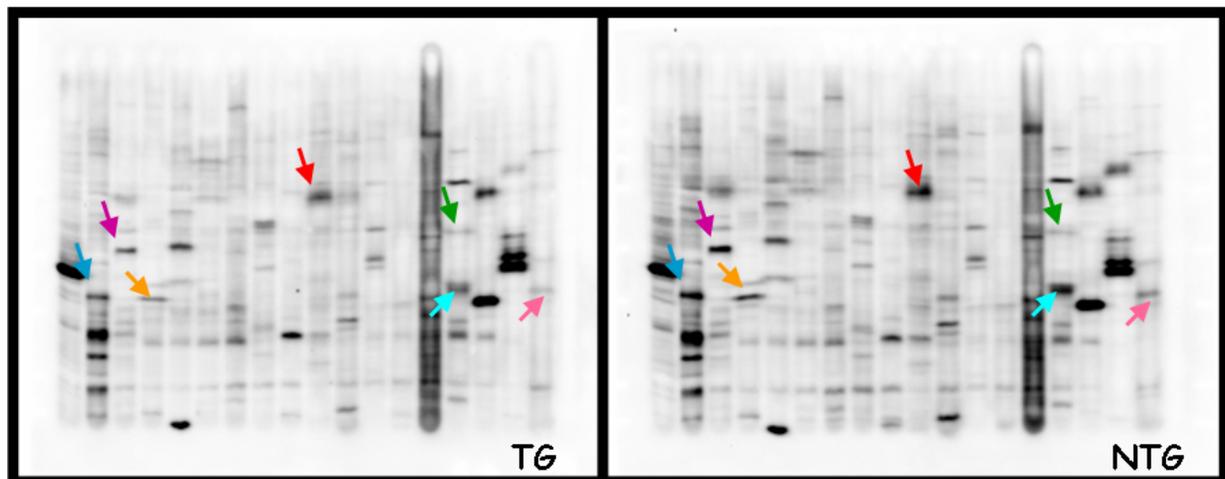


Figure 24: PIK3IP1 overexpression in the liver changes its phospho-protein profile.

Changes in the intensity of phosphorylation signals are evident in PIK3IP1 TG liver tissues (Left) as compared with NTG liver tissues (Right)

Table 2: Phospho-protein changes in PIK3IP1 TG liver tissues as compared to NTG controls

protein name	epitope	Tg:NTG	Expected outcome
RSK1/2	[S380/S386]	-65%	transcriptional regulation
GSK3-beta	[Y216]	-42%	↓Beta catenin; ↓Proliferation
Src	[Y529]	-37%	↓cell adhesion, migration, and cancer progression
MEK1	[S297]	-36%	↓proliferation & differentiation
Erk2	[T185/T187]	-29%	↓survival & growth
Focal adhesion protein-tyrosine kinase (FAK)	[S910]	-27%	↓cell spreading, differentiation, migration & survival
Proline-rich Akt substrate 40 kDa (Akt1S1, PRAS40)	T246	-26%	↓mTOR1 activity
GSK3-alpha	Y279	-25%	↓β-catenin activity
Akt	[S473]	-13%	↓PI3K signaling
Dok2	[Y142]	11%	negatively regulate signal transduction and cell proliferation controlled by cytokines in a feedback loop
Eukaryotic translation initiation factor 2 alpha (eIF2a)	[S51]	23%	Regulation of protein synthesis
PTEN	[S380+T382+S385]	35%	↑PTEN protein stability (Torres et al., 2001)
Insulin receptor (IR)	[Y999]	54%	↑IR signaling (compensatory?)

4.3.4 Comparison of protein phosphorylation between TG and NTG mouse hepatocytes

Similar to the changes observed in vivo, we found that phosphorylation of RSK1/2 [S221/S227], PRAS40 [T246], Erk1 [T202+Y204], Akt1 [S473], p38 MAPK [T180+Y182] and IRS1 [Y612] were reduced in TG mouse hepatocytes as well. At the same time, we observed that

phosphorylation of eIF2a [S51], PTEN [S380+T382+S385] and IR [Y999] were increased in TG mouse hepatocytes (Table 3).

Table 3: Phospho-protein changes in PIK3IP1 TG hepatocytes as compared to NTG hepatocytes

protein name	epitope	TG:NTG	Expected outcome
RSK1/2	S221/S227	-66%	transcriptional regulation
Proline-rich Akt substrate 40 kDa (Akt1S1, PRAS40)	T246	-45%	↓PI3K signaling
Erk1	T202+Y204	-39%	↓survival & growth
PKB/Akt1	S473	-36%	↓PI3K signaling
P38 MAPK	T180+Y182	-33%	↓cell differentiation & growth
IRS1	Y612	-28%	↓PI3K recruitment (Esposito et al., 2001)
p70 ribosomal protein-serine S6 kinase alpha (p70S6K)	T229	52%	↓cell cycle & cell growth
Dok2	Y142	60%	negatively regulate signal transduction and cell proliferation controlled by cytokines in a feedback loop
Src	Y418	83%	Src activation
Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK); (JNK1+JNK2)	T183/Y185	100%	JNK pathway
Eukaryotic translation initiation factor 2 alpha (eIF2a)	S51	119%	Regulation of protein synthesis

4.4 DISCUSSION

In the present study, we demonstrated that endogenous PIK3IP1 expression is negatively correlated with tissue PI3K activity. This is a very interesting phenomenon, because it suggests that PIK3IP1, a negative PI3K regulator, may be a determinant factor of tissue PI3K activity.

This can be explained by the fact that PIK3IP1 directly binds with p110 catalytic subunit of PI3K and suppress its activity.

We also demonstrated that overexpression of PIK3IP1 in hepatocytes dramatically changes protein phosphorylation both in vivo and in vitro. Noticeably, many kinases that we examined in this study showed decreased phosphorylation in PIK3IP1 TG liver tissues and hepatocytes, suggesting that PIK3IP1 widely suppresses proliferation and survival through these pathways. Interestingly, most of the proteins demonstrating changes in phosphorylation by PIK3IP1 overexpression are direct or indirect targets of the PI3K pathway (such as Akt, GSK3, PRAS40, Erk1 and JNK1/2).

In conclusion, we confirmed that PIK3IP1 mRNA abundance is negatively correlated with PI3K activity in the mural tissues. Furthermore, hepatic PIK3IP1 overexpression altered the hepatic phospho-proteome both in vivo and in vitro, probably through suppression of PI3K/Akt pathway. These observations help to explain PIK3IP1's activity to dampen proliferation and survival, and provide further evidence as to how PIK3IP1 reduces HCC tumorigenesis we observed in PIK3IP1 TG mice.

5.0 PIK3IP1 OVEREXPRESSION INDUCES HYPERGLYCEMIA AND OBESITY

5.1 INTRODUCTION

Insulin signaling is a critical component in the maintenance of whole-body glucose and fat homeostasis. Insulin activates the membrane receptor which in turn activates insulin receptor substrate-1 (IRS-1) by phosphorylation (Dresner et al 1999; Yu et al 2002). The liver plays a key role in insulin induced glucose metabolism in two ways: 1). By controlling glucose uptake through glycogenesis; and 2) by releasing glucose via glycogenolysis and gluconeogenesis. Insulin stimulates glycogen synthesis by activating glycogen synthase (GS) through dephosphorylation. Akt-mediated inactivation of GSK-3 contributes to a reduction in the net phosphorylation of GS and a further induction of glycogen synthesis (Whiteman et al., 2002). Therefore, gain-of-function in PI3K leads to hypoglycemia, which is the main manifestation in PTEN knockout mice and mice harboring activated Akt (Stiles B, 2007 and Ono H, et al., 2003).

The PI3K pathway is also involved in liver adipogenesis. Activation of Akt induces synthesis of SREBP-1 and SREBP-2, which will lead to the induction of key enzymes of the cholesterol and fatty acid biosynthesis pathways, such as fatty acid synthase. Therefore, activation of PI3K/Akt induces an increase in the concentration of liver triglyceride (Porstmann et al. 2005; Ono H et al., 2003).

Although hepatocyte-specific Pten knock-out mice and mice overexpressing Akt display steatohepatitis (Horie Y et al., 2004), hepatic overexpression of HGF dramatically ameliorates a high-fat diet-induced fatty liver (Kosone T, et al., 2007). HGF/Met can activate another important factor of energy metabolism, the Ras/Raf/MEK/ERK kinase cascade. Erk1-MAPK mediated phosphorylation of PPAR γ associated with adipogenesis in fat cells (Hu E, et al. 1996).

Since both PI3K and Ras/MAPK activities are impaired in PIK3IP1 transgenic hepatocytes, we hypothesize that our PIK3IP1 TG mice may have disturbed glucose and triglyceride metabolism.

5.2 MATERIALS AND METHODS

5.2.1 Animals

PIK3IP1 TG and NTG male littermates aged 8-10 months old were used for the experiments. For metabolic measurements, animals were fasted overnight. Blood samples were then taken for assessment of glucose and triglycerides by using manufactured kits. For tissue collection, mice were fasted overnight and blood was collected from cardiac puncture after the measurement of the body weight. And then, the visceral fat was taken out from the abdomen and weighted. Liver tissues were flash-frozen in liquid nitrogen for protein analysis or liver triglyceride analysis.

5.2.2 Western blot analysis

Protein sample preparation and SDS/PAGE were performed as mentioned in chapter 2.2.5. Blots were probed with phospho-insulin receptor β (Tyr1146) Antibody (Cell Signaling Technology, Inc., Cat#: 3021) and the same blots were reprobed with insulin receptor β antibody (Santa Cruz Biotechnology Inc., Cat#: SC-711).

5.2.3 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.

5.3 RESULTS

5.3.1 Mice overexpressing PIK3IP1 in hepatocytes display increased body weight and visceral fat deposition

To identify the possible role of PIK3IP1 in metabolism, we performed a cohort study. Five TG and five NTG male littermates, aged 8-10 months, were assessed for body weight and visceral fat weight. As a result of PIK3IP1 overexpression in the liver, we found a 3-fold increase of visceral fat deposition in TG males (Figures 25 B&C). We also observed a significant increase of the body weight in our PIK3IP1 TG mice (Figure 25A, open bar). Taken together, these results suggest that PIK3IP1 overexpression in the liver is associated with deregulation of fat metabolism and can lead to visceral obesity.

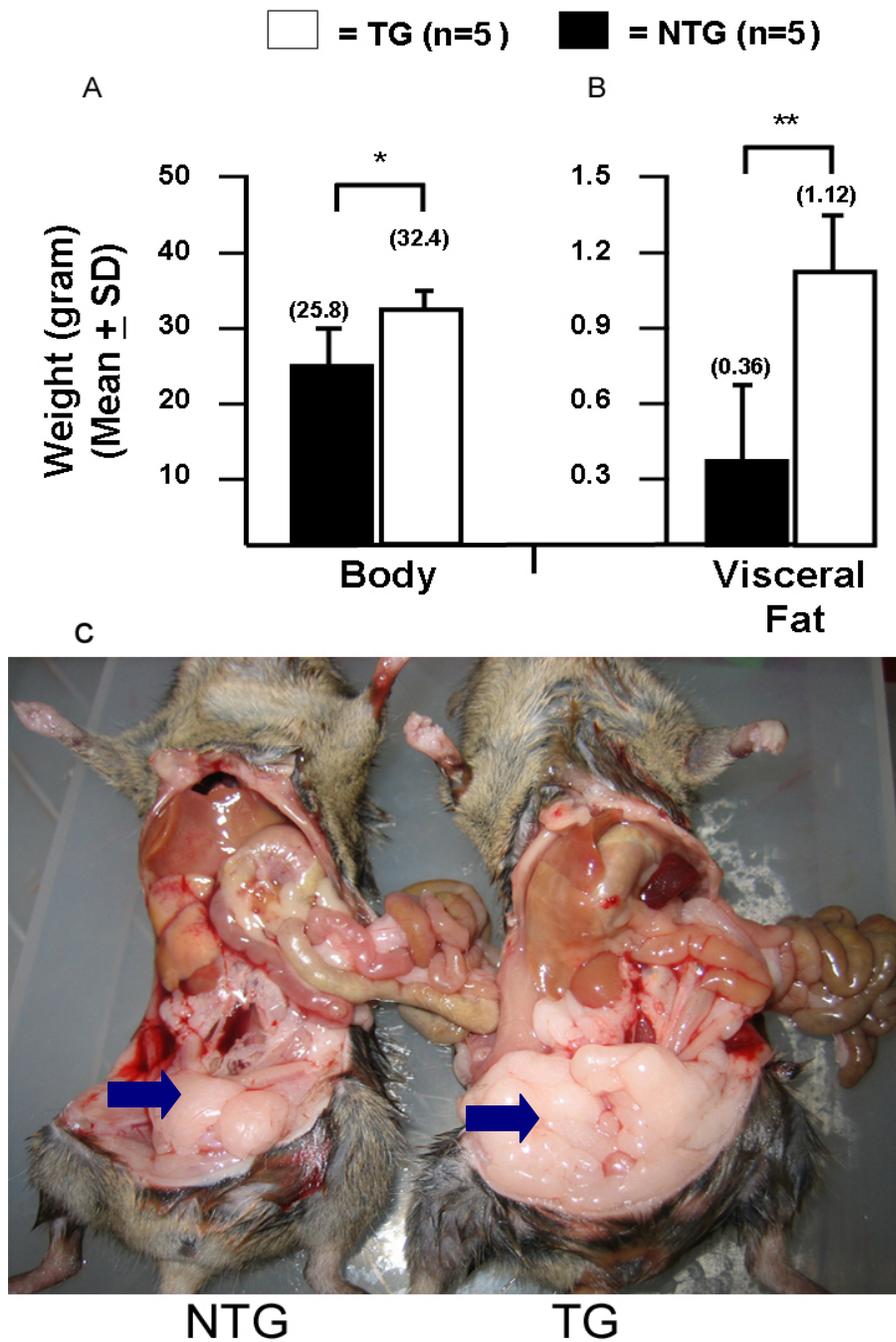


Figure 25: Mice overexpressing PIK3IP1 in hepatocytes display increased body weight and visceral fat deposition.

(A) TG mice have increased body weight. $*p=0.016$; and (B) increased visceral fat. $**p=0.0003$. (C) Images depicting visceral fat from PIK3IP1 NTG (Left) and TG (Right) mice.

5.3.2 Serum fasting glucose is elevated in mice with hepatic overexpressing of PIK3IP1

Because both the body weight and the visceral fat weight are related to insulin resistance, we then evaluated serum fasting glucose between TG and NTG littermates from the same cohort. PIK3IP1 overexpression was associated with an increase in fasting serum glucose levels in the TGs (Figure 26). This suggests that PIK3IP1 may contribute to insulin resistance.

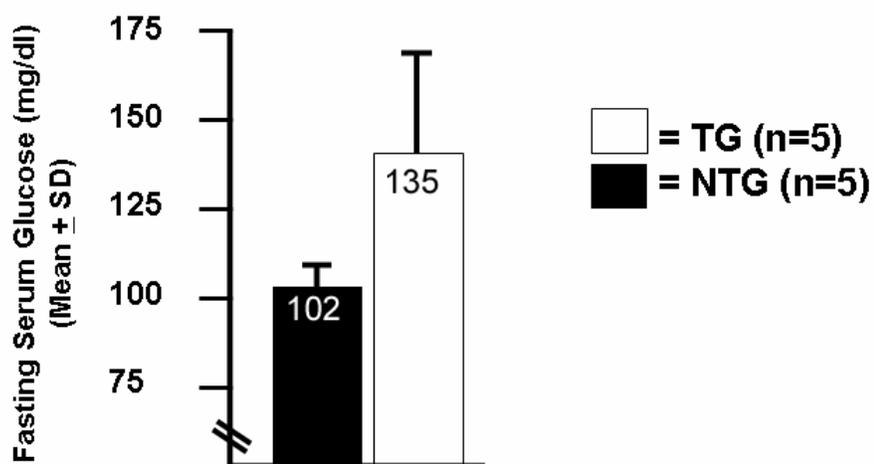


Figure 26: Fasting serum glucose levels are elevated in PIK3IP1 TG mice (open bar).

5.3.3 PIK3IP1 overexpression increased insulin receptor (IR) phosphorylation

Because IR is the key receptor responsive for insulin signaling, we compared the phosphorylation pattern of IR between TG and NTG livers. In TGs, IR phosphorylation was significantly higher than that in NTGs (Figure 27).

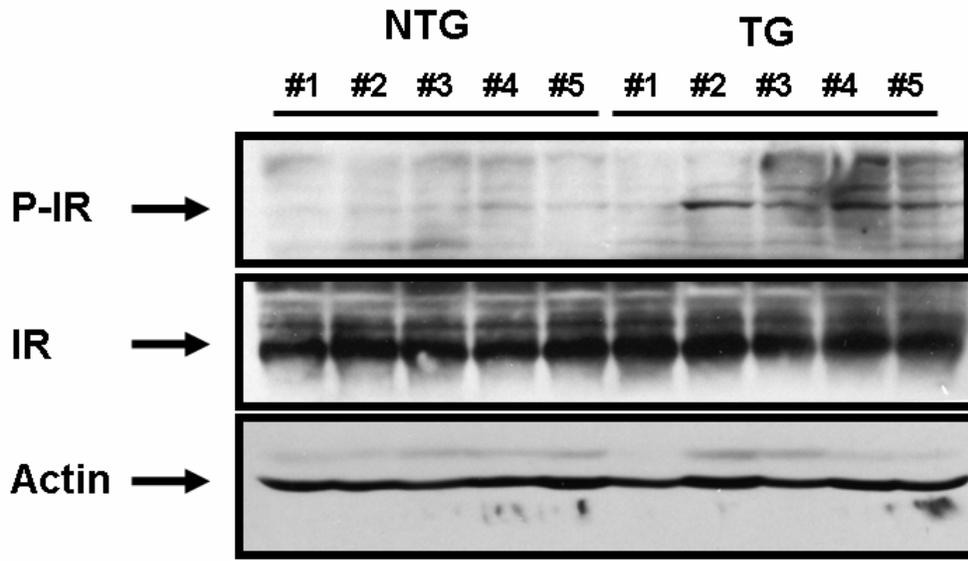


Figure 27: PIK3IP1 overexpression increased phosphorylation of insulin receptor.

A Western blotting analysis showed that IR phosphorylation is increased in Tg livers.

5.4 DISCUSSION

IR and IRS proteins activate PI3K which leads to Akt stimulation. This IR-IRS-PI3K-Akt axis is known to play a critical role in insulin-induced metabolism (Saltiel AR et al., 2001; Whiteman E et al., 2002; Miyake K et al., 2002) and the homeostasis of which will be altered if the components are modulated or disrupted.

Overexpression of PIK3IP1 in the liver resulted in hyperglycemia and obesity. These indices are strong determinants for diabetes and insulin resistance. PIK3IP1 suppresses PI3K activity, which may inhibit the transport of the blood glucose into the liver. One downstream protein of PI3K/Akt pathway is GSK-3. Insulin-stimulated protein kinase B (PKB/Akt) and GSK-3beta phosphorylation, therefore reduce hepatic glucose output and induce hepatic glycogen synthesis. GSK-3 activity is elevated in human and rodent models of diabetes, and

various GSK-3 inhibitors improve glucose tolerance and insulin sensitivity in rodent models of obesity and diabetes. As shown in our phospho-protein array study, PIK3IP1 overexpression inhibit Akt induced GSK-3 phosphorylation (Table 2) and IRS phosphorylation (Table 3). Therefore, PIK3IP1 likely increases blood glucose by inhibiting the PI3K/GSK-3 pathway.

Surprisingly, the abundance of tyrosine phosphorylated IR was upregulated in TGs. We confirmed this observation by WB. This finding is likely explained by the fact that hepatic signaling downstream of IR at the level PI3K is dampened by PIK3IP1 in the TGs. Thus, it is possible that IR hyperphosphorylation reflects a compensatory mechanism in TGs to overcome resistance at the level of PI3K. It would thus follow that PIK3IP1 TGs are hyperinsulinemic. This possibility is one that we will explore in the specific aims of our application.

In conclusion, in the present study, we demonstrated that overexpression of PIK3IP1 in the liver dramatically increased visceral fat, liver fat, and serum fasting glucose. Further studies are needed to elucidate whether this defect is caused by the suppressive effect of PIK3IP1 on IR/PI3K pathway and/or HGF/Met/Erk pathway.

6.0 DISCUSSION

6.1 SUMMARY

The objective of this dissertation was to elucidate the role of PIK3IP1, a negative PI3K regulator, in hepatic tumorigenesis and metabolism. We successfully generated a transgenic mouse model with hepatic overexpression of PIK3IP1 level as early as one month after birth. The high PIK3IP1 level in the liver significantly suppressed PI3K pathways as shown by KinexusTM phospho-protein assay and PI3-kinase assay.

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 (Zhu et al., 2007). However, we had not elucidated the role of PIK3IP1 in controlling PI3K activity in vivo. This study represents the first time that hepatic overexpression of PIK3IP1 can suppress tumorigenesis of hepatocellular carcinoma, mainly through curbing PI3K pathway.

In addition, hepatic overexpression of PIK3IP1 leads to hyperglycemia and high visceral fat deposition. This indicates that PIK3IP1 may contribute to insulin resistance, and the higher order implications of the findings are the focus of future studies.

6.2 FUTURE DIRECTIONS

Based on the current results, there are several directions which are critical for us to investigate. First, we want to know how the effect of PIK3IP1 loss-of-function to liver homeostasis. Since PIK3IP1 is a negative regulator of PI3K, loss of PIK3IP1 should increase PI3K activity. This could result in upregulated replication and survival of hepatocytes and could induce HCC tumorigenesis. Furthermore, once PIK3IP1 is knocked out, elevated PI3K activity in the liver may also change glucose and triglyceride metabolism. This will probably induce hypoglycemia, since insulin signaling through PI3K may be enforced when PIK3IP1 expression is switched off in the liver.

The second direction we would like to follow is the role of PIK3IP1 in liver regeneration. The Akt/PKB pathway has been shown to be activated during liver regeneration and pancreatic regeneration. Phospho-Akt levels increased as early as 30 min. after two thirds partial hepatectomy (PHx) in mice, suggesting a possible mechanism of PI3K in liver regeneration (Hong et al., 2000). However, the role of PI3K in liver regeneration still remains unclear. A recent paper by Jackson et al. shows that wortmannin administration in mice dampens liver regeneration following 2/3 Phx at the second day time point. The effect of siRNA treatment to knock down p85 alpha or p110 alpha is slightly more dramatic resulting in a more pronounced inhibition of liver regeneration. Hepatic resection is associated with rapid proliferation and regeneration of the remnant liver lobes. Many growth factors and cytokines, most notably hepatocyte growth factor, epidermal growth factor, transforming growth factor-alpha, interleukin-6, tumor necrosis factor-alpha, insulin, and norepinephrine, appear to play important roles in this process. Therefore, PIK3IP1 may be involved in liver regeneration as a negative regulator. Our future study will focus on: i) defining the involvement of the PI3K/Akt pathway in hepatic regeneration, and ii) determining the effects of PI3K inhibition by PIK3IP1 on hepatic regeneration following partial hepatectomy.

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