The Requirement of Hexokinase 2 in The Process of Tumorigenesis: Implications for Cancer Therapy

BY

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THESIS

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TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION……1
1. Glucose metabolism and aerobic glycolysis……1
2. Glucose metabolism is one of the major sources for biosynthetic precursor synthesis……2
3. Hexokinases……6
4. Hexokinase-2 and cancer……10
5. Targeting metabolism for cancer therapy: Hexokinase 2 inhibition a rational therapeutic target……12

II. MATERIAL AND METHODS……15
1. Mice stains and husbandry……15
2. Lung tumor induction in K-Ras^{LSLG12D} mice……16
3. Liver tumor induction by DEN……16
4. Xenograft assays……16
5. Histopathology and Immunohistochemistry……17
6. Measurements of BrdU labeling in tumor sections……18
7. Tamoxifen treatment……18
8. Cell lines……18
9. Retrovirus and lentivirus production and infection……19
10. Isolation and establishment of cell lines from mice……19
11. Anchorage independent growth assay……20
12. Proliferation assays……21
13. Immunoblotting……21
14. Glucose consumption assay……21
15. Hexokinase activity assay……22
16. Metabolites extraction and analysis……22
17. ^{14}C Glucose Incorporation into DNA……23
18. Statistical analysis……23

III. RESULTS……24
1. The expression of hexokinase 2 is induced in human cancer tissues and cells and is required for tumorigenicity of the human cancer cells……24
(A) Background……24
(B) Results……25
1. HK2 is specifically expressed in human cancer tissues……25
2. Stable shRNA mediated knockdown of HK2 attenuates the tumorigenicity of human cancer cells……33
2.1 Design and validation of HK2 specific shRNA in human cancer cells……33
2.2 Expression of stable HK2shRNA affects the tumorigenicity of human cancer cells *in vitro*……36
2.2.1 Effect on Human Hepatocellular carcinoma (HCC) cell lines……36
2.2.2 Effect on human non-small cell lung cancer cells……41
2.2.3 Effect on human breast cancer cells……44
2.3 Expression of stable HK2shRNA affects the tumorigenicity of human cancer cells *in vivo*……47

TABLE OF CONTENTS (continued)

CHAPTER PAGE

2.3.1 HK2 is required for tumor formation *in vivo*……47
2.3.2 HK2 is required for tumor maintenance *in vivo*……50
2. Hexokinase 2 is required for oncogenic transformation *in vitro* and tumor initiation of different mouse models of cancer *in vivo*……58
(A) Background……58
(B) Results……59
1. HK2 conditional knockout mouse……59
2. HK2 is required for oncogenic transformation of Mouse embryonic fibroblasts……62
3. Effect of Hexokinase 2 deletion in tumor initiation in mice models of cancer……72
3.1 HK2 is required for tumor initiation in a mice model of NSCLC……72
3.2 HK2 is required for tumor initiation in mice model of breast cancer……80
3.3 Genetic ablation of HK2 reverses tumorigenic potential of Neu transformed breast cancer cells……86
3.4. HK2 deletion impairs liver carcinogenesis in mice……91
3. Systemic HK2 deletion in adult mice does not cause an overt phenotype but reduces tumor burden of lung cancer……95
(A) Background……95
(B) Results……95
1. Systemic deletion of HK2 is tolerated in mice without any overt physiological consequences……95
2. Systemic ablation of HK2 is therapeutic in a mouse model of lung cancer……101
4. Molecular mechanisms underlying how HK2 affects tumorigenesis……107
(A) Background……107
(B) Results……108
1. HK2 efficiency in K-Ras^{G12D}-NSCLC cells impairs the downstream metabolites...108
1.1 Nucleic acid biosynthesis pathway……108
1.2 Precursors of fatty acid biosynthesis……115
2. Glutamine-derived carbon incorporation into TCA cycle intermediates is reduced in K-Ras^{G12D}-NSCLC cells following HK2 deletion……115

IV. DISCUSSION……125

V. REFERENCES……135

VI. VITA……142

LIST OF FIGURES

FIGURE PAGE

1. The common metabolic pathways that support anabolic growth in proliferating cells……5
2. Glucose phosphorylation and Hexokinases……9
3. HK2 expression is elevated in Human Hepatocellular Carcinoma……28
4. HK2 expression is elevated in Human Non-small cell carcinoma (NSCLC) and correlated with the aggressiveness of the disease……30
5. HK2 expression is elevated in Human breast cancer and correlated with the aggressiveness of the disease. ……32
6. Validation of HK2 specific shRNA in human cells……35
7. HK2 is required for tumorigenic potential Hepatocellular carcinoma cell lines……38
8. HK2 silencing reverses tumorigenesis of NSCLC cells *in vitro*……43
9. HK2 silencing reverses tumorigenesis of human breast cancer cells *in vitro*……46
10. HK2 is required for tumorigenic potential of cancer cell lines *in vivo*……49
11. HK2 is required for tumor maintenance of Human NSCLC cell lines *in vivo*……53
12. Characterization of HK2 conditional knockout mice……61
13. HK2 expression is elevated by oncogenic Ras and it is required for oncogenic transformation of mouse embryonic fibroblasts (MEFs)……65
14. Genetic ablation of HK2 reduces the proliferation of primary K-Ras^{G12D} mutated mouse NSCLC cells in–vitro……71
15. HK2 is required for the development of oncogenic K-Ras driven non-small cell lung cancer (NSCLC) in mice, and its deletion extends the lifespan of these mice……75
16. HK2 is required for the development of ErbB2/Neu-driven mammary tumors in mice……83
17. The deletion of HK2 reverses the oncogenic potential of mouse mammary tumor cells derived from MMTV-NeuT-HK2$^{f/f}$ mice……88
18. Liver specific knockout of HK2 reduces tumor burden in chemical carcinogen induced HCC in mice……94
19. Systemic ablation of HK2 in adult mice dose not cause any overt phenotype……98
20. Systemic deletion of HK2 in the mouse is therapeutic for oncogenic K-Ras driven NSCLC……104
21. Characterization of the K-Ras^{G12D} NSCLC cell lines used in the metabolomic analysis……112
22. The effect of HK2 deletion in K-Ras^{G12D} NSCLC cells on the intracellular levels of metabolites derived from glucose or glutamine……114
23. The effect of HK2 deletion on glutamine metabolism in Ras transformed cells……122
24. Schematic illustration depicting metabolomics changes induced by the deletion of HK2 in K-Ras^{G12D}NSCLC cells……124

LIST OF ABBREVIATIONS

1,3BPG	1,3 bisphosphoglecerate
^{18}FDG	Fluoro-deoxy Glucose (18)
2DG	2-Deoxy glucose
2DG-6P	2-Deoxy-6-phosphate
2PG	2 phosphogecerate
3PG	3 phosphoglecerate
AKT	Ak mouse strain, transforming
AMP	Adenosine-5'-monophosphate
AMPK	5'-AMP activated kinase
Asp	Aspartate
ATP	Adenosine-5'-triphosphate
BSA	Bovine Serum Albumin
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
CO_2	Carbon dioxide
CTP	Cytidine -5'-triphosphate
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DEN	Diethylnitrosamine
DHAP	Dihydroxy acetone phosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
F1,6P	Fructose 1, 6-bis phosphate
F6P	Fructose 6-phosphate
FBS	Fetal Bovine Serum
G3P	Gleceraldehyde 3-phosphate
G6P	Glucose 6-phosphate
G6PDH	Glucose 6-phosphate dehydrogenase
GCK	Glucokinase
GFP	Green fluorescent protein
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HBP	Hexosamine biosynthetic pathway
HCC	Hepatocellular carcinoma
HCl	Hydrogen chloride
HER2	Human Epidermal Growth Factor Receptor 2
HIF1	Hypoxia inducible factor-1
HK	Hexokinase
i.p.	Intraperitoneally
IHC	Immunohistochemistry

LIST OF ABBREVIATIONS (continued)

LA2	Latent activated 2
Lac	Lactate
LC	Liquid Chromatography
LKB1	Liver Kinase B1
LSL	Lox-stop-Lox
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse embryonic fibroblast
mRNA	messenger Ribonucleic acid
MS	Mass Spectroscopy
mTOR	Mammalian Target of Rapamycin
NADP	Nicotianamide adeninine diphosphate-oxidized
NADPH	Nicotianamide adeninine diphosphate-reduced
NCI	National cancer institute
NIC	Neu-IRES-Cre
O2	Oxygen
OAA	Oxaloacetate
PBS	Phosphate-buffered saline
PEP	Phosphoenol pyruvate
PET	Positron Emission Tomography
PI3K	Phosphatidylinositol-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PPP	Pentose phosphate pathway
pSer	Phosphoserine
PVDF	Polyvinylidene fluoride
Pyr	Pyruvate
qRT-PCR	Quantitative real-time polymerase chain reaction
R5P	Ribose-5-phosphate
RAS	Rat sarcoma
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
Ru5P	Ribulose-5-phosphate
S7P	Sedoheptulose 7-Phosphate
shRNA	Small hairpin RNA
TCA	Tri-carboxylic acid
UBC	UbiquitinC
UTP	Uridine-5'-triphosphate
WT	Wild type

CHAPTER-I

INTRODUCTION

1) Glucose metabolism and aerobic glycolysis

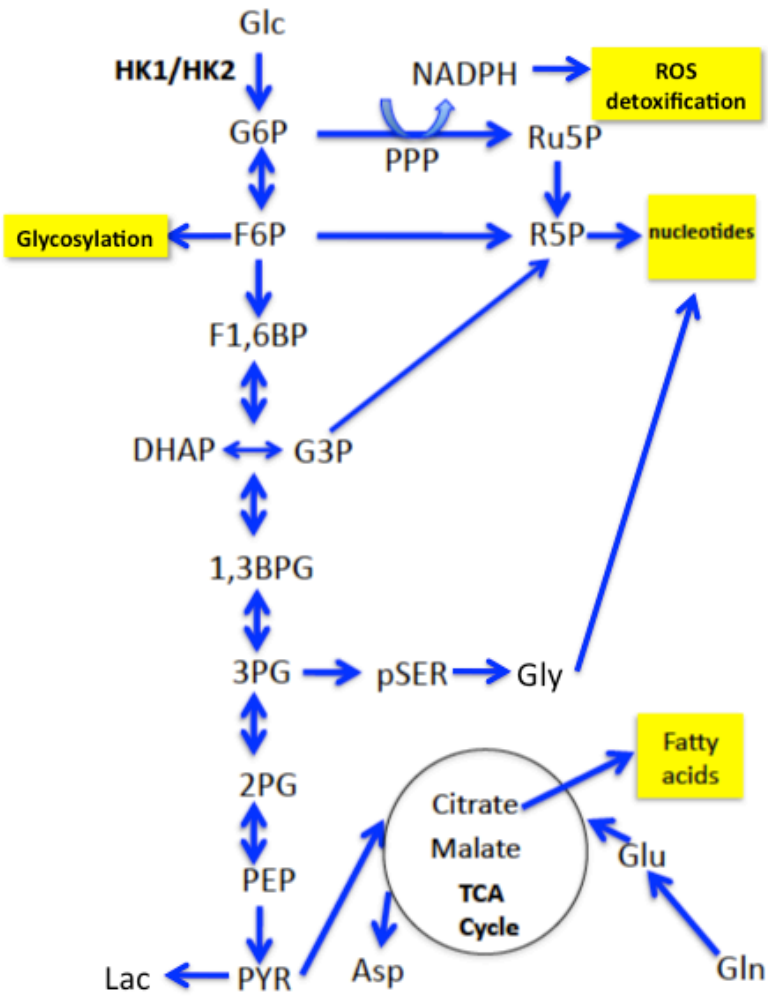
Living organisms need nutrients such as glucose and glutamine in order to maintain their metabolic homeostasis. These nutrients provide energy and support essential anabolic processes such as nucleic acid, protein, and lipid synthesis. In rapidly dividing cells there is a higher requirement of glucose and glutamine for generation of biosynthetic precursors to synthesize the building blocks that support anabolic processes (Lunt and Vander Heiden, 2011; Schulze and Harris, 2012). In normal conditions, most mammalian tissues utilize glucose as the main source of energy, with few exceptions, such as in heart and resting skeletal muscle, where fatty-acid oxidation provides ATP. When oxidized in the presence of oxygen (O_2), a single molecule of glucose generates 38 molecules of ATP. In this process, glycolysis produces 2 molecules of ATP and the remaining 36 are produced by oxidative phosphorylation. When oxygen availability is limited and oxidative phosphorylation is impaired, elevated glucose metabolism through glycolysis is the primary source of energy in the mammalian cell. In the 1950s, German scientist, Otto Warburg, reported that cancer cells exhibit a hyperactive glycolysis under aerobic conditions and is popularly known as the "Warburg effect" (Warburg, 1956). Initially it was believed that the glycolytic phenotype of cancer cells was because of the mitochondrial dysfunction, but careful observations revealed that most tumor cells have functional mitochondria, suggesting that the glycolytic phenotype is not simply an adaptation, but rather is an inherent property of tumor cells (Zu and Guppy, 2004). Not only cancer cells, but also proliferating normal cells such as lymphocytes (Wang et al., 1976) and thymocytes (Brand, 1985) exhibit this phenomena.

2) Glucose metabolism is one of the major sources for biosynthetic precursor synthesis.

If increased ATP production is not the main reason for hyperactive glucose metabolism then why do proliferating cells demonstrate such high preference for it? This question can be explained by the fact that glucose metabolism provides the metabolic precursors to synthesize proteins, nucleic acids, and lipids which support the biosynthetic demands of proliferation (Fig.1A). Glucose is transported into cells by a series of glucose transporters and is converted to glucose-6-phosphate (G-6P) by a committed phosphorylation event catalyzed by hexokinases (Wilson, 1995) (Hexokinases are discussed in detail in the next section). G-6P is the central molecule for many biosynthetic pathways and is subsequently routed to different pathways according to the requirements of the cell (Fig. 1B). For example, through several metabolic steps, glucose generates pyruvate, and in cancer cells, the majority of glucose is metabolized to form lactate. Pyruvate enters the mitochondria and is utilized in the TCA cycle that generates NADH for oxidative phosphorylation. Alternatively, G6-P can be converted to fructose-6-phosphate which is utilized in the hexosamine pathway that supports glycosylation of proteins (Lunt and Vander Heiden, 2011). Recently it was shown that glucose metabolism coordinates glutamine metabolism to support the glycosylation of growth factor receptors in cells (Wellen et al., 2010). G-6P can also be metabolized in the inositol synthesis pathway, which is not well studied in mammalian cells. The Pentose phosphate pathway (PPP) is initiated by G-6P through the enzyme Glucose-6-phosphate dehydrogenase (G6PDH). The PPP produces NADPH, which is required for glutathione regeneration. Glutathione is required for reactive oxygen species (ROS) detoxification inside cells, which can be resulted largely by hyperactive proliferation, as well as by metabolic stress (Jeon et al., 2012), which are two main characteristics of cancer. Therefore, glucose metabolism through the pentose

phosphate pathway in cancer cells remains an important mechanism of survival. In addition, NADPH is utilized in lipid synthesis (Vander Heiden et al., 2009). The pentose phosphate pathway can be operated by two modes, oxidative and non-oxidative. Only the oxidative mode generates NADPH, but both modes generate Ribose-5-Phosphate (R-5P) to synthesize ribonucleotides (Fig. 1A). Phosphoribosyl-α-pyrophosphate (PRPP), an activated form of ribose-5-phosphate that comes from glucose, contributes half of the purine nucleotide carbons. Two additional carbons of purine are donated from glycine, which is also generated by an intermediate of glucose metabolism. De-novo pyrimidine synthesis requires PRPP and aspartate, the later can be synthesized from oxaloacetate made in the TCA cycle, which is also supported by glucose metabolism. Therefore, glucose metabolism plays a significant role in both de-novo purine and pyrimidine synthesis (Lunt and Vander Heiden, 2011). Experiments using isotopomers of glucose demonstrated that serine and glycine are synthesized from 3-phosphoglycerate, a metabolic intermediate of glycolysis. Disruption of this pathway is detrimental for cancer cells underscoring the importance of this glucose-serine-glycine axis for cancer cells (Locasale et al., 2011; Possemato et al., 2011). Apart from these two amino acids, alanine can be generated from pyruvate, the end product of the glycolysis pathway. Glucose metabolism also supports de-novo lipid synthesis (Vander Heiden et al., 2009). Acetyl-CoA, the backbone of fatty acids is generated form citrate, which is generated from both glucose and glutamine metabolism. Using isotopomers of glucose it was shown that the glioblastoma tumors *in vivo* derive a majority of the citrate from glucose metabolism (Marin-Valencia et al., 2012). Thus, hyperactive glucose metabolism in cancer cells is required for NADPH and fatty acid synthesis that are used to generate sufficient lipids required for highly proliferative cancer cells. Hence, the major function of enhanced glucose metabolism is to provide macromolecular precursors for nucleic acid, protein, and lipid synthesis in proliferating cells.

Figure 1. The major metabolic pathways that support anabolic growth in proliferating cells. Schematic diagram of glucose and glutamine metabolism to generate precursors for many biosynthetic pathways. Abbreviations are: Glc (Glucose), G6P (Glucose 6-phosphate), F6P (Fructose 6-phosphate), F1, 6P(Fructose 1, 6-bisphosphate), DHAP (Dihydroxy acetone phosphate), G3P (Glyceraldehyde 3-phosphate), 1,3BPG (1,3 Bisphosphoglecerate), 3PG(3 Phosphoglecerate), 2PG(2 Phosphogecerate), PEP (Phospho-enol Pyruvate), Pyr (Pyruvate), Lac (Lactate), Asp (Aspartate), Gln (Glutamine), Glu (Glutamate), pSer (Phosphoserine), Gly (Glycine), R5P (Ribose-5-phosphate), Ru5P (Ribulose-5-phosphate), NADPH (Nicotianamide adenine diphosphate-reduced), TCA cycle(Tri-carboxylic acid cycle), PPP(Pentose phosphate pathway), HK1/HK2(Hexokinase-1/Hexokinase-2).


Glc
HK1/HK2
NADPH
ROS detoxification
G6P
PPP
Ru5P
Glycosylation
F6P
R5P
nucleotides
F1,6BP
DHAP
G3P
1,3BPG
3PG
pSER
Gly
Fatty acids
2PG
Citrate
Malate
TCA Cycle
Glu
PEP
Lac
PYR
Asp
Gln

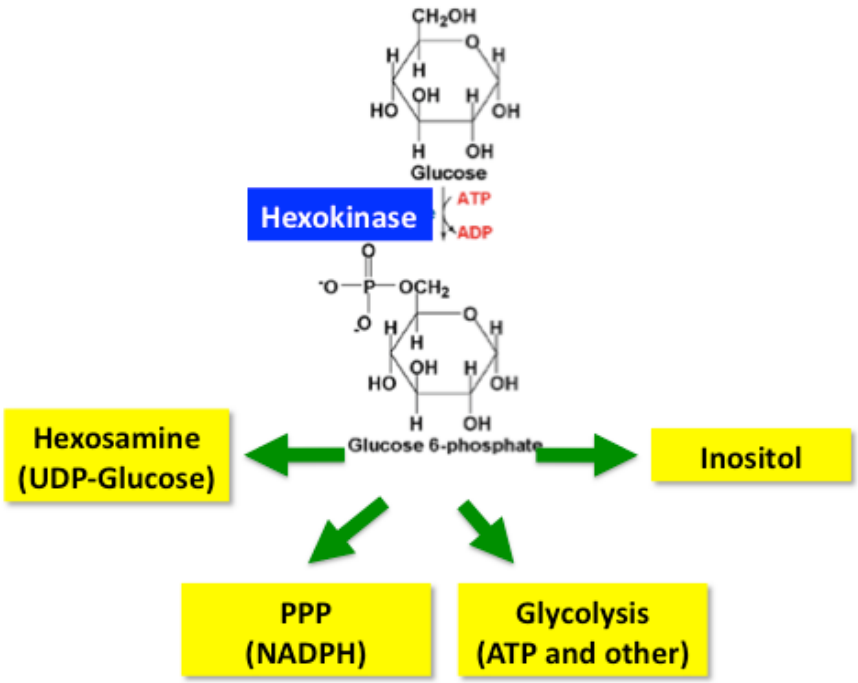
3) Hexokinases

The first committed step in glucose metabolism is the ATP dependent phosphorylation of glucose to glucose-6-phosphate (G-6P), which is catalyzed by Hexokinases (HKs). Hexokinases play a major role in the cellular uptake and utilization of glucose. By catalyzing the phosphorylation of Glc to G6P, hexokinases promote and sustain a concentration gradient that facilitates glucose entry into cells and the initiation of all major pathways of glucose utilization. Therefore hexokinases influence both the magnitude and the direction of glucose flux within cells(Robey and Hay, 2006). Four major hexokinase isoforms, encoded by separate genes, are expressed in mammalian tissues— denoted as HK1, HK2, HK3, and HK4 (also known as glucokinase)(Wilson, 2003). HK1, 2 and 3 share an extensive sequence similarity. The 100 kDa enzyme HK1, 2 and 3 are believed to be evolved from a gene encoding an ancestral 50 kDa HK by gene duplication and fusion forming two identical hemidomains called C-terminal and N-terminal. HK1, HK2, and HK3 are high affinity isoforms and are sensitive to a feedback inhibition by their principal reaction product, glucose-6-phosphate(Wilson, 2003). The C-terminal and N-terminal of HK2 influence each other by regulating the kinetic properties and feed back inhibition. The high affinity HK1 and HK2 bind to the outer surface of mitochondria by their N-terminus 15 amino acids. This mitochondrial binding properties has been implicated in maintaining the integrity of mitochondria and in cell survival by growth factors. HK3 has been reported to be inhibited in normal physiological concentrations of glucose. Interestingly the sub cellular localization of HK3 is found in nuclear periphery and the functional significance of this localization is unknown. HK4 or Glucokinase is a low affinity hexokinase and insensitive to product inhibition and play a significant role in systemic glucose homeostasis. Apart from their size and kinetic properties all these four isoforms have been shown to have different tissue distribution. HK1 is the ubiquitous hexokinase and present majority organs in the body where as its expression is the highest in the brain and kidney. Glucokinase is

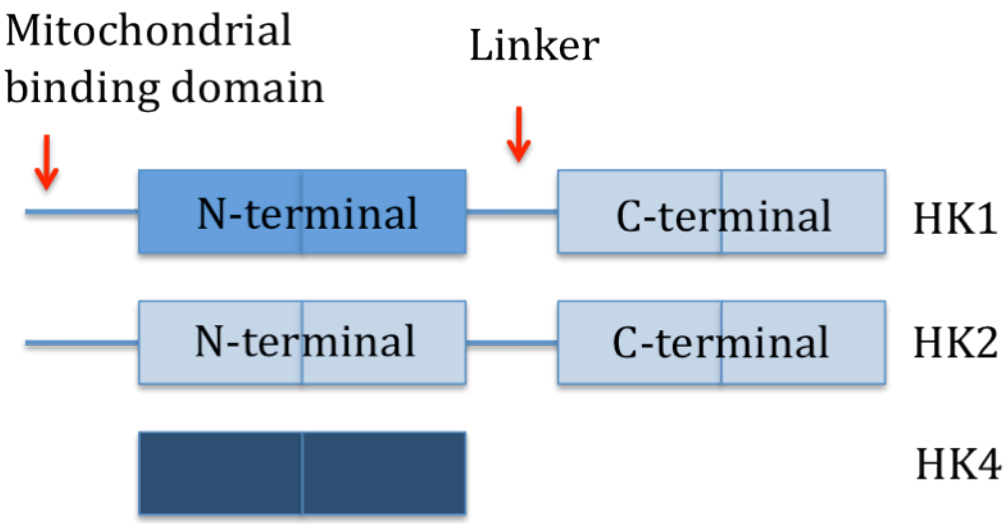
expressed in liver and pancreas serving as a “Glucose sensor”(Cardenas et al., 1998). HK3 has been shown to be an isoform of the myeloid lineage(Wilson, 1995). HK2 is abundantly expressed in embryonic tissues at relatively high levels, it is expressed only in limited number of adult tissues such as adipose, and skeletal and cardiac muscles (Wilson, 2003). However using mice with heterozygous knockout of HK2 and over-expression of HK2 it was shown that HK2 has an important role in exercise induced glucose uptake in skeletal muscle. Notably, it was reported that mice with 50% germ line deletion of HK2 do not have a marked change in fed glucose levels under normal chow diet (Heikkinen et al., 1999). These mice do display hyperinsulinemia during hyperinsulinemic-euglycemic clamp especially if combined with a high fat diet, and also display reduced endurance during exercise (Fueger et al., 2003).

Figure. 2 Glucose phosphorylation and hexokinases. (A) Glucose is converted to Glucose-6-phosphate by a transfer of a phosphate group from ATP by hexokinases. Glucose-6-phosphate utilized in many metabolic pathways. (B) Schematic diagram of HK1, HK2 and HK4. HK1 and two has two hemi domains (N-terminal and C-terminal) connected by a linker region. HK1 and HK2 bind to mitochondria by a hydrophobic stretch of 15 amino acid residues present in the N-terminal. In contrast HK4 consists of only one domain and does not bind to the mitochondria.

(A)


Glucose
Hexokinase
ATP
ADP
Glucose 6-phosphate
Hexosamine
(UDP-Glucose)
Inositol
PPP
(NADPH)
Glycolysis
(ATP and other)

(B)


Mitochondrial
binding domain
Linker
N-terminal
C-terminal
HK1
N-terminal
C-terminal
HK2
HK4

4) Hexokinase-2 and cancer

Hyperactive glycolysis observed in tumor cells is orchestrated by a series of glycolytic enzymes. Several alterations in these enzymes take place in their mRNA expression and post-translational modification level. The expression pattern of hexokinase isoenzymes has been reported to be altered in cancer cells(Mathupala et al., 2006). In the majority of cancer cells and tumors the expression of HK2 is elevated whereas the corresponding normal tissues do not express this isoform. Interestingly, there is an isoform-switching event that occurs in the human liver cancer cells. Normal adult liver expresses only glucokinase, but during HCC, glucokinase expression is suppressed and expression of HK2 is elevated (Mathupala et al., 2006). The higher expression of HK2 occurs at the transcriptional level, which was first demonstrated by comparing the rat liver and rat ascites hepatoma (Rempel et al., 1996). Later by functional analysis of the HK2 promoter in rat hepatoma cells it was reported that insulin, glucose, hypoxia, cAMP analogs are several modulators of HK2 promoter(Mathupala et al., 1995). Elevated expression of HK2 appears to be associated with many types of human cancer including HCC, NSCLC, osteosacrcoma, melanoma, breast carcinoma, leukemia, lymphoma, squmose cell carcinoma and glioblastoma. In the malignant cancer cells, expression of high level of HK2 (Mathupala et al., 2001; Shinohara et al., 1994) distinguishes them from the normal differentiated cells, and is, at least in part responsible for the accelerated glucose flux. The high level of HK2 expression and activity in glycolytic cancers is manifested by the use of positron emission tomography (PET) to visualize tumors. PET is used following injection of the labeled glucose analog, [^{18}F] fluoro-2-deoxyglucose (FDG), which is then taken up by glycolytic cancer cells and phosphorylated by hexokinase to form FDG-phosphate, which can be detected by PET. Tumor samples from breast cancer patients show a strong expression of such as glucose transporter-1 (Glut-1), HK2 (Brown et al., 2002) and that is used to classify different stages

of tumor with the help of PET imaging (Schirrmeister et al., 2001). Notably, investigations on drug resistance phenotypes of breast cancer cells showed that up regulated glucose metabolism, and especially HK2 expression are associated with the chemoresistance phenotypes in breast cancer cells (Kaplan et al., 1990; Komurov et al., 2012; Lyon et al., 1988). Therefore, HK2 may be a genetic modifier of drug resistance that can be therapeutically exploited.

MAPK and PI3K signaling, which are frequently activated in human cancers have been shown to up regulate HK2 expression (Osawa et al., 1996). c-Myc oncogene directly binds to the promoter and elevate the expression of HK2. HIF1 alpha the master regulator of hypoxic response, also increases the HK2 mRNA during hypoxia(Dang, 2007) .

If all isoforms of hexokinases have the capacity to phosphorylate glucose then why HK2 is over-expressed in cancer? From a metabolic standpoint, HK2 is the most efficient enzyme among other hexokinases for formation of G-6P, which is utilized in various anabolic pathways in cancer cells. (Mathupala et al., 2006). HK2 is one of the low affinity isoform of hexokinases. As two hemi-domains of HK2 are catalytically active, it was speculated that HK2 has the highest capacity to produce more G-6P compared to other hexokinases. Again by its high glucose phosphorylation capacity HK2 generates a concentration gradient of glucose, which helps to increase the magnitude of glucose flux into the cells. Like HK1, HK2 associates with the outer mitochondrial membrane (OMM) by binding to the porin channels. That's how HK2 couples the glycolysis to the oxidative phosphorylation by having a preferential access to the ATP generated in mitochondria(Robey and Hay, 2006). Binding of HK2 to the OMM protects the cells from growth-factor withdrawal and oxidative stress induced apoptosis(Majewski et al., 2004a; Sun et al., 2008). Hence over expression HK2, might help cancer cells to evade apoptosis. Therefore HK2 is selected over other isoforms of HK to be elevated in cancer.

5) Targeting metabolism for cancer therapy: Hexokinase 2 inhibition a rational therapeutic target.

The majority of current drugs for treatment of cancer are targeted against the pathways those are shown to be altered by mutational activation of oncogenes and inactivation of tumor suppressor genes. The traditional targeted chemotherapy is not always successful in patients because of many additional mutations develop during the evolution of the cancer or during the treatment, which ultimately leads to drug resistance. It is important to note that although all the oncogenic mutations are different in nature, but all of them rewire the metabolic pathways and they are dependent on them for proliferation (Locasale et al., 2009). Therefore targeting metabolic pathways holds a therapeutic potential to treat cancer. In fact one the first line of modern chemotherapy anti-folates, which were used against child acute lymphoblastic leukemia is a classical example of targeting metabolism for cancer therapy(Vander Heiden, 2011). Although expression of many metabolic enzymes has been altered in cancer and required for survival and proliferation, but it raises concern on normal body physiology if targeted for cancer therapy. Among many tumor-associated metabolic enzymes, HK2 is a suitable target because of the limited tissue distribution in adult. Several clinical trials have been done to inhibit glycolysis through 3-bromopyruvate and 2-DG, as potential hexokinase inhibitors in cancer. The glucose analogue 2DG when phosphorylated by hexokinase to 2DG-P, which accumulates in the cell and inhibits all isoforms of hexokinases like the natural product G6-P. Because of the unacceptable toxicity in humans or experimental animals, use of 2DG for cancer therapy was terminated (Cheong et al., 2012). As a pan hexokinase inhibitor, 2DG-6P it can inhibit HK1, which is constitutively expressed, and cause unwanted toxicity when administered systemically. Many studies showed that 2DG not only inhibits HK2 but also many other processes like glycosylation (Yu and Kim, 2010) or mTOR inhibition(Dennis et

al., 2001) or high level of AMPK activation(Inoki et al., 2003) which are implicated in many normal physiological processes. Therefore HK2 isoform specific approaches should be considered to target it as a cancer therapy.

Using RNAi against HK2, Wolf et al showed that glioblastoma tumors are dependent on HK2 *in vitro* and *in vivo*. Employing conditional knockout mice of HK2, a study demonstrated that HK2 ablation can delay tumor formation in autochthonous model of medulablastoma induced by sonic hedgehog activation(Gershon et al., 2013). But the developmental problems associated by HK2 ablation in non-tumor brain can be accounted for the phenotypes seen in that tumor model. Therefore a systematic study is required to address the following question before HK2 can be proven as a suitable target for cancer therapy. 1) If HK2 can inhibit tumor initiation developed during adult phase of an animal i.e. a situation generally seen in human. 2) Can inhibition of HK2 after tumor formation will have any effect on tumor progression? 3) Whether the effect of HK2 ablation on tumor initiation can be generalized to other kind of tumors. 4) What is the mechanism by which HK2 ablation inhibits tumorigenesis?

Addressing all these important questions pertinent to HK2 tumor biology, here we provide a comprehensive evidence of essential requirement of HK2 in the process of tumorigenesis. We showed that Hexokinase 2 (HK2) isoform is expressed at high level in cancer cells and only in limited number of normal adult tissues both in human and mice. Using HK2 conditional knockout mice, we showed that HK2 is required for both tumor initiation and progression in mouse models of K-Ras-driven non-small cell lung cancer (NSCLC), and ErbB2-driven breast cancer despite HK1 presence. Similarly HK2 ablation inhibits the neoplastic phenotype of human NSCLC and breast cancer cells *in vitro* and *in vivo*. Systemic deletion of HK2 is therapeutic in NSCLC mouse model without adverse physiological consequences. HK2 deletion in K-Ras^{G12D}-NSCLC cells diminished glucose-

derived ribonucleotides, phosphoserine, and fatty acids synthesis, and unexpectedly suppressed glutamine-derived carbon utilization in anaplerosis. Thus, HK2 is required for pivotal metabolic pathways in cancer cells. Importantly, these results provide a proof of concept that HK2 could be systemically ablated for cancer therapy.

CHAPTER-II

MATERIALS AND METHODS

1. Mice stains and husbandry

Hexokinase 2 conditional knock out ($HK2^{f/f}$) mice were generated as described in the text. The primers used to genotype $HK2^{f/f}$ are C667: CCCCTTCGCTTGCCATTAC, C668: TGTCTTGGCTCAGATGTGAC and 854: AACCACGACGCCCAATGATTTAG. PCR amplification of genomic DNA by C667 and C668 generates a product of 399bp for Flox/Flox allele and 344 for WT/WT allele. If recombination of loxP allele occurs by Cre recombinase then PCR amplification of genomic DNA by A854 and C667 generates a PCR product of around 800bp. K-Ras $^{LSL\text{-}G12D}$, and MMTV-Neu-IRES-Cre (MMTV-NIC) mice were described before (Jackson et al., 2001; Ranger et al., 2009). K-Ras$^{LA2\text{-}G12D}$ (strain # 01BM3) (Johnson et al., 2001) obtained from the NCI-Mouse Models of Human Cancer Consortium. Ubc-Cre-ERT2 (stock # 008085) MMTV-NeuT (stock # 005038), Albumin-Cre (stock#003574) mice were purchased from the Jackson laboratory. K-Ras $^{LSL\text{-}G12D}$ and $HK2^{f/f}$ mice were crossed to generate K-Ras $^{LSL\text{-}G12D}$;$HK2^{f/WT}$ mice and subsequent intercrossing was done to generate K-Ras $^{LSL\text{-}G12D}HK2^{f/f}$ and K-Ras$^{LSL\text{-}G12D}HK2^{WT/WT}$ strains. MMTV-NIC mice with FVB background were bred to $HK2^{f/f}$ mice, that were backcrossed to FVB background for 5 generations, to generate compound heterozygotes, that were then intercrossed to obtain MMTV-NIC;$HK2^{f/f}$ (HK2-NIC) or MMTV-NIC;$HK2^{+/+}$ (WT-NIC) mice. MMTV-NeuT;$HK2^{f/f}$ mice were generated by crossing $HK2^{f/f}$ and MMTV-NeuT strains, which were in FVB background. $HK2^{f/f}$, K-Ras$^{LA2\text{-}G12D}$ and UBC-Cre^{ERT2} mice were intercrossed to obtain following genotypes: $HK2^{f/f}$;UBC-Cre^{ERT2}, K-Ras$^{LA2\text{-}G12D}$;$HK2^{f/f}$, K-Ras$^{LA2\text{-}G12D}$; $HK2^{f/f}$;UBC-Cre^{ERT2}, and K-Ras$^{LA2\text{-}G12D}$;UBC-Cre^{ERT2}. HK2 f/f mice were crossed with Albumin-cre to achieve liver specific deletion of HK2. All the experiments on animals were approved by the University of Illinois at Chicago

Institutional Animal Care and Use Committee.

2. Lung tumor induction in K-Ras^{LSLG12D} mice

In 8-10 weeks old K-Ras$^{LSL-G12D}$ mice autochthonous lung tumors were induced by adenovirus Cre recombinase in as described previously (Weinberg et al., 2010). Briefly, the mice were anesthetized with xylazine-ketamine and intubated with a 20-gauge angiocath. With a Hamilton syringe, 5×10^7 pfu adenovirus in 50% bovine surfactant (Infrasurf; Forest Pharmaceuticals) balanced Tris-EDTA buffer was instilled through an angiocath. The total amount of virus was intubated in two equal aliquots 3-5 min apart. After intubation the animals were given pain killer (Buprenex) and monitored till their recovery from anesthesia and attain normal breathing. Any animal that became sick within one week of the procedure was discarded from the study. Sixteen weeks after intubation, the lungs were analyzed for tumors appearance. For survival curve experiments mice were monitored till their death or labored breathing attaining humane end-point criteria.

3. Liver tumor induction by DEN in mice

For liver cancer induction in mice, two weeks old male pups were given a single dose of diethylnitrosamine (DEN) at a dose of 25mg/kg. After 9 months the mice were sacrificed and the liver was analyzed for estimation of tumors. Tumors more that 0.1 mm in size was counted for analysis.

4. Xenograft assays

All male and female athymic nude mice (6–8 weeks old) were purchased from Charles River Laboratories. Ras transformed control cells, HK2 knockout cells, HK2 knockout cells expressing WT or kinase dead (SA) mutant of rat HK2 ($0.75\text{-}1.0\times10^6$ in 0.1

ml PBS) were injected subcutaneously into both the left and right flanks of each mouse. Cell suspensions of 6×10^6 MDA-MB-453 (control or HK2 knockdown) or of 1.5 x 10^6 MMTV-NeuT (Control or HK2 knockout) in 0.1ml of 1:1 PBS/matrigel were orthotopically injected into the 4th mammary fat pads (left and right) of female mice. For doxycycline-inducible experiments, NCI-H460 cells (0.75×10^6 in 0.1 ml of PBS) expressing doxycycline inducible shRNA constructs were subcutaneously injected into male nude mice. Once tumors were reached ~ 65 mm^3 volume, the mice were randomly assigned into different groups and fed with regular chow or doxycycline chow (200mg/kg: Bioserve) as explained in the text. The tumor size was measured weekly or bi-weekly, and the volume was calculated as V (in mm^3) = a×bxc, where a is the longest diameter, b is the shortest diameter and c is the height. When the tumor size reached the end-point criterion (a diameter greater than 2 cm), the mice were sacrificed.

5. Histopathology and Immunohistochemistry

For histopathology analyses, tissues were collected and quickly fixed with 10% buffered formalin overnight and preserved with 70% ethanol. Paraffin embedded tissues were processed and 5-micrometer slides were prepared for hematoxilin and eosin (H&E) or immunostaining. For lung tumors analyses all the lung lobes were separated and total tumor burden was calculated from H&E stained slides using aperio image scope software, and by calculating ratio of tumor area to total lung area. For immunostaining, antigen retrieval was done with 10mM citrate buffer (pH 6.0) boiling at 95^0 C. Detection was done by ABC-DAB kits (vector labs). Anti-BrdU mouse monoclonal (Dako# M0744), anti-HK2 rabbit monoclonal (cell signaling # 2867) were used for immunostaining. Tissue microarrays were purchased from US Biomax Inc.

6. Measurements of BrdU labeling in tumor sections

For BrdU labeling experiments, mice bearing xenograft tumors were injected intraperetonealy with BrdU (sigma) in PBS was injected (50mg/kg), 2 hrs prior to tissues collection (Nogueira et al., 2008). Mice with autochthonous lung tumors were injected with BrdU (30 mg/kg) 24 h prior to sacrifice (Oliver et al., 2010). After immunostaining, BrdU positive cells in the highest staining area of the tumors were counted as previously described (Chen et al., 2006). At least 6-10 tumor section fields from 3 different mice with the same genotype were taken for the quantification.

7. Tamoxifen treatment

Unless otherwise stated animals were injected with tamoxifen at 8-9 weeks age to recombine HK2 allele using $UBC^{CreERT2}$ system. Tamoxifen (sigma) was dissolved in corn oil and 5 doses (0.5ug/gm b.w) were given in every alternate day as previously described (Ruzankina et al., 2007).

8. Cell lines

NCI-H460, NCI-H1299, NCI-H727, NCI-H358, T47D, MDA-MB-453, BT474, LA2-1B, and LA2-2 cells were grown in RPMI-1640/10% FBS/1% pen-strep media. MCF-10A cells were maintained in DMEM/F12 with the supplements. MDA-MB-231, 293FT, phoenix-ecotropic cells, and Mouse embryonic fibroblasts (MEFs) were maintained in DMEM/10% FBS/1% pen-strep media. All the cell lines were grown at 37 ^{0}C, 5% CO_2 incubator unless otherwise mentioned.

9. Retrovirus and lentivirus production and infection

For stable shRNA expression, either pLenti6 or pLKO-puro retroviral vectors were used. Sequences of shRNAs to target HK2 used in these studies are : HK2 shRNA1: 5'-GCATATGATCGCCTGCTTATT -3',HK2 shRNA2: 5'-GCAGAAGGTTGACCAGTATCT-3', and HK2 shRNA3: 5'-CCAAAGACATCTCAGACATTG-3'. shRNA1 targets mouse and shRNA2 and 3 target human HK2. The oligonucleotides were cloned into pLenti6 lentiviral vector using BLOCK-iT Lentiviral RNAi Expression System (Invitrogen). The pLenti6 expressing ShRNA3 was chosen for all subsequent studies. In some cases the pLKO.1 retroviral vector containing shRNA3 was used. Oligonucleotides for shRNA3 or scrambled oligonucleotides were cloned into Tet-pLKO-puro (adgene plasmid# 21915) for the inducible shRNA system. Rat HK2 WT or kinase dead (SA: S155A/S603A) (Tsai and Wilson, 1996) mutant were expressed from pLenti-D-TOPO vector (Invitrogen). Ecotropic and amphotropic retrovirus production was done as previously described (Skeen et al., 2006). Lentiviruses were made in 293FT cells using virapower lentiviral system (invitrogen) according to manufacturer's protocol. Viruses were collected 48-60 hr after transfection and overnight infection was done to target cells (in a 6cm plate) using polybrene (8ug/ml). Cells were selected using 10ug/ml of blasticidin or 1ug/ml of puromycin for 4-6 days using a mock infection plate as reference. Cells were expanded for two passages in drug free media and frozen down for subsequent use. Early passage cells were used for every experiment.

10. Isolation and establishment of cell lines from mice

HK2 flox/flox ($HK2^{f/f}$) mouse embryonic fibroblasts (MEFs) were isolated from E13.5 day embryos. Primary MEFs were immortalized by pBabe-puro-GSE56 expressing retroviruses expressing dominant negative p53 (DN-p53). Stable polyclonal cell lines were subjected to oncogenic transformation by infection with pBabe-Hygro retroviruses

expressing oncogenic H-RasV12 as was previously described (Skeen et al., 2006). HK2 knockout and control cells were generated by infecting MEFs with adeno-virus expressing Cre or LacZ respectively. For the isolation of MMTV-NeuT mammary tumor cells, the tumors were surgically removed and digested with collagenase (0.1 g collagenase, Sigma C5138) prepared in DMEM at 37^0 C. Digested tumors were washed 3 times with PBS to remove collagenase. The final cell pellets were resuspended in DMEM/F12 and subjected to three differential centrifugations (pulse to 1500 rpm in 10 ml DMEM/F12) to remove single cells and fibroblasts. The cell pellets were then suspended in DMEM/F12 containing 10% FBS, and cells were grown at standard tissue culture condition. Stable polyclonal tumor cell lines were expanded for one to two generations and frozen down for future use. Early passage cells were used to generate NeuT-HK2 F/F or NeuT-HK2 knockout (KO) by a similar procedure using adenovirus Cre as described for MEFs. For isolation of murine lung cancer cells, tumors from $HK2^{f/f}K\text{-}Ras^{LA2\text{-}G12D}$ mice were digested with collagenase as described above cells, and then cultured in RPMI 1640 with 10% FBS to establish stable cell lines. Stable primary tumor cell lines were expanded for one to two generations and frozen down for future use. Since these cells were growth arrested after a few passages, two primary conditional HK2 knockout cell lines (LA2-1B and LA2-2) were immortalized with DN-p53 for long-term culture experiments. HK2 knockout lung cancer cells were generated by a similar procedure using adenovirus Cre as described for MEFs.

11. Anchorage independent growth assay

Anchorage-independent growth was performed as previously described (Skeen et al., 2006). In brief, cells ($10\text{-}25 \times 10^3$) were re-suspended in 10% FBS in respective culture medium containing 0.35% agarose and plated onto a layer of 0.7% agarose-containing medium in a 6-well dish. Soft agar colonies from the entire well were counted after 6 weeks

for transformed MEFs and 3 weeks for human cancer cells. Experiments were performed two to three times in triplicates.

12. Proliferation assays

Proliferation assays were performed by plating cells at low density (25×10^3 cells per 6 cm plate or as stated in the text). Required growth media was changed in every three days. To determine the proliferation rate, cells were trypsinized with 0.05% trypsin at the time points indicated, re-suspended in 1 ml of media and counted using a hemocytometer. Cell number for each time point was determined in triplicate and expressed with ± SD.

13.Immunoblotting

Protein extraction was done in ice-cold lysis buffer (20 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 5 mM iodoacetic acid, 20 nM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail [Roche Diagnostics]). Tissue extracts were done in the same buffer with a tissue homogenizer after thawing frozen tissues collected by LN2 snap freezing.

14. Glucose consumption assay

Glucose consumption assay was done with a glucometer by measuring decrease in glucose in spent media. Briefly cells were grown subconfluently in a 6 cm plate. After 18 hours of growth the spent media was diluted and analyzed for glucose concentration. By comparing a mock plate with same media and without cells and subsequently normalizing to cell number, amount of glucose consumed were determined.

15. Hexokinase activity assay

Hexokinase activity assay was done using a standard glucose-6-phosphate dehydrogenase-coupled spectrophotometric assay (Gottlob et al., 2001) with minor modifications. Briefly, 24 hour after seeding whole cell lysates were prepared in 45 mM Tris-Cl, pH 8.2, 50 mM KH2PO4, 10 mM glucose, 11.1 mM monothioglycerol, 0.5 mM EDTA, 0.2% Triton X-100. The samples were incubated on ice for 1 hour with intermittent vertexing in every 10 mins to break the cells. To determine hexokinase activity, 20 μL of the lysates were added to 220 μL assay mix (final concentrations were 41.7 mM Tris-Cl, pH 8.5, 7.7 mM MgCl2, 4.2 mM glucose, 10.6 mM monothioglycerol, 0.5 mg/ml NADP, 6.7 mM ATP, 1 mM NaPO4, 45 mM KCl, 0.5 mM EDTA, 0.05% Triton X-100). The initial OD (0 minute) was taken at $\lambda = 340$ nm. After addition of 1 U/ml glucose-6-phosphate-dehydrogenase the OD was measured every 2.5 minutes seconds for 10 minutes. The increase in OD reflects the increase in NADPH concentration, and the total hexokinase activity was calculated from the slope of the resulting curve in the log phase. HK activities were normalized to the protein content of the lysate.

16. Metabolites extraction and analysis

Cells were plated 24-hours prior to labeling at 60% confluence in a 6-well dish using a minimal RPMI media (RPMI1640 Base Media no amino acids or sugars present, supplemented with 3% dialyzed FBS, 5mM glucose, 2mM glutamine, essential BME amino acids and non-essential amino acids). The next day media was replaced with minimal RPMI media and the appropriately labeled metabolite. Labeling was stopped by aspirating media, washing three times with freshly made 75mM ammonium carbonate buffer pH 7.4, and immediately adding hot 70% ethanol to extract metabolites. Ethanol extract was transferred to a microcentrifuge tube and centrifuged at max speed (~16k x G) at 4°C for 10

min. Subsequently, supernatant was transferred to a 96-well plate and dried down to for eventual LCMS analysis or and flow injection-time-of-flight mass spectrometry analysis.

17. ^{14}C Glucose Incorporation into DNA

Sub confluent proliferating cells growing in a 6 well plate were treated with full media containing 4 μCi of U-^{14}C glucose or 1-^{14}C glucose. 24 hrs after cells were harvested and DNA isolation was done by a QIAGEN kit according to the manufacturers protocol and concentration was measured by a NanoDrop. Equal volume of DNA was subjected to liquid scintillation counting. Radioactive counts were normalized to the DNA concentration. Data was expressed to the percentage radioactive count of KO cells to the WT cells.

18. Statistical analysis

Statistical analysis was done with unpaired Student's t-tests, Survival curves were analyzed by log-rank test (Mantel-Cox) and data are expressed as SD or SEM as indicated in the figure legend. Unless otherwise indicated all the experiments were performed for at least three times.

CHAPTER-III

RESULTS

(1) The expression of hexokinase 2 is induced in human cancer tissues and cells and is required for tumorigenesity of the human cancer cells.

A) Background

Hexokinases catalyze the 1st committed step in glucose metabolism i.e. conversion of glucose to glucose-6-phosphate (G6P). Mammalian cells express four major isoforms of hexokinases such as HK1, 2, 3 and 4. The different isoforms have a different tissue distribution in mammals. HK1 is constitutively expressed in most mammalian adult tissues with its highest expression in brain. HK3 is reported to be expressed in some specific cells of hematopoietic system. HK4, also known as glucokinase is expressed in insulin responsive tissues like liver and pancreas. HK2, however, is expressed at high level in embryonic tissues and only in limited number of adult tissues such as adipose, skeletal muscles and heart(Wilson, 2003). However, it was reported that many rodent and human cancer cells express high level of HK2, which distinguishes them from the normal cells and which is, at least in part responsible for the accelerated glucose flux. The high level of HK2 expression and activity in glycolytic cancers is manifested by the use of positron emission tomography (PET) to visualize tumors(Vander Heiden et al., 2009). As HK2 is a HIF1 target gene; hypoxic tumor regions are expected to express HK2. Several studies have correlated HK2 expression with the tumor pathophysiology (Oshida et al., 1998; Osthus et al., 2000; Palmieri et al., 2009). In our study we investigated human tumor tissue samples from lung, breast and liver for expression of HK2 isoform and its correlation to cancer. We found the expression of HK2 is significantly elevated in the malignant lesions as compared to the adjacent normal tissues. Therefore we depleted HK2 by shRNA and demonstrated that the inhibition of HK2

expression dramatically inhibit tumorigenic potential of human cancer cells *in vitro* and *in vivo*. Moreover employing an inducible knockdown system for HK2, we showed the important role of HK2 in tumor progression of human NSCLC cells *in vivo.*

B) Results

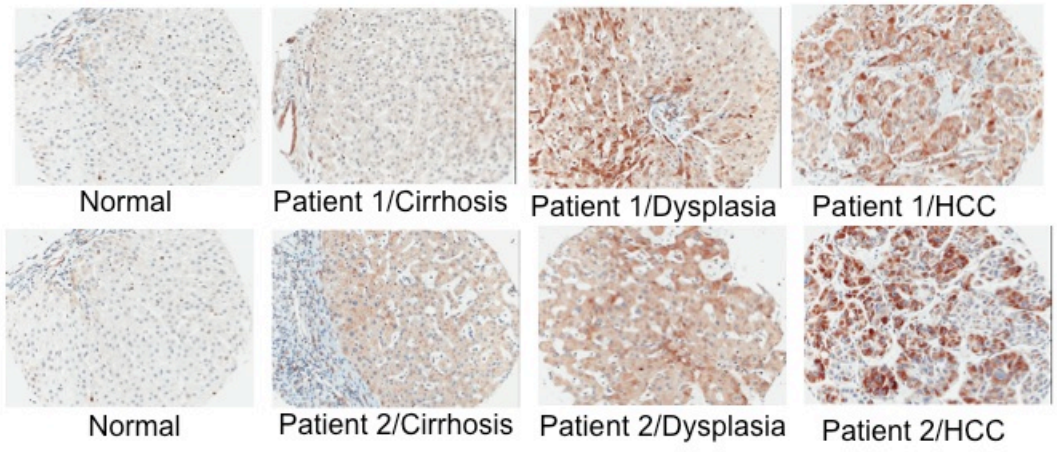
1. HK2 is specifically expressed in human cancer tissues.

In order to analyze the expression of HK2 in primary human tumor samples, we used tissue microarrays (TMAs). We performed immunohistochemical staining for human lung cancer patients with HK2 specific antibody, which was validated for human IHC by the supplier. Immunohistochemical staining showed that HK2 expression is markedly and significantly elevated in tumor samples human hepatocellular carcinoma (HCC) as compared to the normal liver (Fig. 3A). Cirrhosis and dysplasia are pre-clinical stages of HCC in human. HK2 expression pattern is increased in these two pathological stages as well. Although HK2 expression was not detected in normal human liver, interestingly the protein level , as a measured by the intensities of the staining is well correlated with advancement of the disease i.e. cirrhosis to dysplasia and HCC (Fig. 3B). Chronic HCV infection is one of the major causes of HCC in human. A clinical dataset of human HCC caused by HCV infection (Wurmbach et al., 2007)(deposited in Oncomine) reveled that the mRNA level of HK2 is significantly high in cirrhosis, dysplasia and HCC (Fig. 3C). Therefore we conclude that HK2 expression is positively correlated with the pathological stage of human HCC. Like normal liver normal human lungs also do not express the HK2 isoform. Therefore we were interested to verify the HK2 status in human Non-small cell lung cancer (NSCLC) tissue samples a human TMA. As shown in fig. 4A, the HK2 expression is markedly high in NSCLC as compared to adjacent normal lung tissue. Further analysis of the same TMAs that contained samples of various grades of NSCLC revealed that the higher HK2 expression level is positively correlated with the pathological grade of the tumors (Fig. 4B). To further

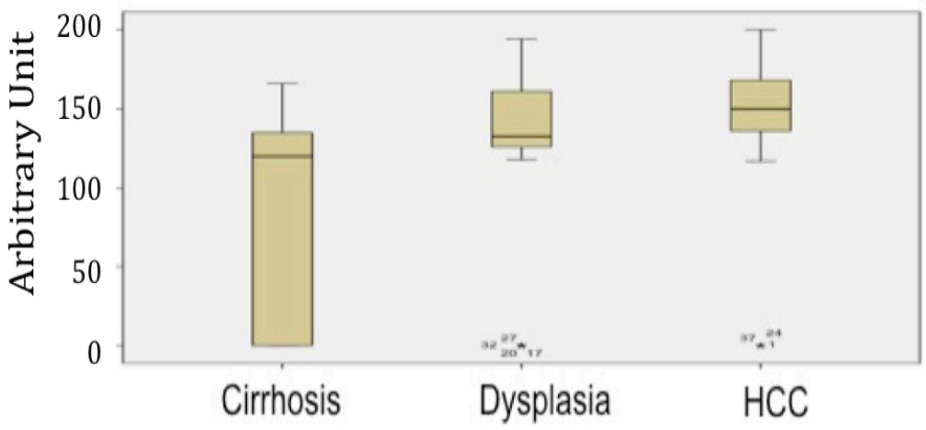
assess the prognostic significance of high HK2 expression in human patients, we analyzed a previously generated microarray dataset comprising a large number of human NSCLC patients(Shedden et al., 2008). We found that high HK2 expression is significantly (p=0.0025) associated with poor prognosis of these individuals (Fig. 4C). As in NSCLC, similar analysis was done for human breast cancer tissue microarrays. HK2 staining is barely detectable in the adjacent stromal tissues isolated from the breast cancer patients, where as the expression is markedly high in cancer tissues (Fig. 5A). Further, comparison of HK2 expression with respect to the different stages of the breast cancer showed that higher HK2 expression level is positively correlated with the pathological stage of the breast tumors (Fig. 5B). Again the analysis of an available well characterized human breast cancer dataset(van de Vijver et al., 2002) showed that high HK2 expression is significantly (p=0.02) associated with high mortality of the patients (Fig. 5C). These experiments and analysis documents that HK2 expression is significantly correlated with the pathophysiology of the human cancers.

Figure. 3 The expression of HK2 is elevated in Human Hepatocellular Carcinoma.
(A) Immunohistochemical staining of HK2 expression in representative human HCC patient samples tissue microarray (TMA) correlates with the pathological grade of the disease. (B) Quantification of HK2 staining intensity in accordance with the pathological grade. A total of 312 TMA samples collected from 151 patients. ($p < 0.01$). The images were generated by Dr. Grace Guzman and Dr. Pei-Zang Xu. (C) The mRNA expression (Log2-median centered intensity) of HK2 in HCV-induced HCC (Wurmbach Liver dataset deposited in Oncomine).

(A)


Normal
Patient 1/Cirrhosis
Patient 1/Dysplasia
Patient 1/HCC
Normal
Patient 2/Cirrhosis
Patient 2/Dysplasia
Patient 2/HCC

(B)


Arbitrary Unit
200
150
100
50
0
Cirrhosis
Dysplasia
HCC

(C)

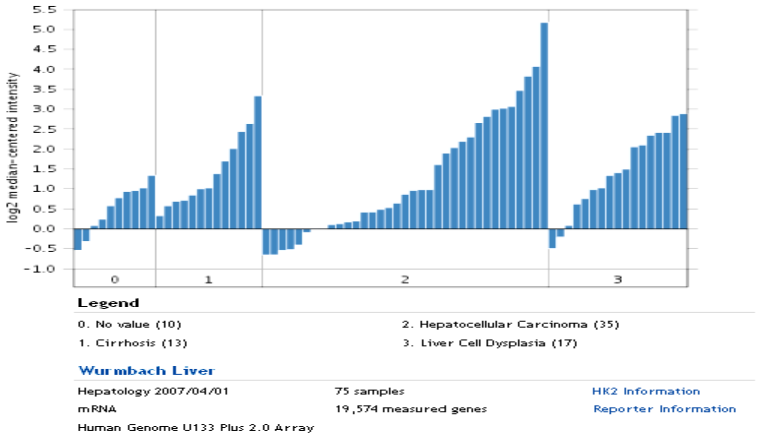
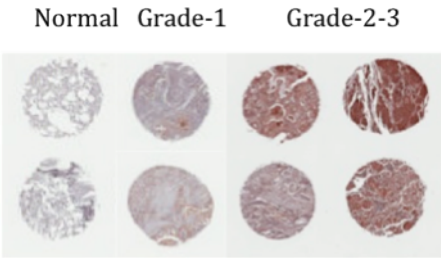
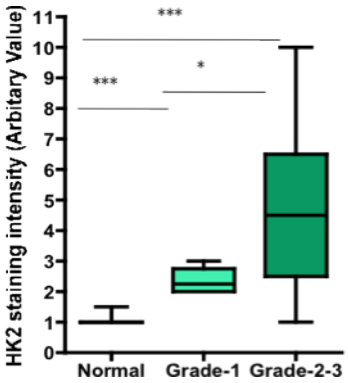

log2 median-centered intensity
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0
1
2
3
Legend
0. No value (10)
1. Cirrhosis (13)
2. Hepatocellular Carcinoma (35)
3. Liver Cell Dysplasia (17)
Wurmbach Liver
Hepatology 2007/04/01
mRNA
Human Genome U133 Plus 2.0 Array
75 samples
19,574 measured genes
HK2 Information
Reporter Information

Figure 4 The expression of HK2 is elevated in Human Non-small cell carcinoma (NSCLC) and correlated with the aggressiveness of the disease. (A) Immunohistochemical staining of HK2 expression in representative human NSCLC patient samples tissue microarray (TMA) correlates with the pathological grade of the disease. (B) Quantification of HK2 staining intensity in accordance with the pathological grade. TMA samples analyzed are: 20 non-tumor, 22 Grade-1, 44 grade-2, and 14 Grade-3. (C) Correlation between HK2 expression level and lung cancer patients survival. Kaplan-Meier survival analysis of patient cohorts analyzed in "Directors challenge dataset"(Shedden et al., 2008). Based on median expression of HK2 patients were separated into High HK2 (above-median: Blue line) and Low HK2 (below median: Black line) expression. Censored events were denoted by circles.

(A)


Normal
Grade-1
Grade-2-3

(B)


HK2 staining intensity (Arbitary Value)
11
10
9
8
7
6
5
4
3
2
1
0
***
***
*
Normal
Grade-1
Grade-2-3

(C)

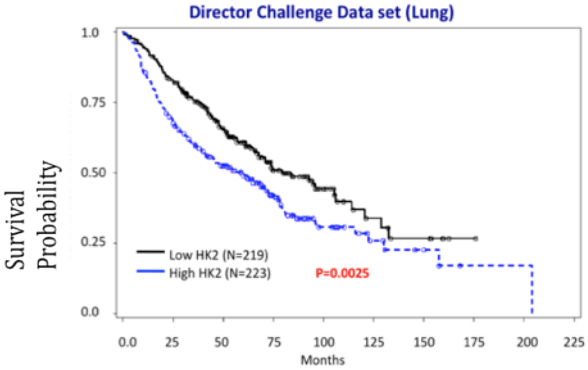
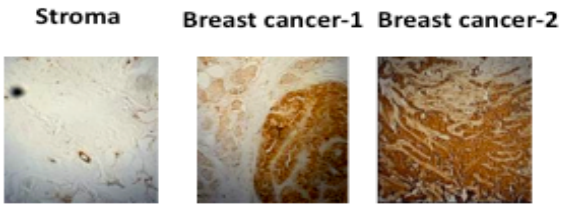
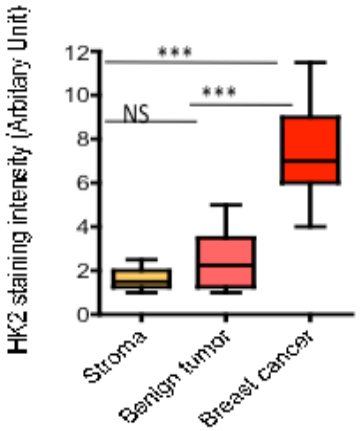

Director Challenge Data set (Lung)
Survival Probability
1.0
0.75
0.50
0.25
0.0
Low HK2 (N=219)
High HK2 (N=223)
P=0.0025
0.0
25
50
75
100
125
150
175
200
225
Months

Figure. 5 The expression of HK2 is elevated in Human breast cancer and correlated with the aggressiveness of the disease. (A) Representative images of immunohistochemical staining of HK2 in human breast cancer samples TMA, showing low expression in the stroma and benign tumors and high expression in cancer samples. (B) Quantification of HK2 staining intensity in the stroma, benign tumors and cancer samples. (Stroma (n=4, Benign (n=8), Tumor (n=15). (C) Correlation between HK2 expression level and breast cancer patient survival. Kaplan-Meier survival analysis of patient cohorts analyzed in "Van de Vijver dataset"(van de Vijver et al., 2002). Based on median expression of HK2 patients were separated into High HK2 (above-median: Blue line) and Low HK2 (below-median: Black line) expression. Censored events were denoted by circles.

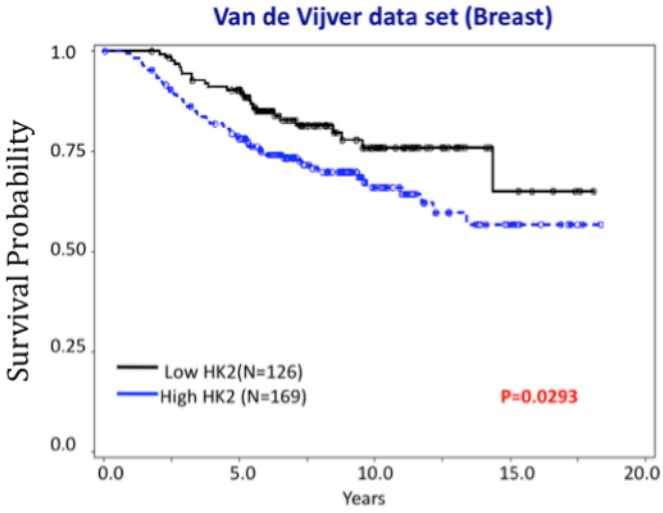
(A)


Stroma
Breast cancer-1
Breast cancer-2

(B)


HK2 staining intensity (Arbitary Unit)
12
10
8
6
4
2
0
***
***
NS
Stroma
Benign tumor
Breast cancer

(C)


Van de Vijver data set (Breast)
Survival Probability
1.0
0.75
0.50
0.25
0.0
Low HK2(N=126)
High HK2 (N=169)
P=0.0293
0.0
5.0
10.0
15.0
20.0
Years

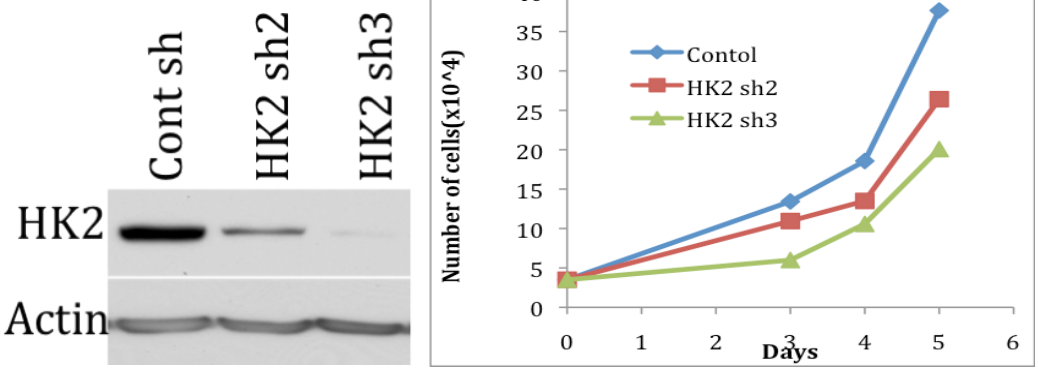
2. Stable shRNA mediated knockdown of HK2 attenuates the tumorigenicity of human cancer cells

2.1 Design and validation of HK2 specific shRNA in human cancer cells.

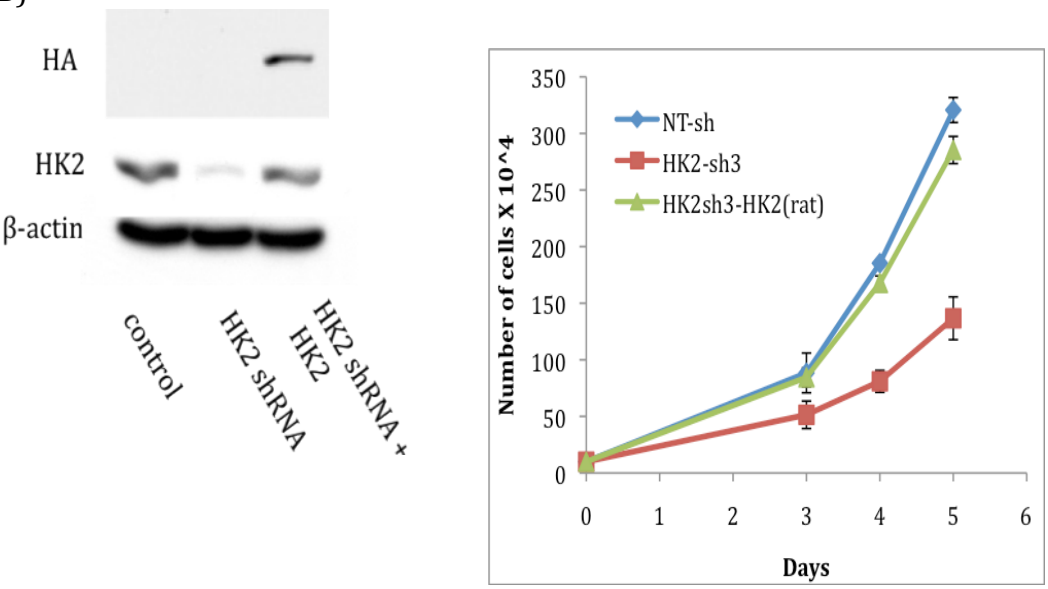
Similar to the HK2 expression in human cancer tissues (Fig. 3-5), I found that HK2 is over-expressed in cancer cell lines established from human or mice tumors. In order to study what is the effect of HK2 attenuation in human cancer cell lines we established a lentiviral stable shRNA knockdown system for HK2. Therefore we designed and cloned two shRNA sequences (named as shRNA2 and shRNA3) into pLenti6-Blast (invitrogen) lentiviral shRNA vector. First the knockdown efficiency of the HK2 shRNAs was verified through western blot in MDA-MB-231 human breast cancer cell line, which expresses a very high level of HK2 protein. A vector expressing shRNA against LacZ was used as a control. We observed that shRNA3 has a better knockdown efficiency than shRNA2 (Fig. 6A). Stable knockdown of HK2 significantly affected the proliferation of MDA-MB-231 cells and the defect in proliferation is correlated to the knockdown efficiency of these two shRNAs (Fig. 6A). Therefore shRNA3 was selected and used in future experiments. In order to rule out the possibilities that the growth disadvantages conferred by HK2 shRNA3 is specific and not due to any off target effect we used a “rescue approach”. Therefore we cloned and expressed the shRNA3 in pLKO-Puro lentiviral vector in NCI-H460 cells human lung cancer cells (Fig. 6B). As expected HK2shRNA3 also attenuated the proliferation of NCI-H460 cells as compared to the control shRNA. Expression of Hemagglutinin (HA) tagged Rat HK2 cDNA (rHK2-HA), which is resistant to shRNA3, through plenti6-blast (invitrogen) expression system rescued the proliferation defect in HK2shRNA3 expressing cells (Fig. 6B). Therefore we concluded that shRNA3 sequence is potent and specific for HK2.

Figure. 6 Validation of HK2 specific shRNA in human cells. (A) Left panel: Immunoblot showing the level of HK2 protein expression in MDA-MB- 231 cells stably expressing LacZ shRNA (as control) or two independent shRNA targeting HK2 (HK2 sh2 and HK2 sh3) The sequences of the oligos are shown in Experimental Procedures. Right panel: Proliferation of MDA-MB-231 after stable HK2 knockdown by two shRNAs against HK2. Proliferation rate of either cells expressing control shRNA or cells expressing HK2 shRNA was determined by seeding equal number of cells followed by counting cell numbers at the indicated time points. B) Left panel: Immunoblot of protein extracts from the indicated cell lines showing expression of HK2. Immunobloting with anti-HA antibodies shows the expression of HA-tagged rat HK2. Right panel: Expression of HA-tagged rat HK2 (resistant to shRNA3) in HK2 knockdown NCI-H460 cells restores the proliferation defects in HK2 knockdown cells. Equal number of indicated cell lines plated in 6 cm plate. Cell numbers were counted 3 days after seeding and data expressed ± SEM.

(A)


Cont sh
HK2 sh2
HK2 sh3
HK2
Actin
Number of cells(x10^4)
Contol
HK2 sh2
HK2 sh3
Days

(B)


HA
HK2
β-actin
control
HK2 shRNA
HK2
HK2 shRNA +
Number of cells X 10^4
NT-sh
HK2-sh3
HK2sh3-HK2(rat)
Days

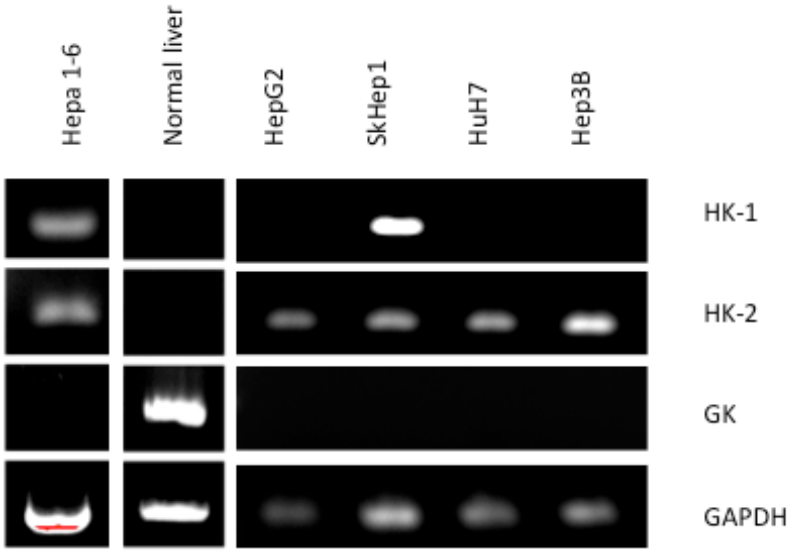
2.2 Expression of stable HK2shRNA affects the tumorigenicity of human cancer cells *in vitro*.

2.2.1Effect on Human Hepatocellular Carcinoma (HCC) cell lines

Liver is one of the most important organs for systemic glucose homeostasis. Normal adult human and mouse liver expresses the low affinity hexokinase, glucokinase. We first analyzed different HK isoforms expression in one murine and several human and HCC cell lines through semi-quantitative reverse transcriptase (RT) PCR. Due to unavailability of a normal human liver hepatocyte cell line, we used mouse liver as a positive control for our experiment. Interestingly all mouse and human HCC cell lines express HK2 whereas the normal murine liver does not express HK2 (Fig. 7A). In contrast to normal liver, which expresses GCK, the HCC cell lines do not express GCK (Fig. 7A). Although the mouse HCC cell line Hepa 1-6 expresses HK1 isoform but none of the human HCC cell liness or normal liver express this isoform. In our analysis we found Sk-Hep1 cell line express HK1 but from the literature we found out Sk-Hep1 is not a true HCC cell line rather it's a cell line of endothelial origin. Therefore we silenced HK2 expression in the HCC cell lines HepG2, Huh7 and Hep3B by HK2shRNA targeting human HK2 (Fig. 7B). When compared to the control knockdown cells, HK2 knockdown cells were significantly impaired in proliferation (Fig. 7C). The anchorage independent growth of these cells was dramatically impaired by the knock down of HK2 (Fig. 7D). Since, Hepa 1-6 is a mouse cell line we employed a different HK2shRNA for the mouse HK2. Surprisingly, HK2 knockdown impaired the tumorigenic potential of these cells despite the presence of HK1, suggesting that HK1 cannot compensate for the loss of HK2 (Fig. 7C). Therefore, HK2 plays a critical role in the proliferation and the tumorigenesisty of HCC cell lines *in vitro*.

Figure. 7 HK2 is required for tumorigenic potential Hepatocellular carcinoma cell lines. (A) Semi-quantitative RT-PCR profile of HK2 isoforms in human and murine HCC cell lines. Adult mouse liver was used as normal liver. (B) Immunoblot of HK2 knockdown in HCC cell lines. HK2 shRNA2 was used for the human cells. For Hepa 1-6 cells HK2 shRNA1 targeting mouse HK2 was used.

(A)


Hepa 1-6
Normal liver
HepG2
SkHep1
HuH7
Hep3B
HK-1
HK-2
GK
GAPDH

(B)

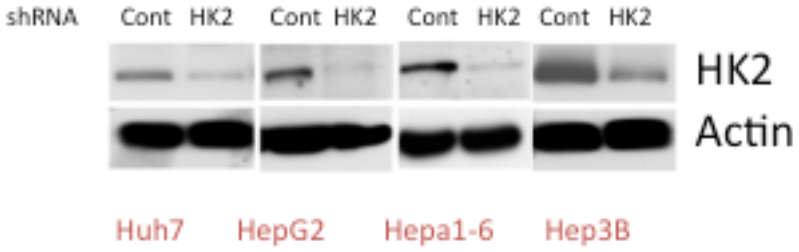
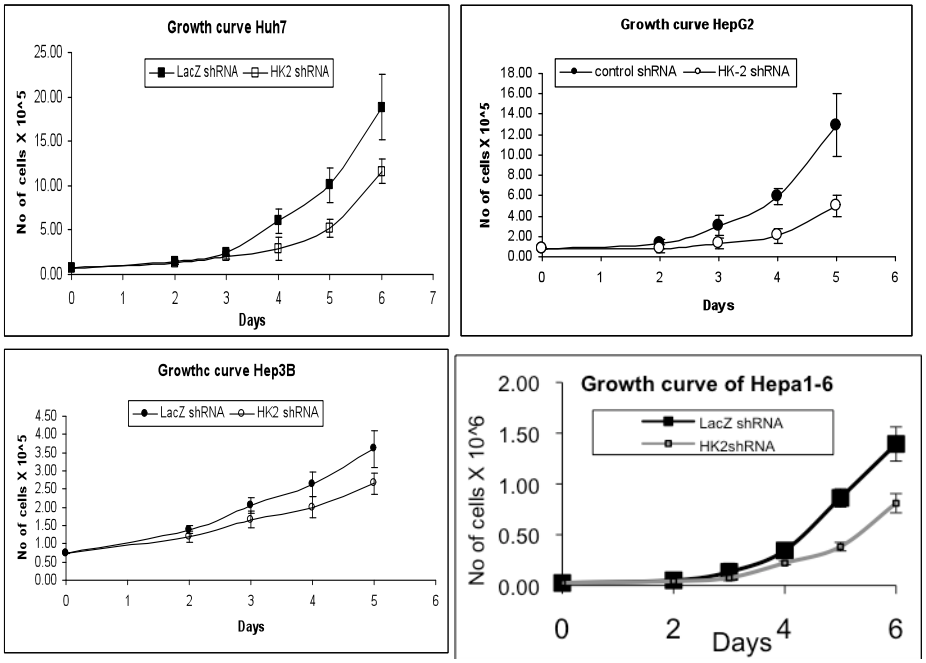
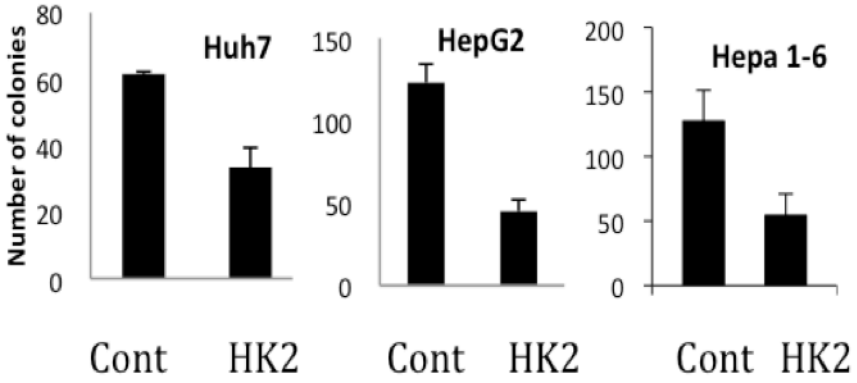

shRNA
Cont HK2 Cont HK2 Cont HK2 Cont HK2
HK2
Actin
Huh7
HepG2
Hepa1-6
Hep3B

Figure. 7 (continued) HK2 is required for tumorigenic potential Hepato cellular carcinoma cell lines. (C) HK2 knockdown attenuates the proliferation of human and mouse HCC cells *in vitro*. Equal number of control and HK2 knockdown cells were seeded in a 6 cm plate and plotted as number of cells after counting everyday two days after plating. (D) HK2 silencing impairs anchorage independent growth of HCC cells. Anchorage-independent growth was assessed by the ability to form soft-agar colonies. Equal numbers of control or HK2 knockdown cells were subjected to soft-agar assay and colonies were counted after three weeks. Data is expressed as the mean of three independent experiments (± SEM).

(C)


Growth curve Huh7
LacZ shRNA
HK2 shRNA
No of cells X 10^5
Days
Growth curve HepG2
control shRNA
HK-2 shRNA
No of cells X 10^5
Days
Growthc curve Hep3B
LacZ shRNA
HK2 shRNA
No of cells X 10^5
Days
Growth curve of Hepa1-6
LacZ shRNA
HK2shRNA
No of cells X 10^6
Days

(D)

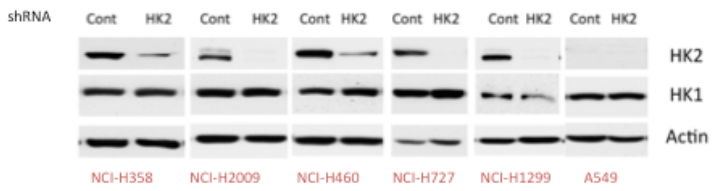

Number of colonies
Huh7
HepG2
Hepa 1-6
Cont
HK2
Cont
HK2
Cont
HK2

2.2.2 Effect on human non-small cell lung cancer cells

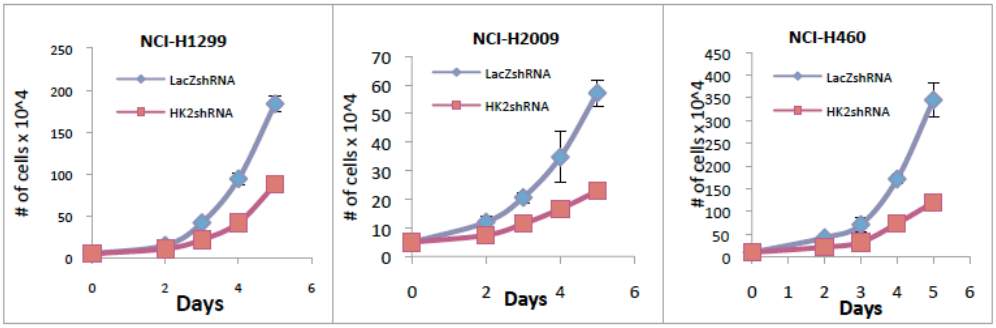
Human NSCLC frequently harbor oncogenic mutations of K-Ras along with other mutations. Oncogenic K-Ras has been shown to increase glucose metabolism in transformed cells. Because we found that HK2 expression is significantly elevated in human NSCLC samples, we verified the effect of HK2 knockdown in human NSCLC cell lines. Stable expression of HK2 shRNA was done in a panel of human NSCLC cell lines (Fig. 8A). Although HK2 expression was substantially attenuated by this shRNA, HK1 level, which is also expressed in these cancer cells, was not changed (Fig. 8A). The knockdown of HK2 significantly and markedly decreased the proliferation of three human NSCLC cell lines tested despite HK1 expression (Fig. 8B). Importantly, the knockdown of HK2 markedly attenuated the ability of all NSCLC cell lines tested to grow in an anchorage-independent manner (Fig. 8C). The anchorage independent growth of A549 lung cancer cell line that expresses an undetectable level of HK2 was not significantly affected (Fig. 8C). These results suggest that cancer cells that express high level of HK2 are highly dependent on it for their tumorigenic potential. Interestingly, like NSCLC cancer cells, a pancreatic cancer cell line that harbors a K-Ras mutation is also dependent on HK2 for efficient colony formation on soft-agar (Fig. 8D). Thus most of the human cancer cell lines those also express oncogenic K-Ras also express HK2 and required for oncogenecity *in vitro* regardless of their tissue of origin.

Figure. 8 HK2 silencing reverses tumorigenesis of NSCLC cells *in vitro*. (A) Immunoblot showing the level of HK2 and HK1 protein expression in a panel of human NSCLC cells stably expressing either shRNA targeting HK2 or LacZ shRNA as control. B) Proliferation of human lung cancer cell lines after stable HK2 knockdown. Proliferation rate of either cells expressing control shRNA or cells expressing HK2 shRNA was determined by seeding equal number of cells followed by counting cell numbers at the indicated time points (C). HK2 silencing impairs anchorage independent growth of human NSCLC cells. Equal numbers of either control or HK2 knockdown cells were subjected to soft-agar assay, and colonies were counted after three weeks. Data are expressed as the mean of three independent experiments ± SEM. (D) Right panel: HK2 silencing impairs anchorage independent growth of MiaPaca-2 human pancreatic cancer cells. Left panel: Immunoblot showing the level of HK2 and HK1 protein expression in MiaPaca-2 cell line stably expressing either shRNA targeting HK2 or LacZ shRNA as control.

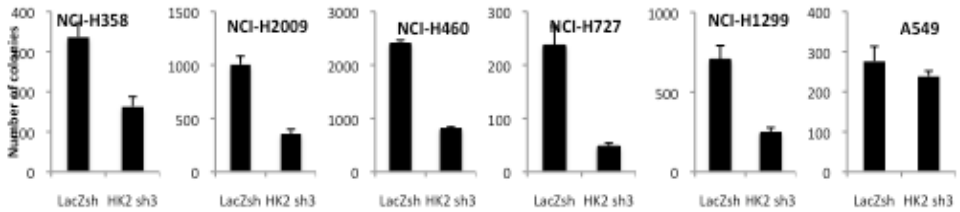
(A)


shRNA
Cont HK2 Cont HK2 Cont HK2 Cont HK2 Cont HK2 Cont HK2
HK2
HK1
Actin
NCI-H358
NCI-H2009
NCI-H460
NCI-H727
NCI-H1299
A549

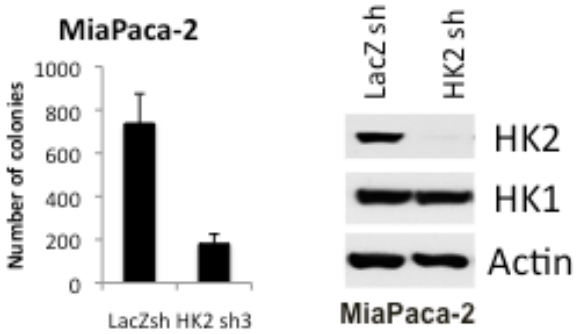
(B)


NCI-H1299
NCI-H2009
NCI-H460
LacZshRNA
HK2shRNA
# of cells x 10^4
Days

(C)


NCI-H358
NCI-H2009
NCI-H460
NCI-H727
NCI-H1299
A549
Number of colonies
LacZsh HK2 sh3

(D)

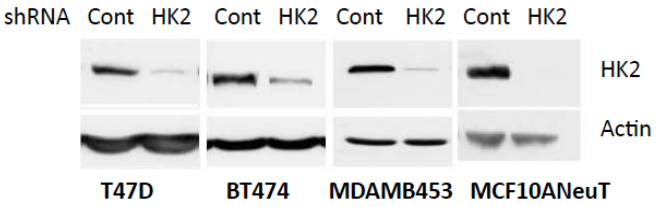

MiaPaca-2
Number of colonies
LacZsh HK2 sh3
LacZ sh
HK2 sh
HK2
HK1
Actin
MiaPaca-2

2.2.3 Effect on human breast cancer cells

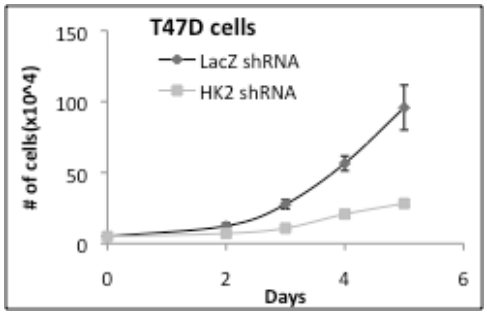
In our TMA analysis of human breast cancer patients we found there is an elevated expression of HK2 in cancer specimens. This prompted us to verify if HK2 is required for the oncogenic potential of human breast cancer cells. We knocked down HK2 in T47D, BT474, MDA-MB-453 and MCF10A-NeuT (expressing an activated Her2/Neu) human breast cancer cell lines (Fig. 9A). Similar to our observation in human lung cancer cell lines, stable knockdown of HK2 in these breast cancer cell lines significantly affected the proliferation in tissue culture conditions (Fig. 9B). Moreover, the HK2 shRNA expression severely compromised the growth of the tested cell lines in an anchorage-independent manner (Fig. 9C). Therefore we conclude that there is a critical role of HK2 in proliferation and anchorage independent growth of human breast cancer cells.

Figure. 9 HK2 silencing reverses tumorigenesis of human breast cancer cells *in vitro*. (A) Immunoblot showing the level of HK2 and HK1 protein expression in a panel of human breast cancer cells stably expressing either shRNA targeting HK2 or LacZ shRNA as control. B) Proliferation of T47D human breast cancer cell lines after stable HK2 knockdown. Proliferation rate of either cells expressing control shRNA or cells expressing HK2 shRNA was determined by seeding equal number of cells followed by counting cell numbers at the indicated time points (C). HK2 silencing impairs anchorage independent growth of human breast cancer cells. Equal numbers of either control or HK2 knockdown cells were subjected to soft-agar assay, and colonies were counted after three weeks. Data are expressed as the mean of three independent experiments ± SEM.

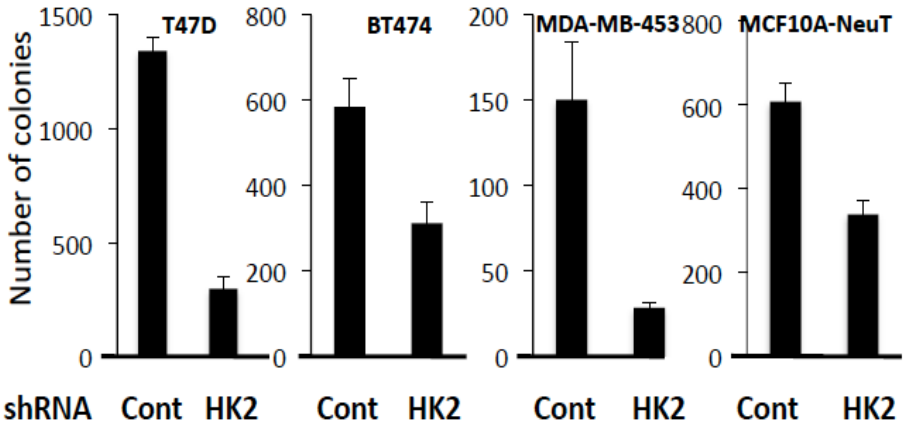
(A)


shRNA Cont HK2 Cont HK2 Cont HK2 Cont HK2
HK2
Actin
T47D
BT474
MDAMB453
MCF10ANeuT

(B)


T47D cells
LacZ shRNA
HK2 shRNA
# of cells(x10^4)
150
100
50
0
0
2
4
6
Days

(C)


Number of colonies
T47D
BT474
MDA-MB-453
MCF10A-NeuT
1500
1000
500
0
800
600
400
200
0
200
150
100
50
0
800
600
400
200
0
shRNA Cont HK2 Cont HK2 Cont HK2 Cont HK2

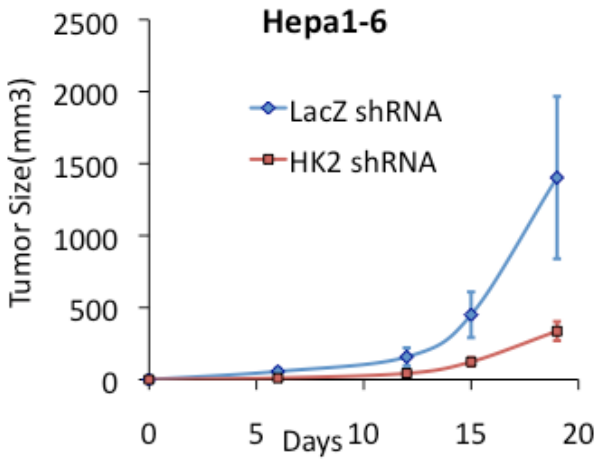
2.3 Expression of stable HK2shRNA affects the tumorigenicity of human cancer cells *in vivo*.

2.3.1 HK2 is required for tumor formation *in vivo*.

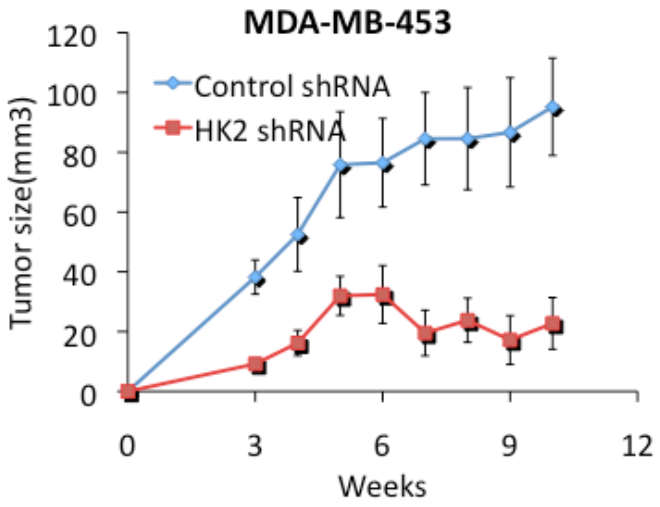
The growth environment of standard tissue culture condition is very different from the microenvironment of the tumors *in vivo*. There are some metabolic genes required for the tumorigenicity in normal tissue culture condition but dispensable i*n vivo* (Possemato et al., 2011). We verified if HK2 is required for tumor formation *in vivo*. Stable HK2 knockdown Hepa 1-6 (HCC) cells and control knockdown cells were subcutaneously injected in male nude mice and tumor growth was followed by caliper measurement till the humane endpoint criteria. As compared to the control knockdown cells the tumor growth was significantly retarded in HK2 knockdown Hepa 1-6 cells (Fig. 10A). Notably, the presence of HK1 even could not compensate the deficiency of HK2 *in vivo*. At the end of the experiment the tumor size in HK2 knockdown cells was around 4.5 times smaller than the control knockdown cells (Fig. 10A). Therefore HK2 is required for growth of human HCC cells *in vivo*. Tumor tissue microenvironment has a profound effect on the growth of the tumors. Therefore we wanted to find out if HK2 required for tumor formation and growth in an orthotropic model of breast cancer. Breast cancer was chosen because we can easily transplant and monitor the tumor growth in an orthotropic tissue environment. The orthotropic tumor growth was dramatically impaired in by the knockdown of HK2 in MDA-MB-453 breast cancer cells that display ErbB2 amplification (Fig. 10B). These results indicate the essential requirement of HK2 in tumor initiation of human cancer cells *in vivo*.

Figure 10. HK2 is required for tumorigenic potential of cancer cell lines *in vivo*. (A) knockdown of HK2 in Hepa 1-6 murine liver cancer cell lines significantly affects the tumor growth in nude mice. (Control or HK2 knockdown cells in(2 x 10^6 cells in 0.1 ml PBS) was subcutaneously injected to opposite flanks of the nude mice. Tumor growth was measured by caliper measurement). HK2 silencing in MDA-MB-453 cells inhibit orthotopic tumor growth. Equal numbers of MDA-MB-453 cells (6 x 10^6 cells in 50% matrigel) stably expressing either HK2 shRNA or control shRNA were injected into the fat pad of the mammary glands of female athymic nude mice.

(A)


Hepa1-6
LacZ shRNA
HK2 shRNA
Tumor Size(mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
Days

(B)


MDA-MB-453
Control shRNA
HK2 shRNA
Tumor size(mm3)
120
100
80
60
40
20
0
0
3
6
9
12
Weeks

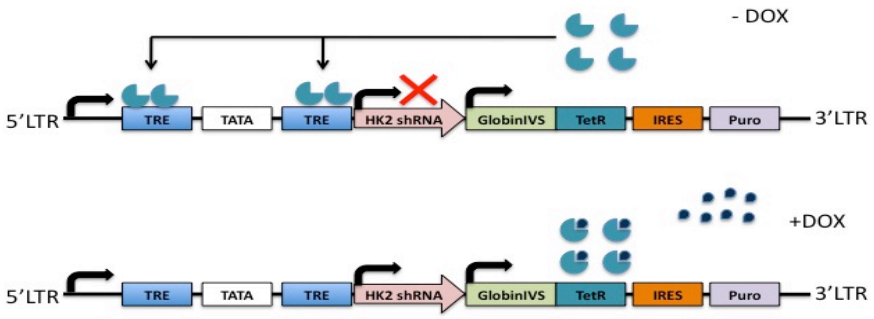
2.3.2 HK2 is required for tumor maintenance *in vivo*.

The above-described experiments showed that HK2 is required for growth of human cancer cells *in vivo*. Next we verified whether HK2 is required for maintenance of already established tumors in vivo. Therefore we established an inducible shRNA knockdown system for HK2. For this purpose, we used the Tet-On-PLKO-puro vector (Wiederschain et al., 2009) (adgene plasmid# 21915) where shRNAs can be expressed in a doxycycline (DOX)/Tetracycline (TET) inducible manner (Fig.11A). This single vector has all the component required for robust RNAi expression for mammalian cells which bypasses the two-step method used in tet-on or tet-off systems. Here TetR protein is constitutively expressed and represses the shRNA expression for the target gene. When the cells are exposed to DOX or TET the shRNA expression is activated and which results in the target gene knockdown (Fig.11A). The shRNA3 oligos, which was used for stable shRNA system, was cloned in Tet-On-PLKO-puro vector. A control vector was generated by cloning a scramble shRNA in the same vector. This system was established in NCI-H460 NSCLC cell line. As shown in Fig. 11B the HK2 shRNA is not expressed in the absence of DOX and addition of DOX efficiently silence HK2 protein expression whereas the presence or absence of DOX does not change the HK2 protein level in the cells expressing scrambled shRNA (control). When DOX was added to the growth media, the proliferation rate of NCI-H460 cells expressing HK2 shRNA was significantly retarded as compared to the non-DOX media (Fig.11C). The proliferation of inducible scramble shRNA expressing cells were unaffected by presence or absence of DOX, ruling out the possibility that the growth inhibition is due to DOX mediated toxicity. The inducible knockdown reduced the proliferation rate of these cells to a similar extent as the stable knockdown (Fig. 11C, Fig. 8B) with a modest increase in cell death (Fig. 11D). NCI-H460 cells expressing inducible HK2 shRNA were then inoculated into nude mice. When tumors reached approximately 65mm^3 in volume, the

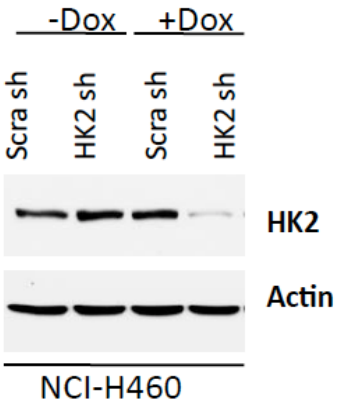
mice were randomly assigned into two groups. The experimental mice groups were subjected to DOX in the diet where was control mice were continued the same chow diet. As shown in Fig. 11E, the induction of HK2 silencing by DOX substantially attenuated the growth of the tumors. We ruled out the possibility that the attenuated tumor growth is due to a potential DOX toxicity since a similar attenuated tumor growth occurred when we compared the tumor growth of cells expressing DOX-inducible HK2 shRNA to that of cells expressing DOX-inducible scrambled shRNA after DOX treatment (Fig. 11F). Tumors isolated from treated and untreated mice, at the endpoint of the study, showed no significant change in HK2 level (Fig. 11G), suggesting that the cells, which gave rise to these tumors escaped the knockdown. We therefore analyzed the tumors one week after DOX treatment, and found a substantial silencing of HK2 expression (Fig. 11H), and reduced BrdU incorporation (Fig. 11I, 11J), further supporting that the tumors observed at a later stage were derived from cells that escaped the knockdown of HK2. Because of the remarkable effect of HK2 silencing on tumor growth, and because the tumors that appeared after induction of HK2 silencing escaped silencing, we predict that a more effective HK2 silencing would completely block tumor growth.

Figure. 11. HK2 is required for tumor maintenance of Human NSCLC cell lines *in vivo*. (A) Schematic diagram of inducible HK2 shRNA system. (Adapted from (Wiederschain et al., 2009)). This single system constitutively expresses TetR repressor, which binds to Tet operator and inhibits HK2 shRNA expression in the absence of doxycycline (DOX). In the presence of DOX the repressors bind to DOX and no longer bind to TetO promoter, hence HK2 shRNA gets expressed. (B) Cells expressing an inducible control (Scramble) or HK2 shRNA were exposed to vehicle or 500ng/ml doxycycline (DOX) for 96 hrs prior to analysis. Immunoblot showing HK2 protein level efficiently silenced only upon addition of DOX in inducible HK2 shRNA expressing cells. (C) The inducible knockdown of HK2 attenuates the proliferation rate of NCI-H460 cells. Cells expressing either inducible scrambled shRNA (Tet-Scra) or HK2 shRNA (Tet-HK2) were treated with 500ng/ml doxycycline, and after 96 hours equal number of cells were replated and growth curve was generated by counting cells at the indicated time points. (D) Inducible HK2 shRNA expression induces a mild cell death in NCI-H460 cells. Equal number of either Tet-Scramble expressing cells or Tet-HK2 shRNA expressing cells were plated in triplicate in a 6 well plate. 96 hrs after 500ng/ml doxycycline treatment, the plates were fixed and apoptotic cells were counted by DAPI staining. A representative experiment was shown with ± SD.

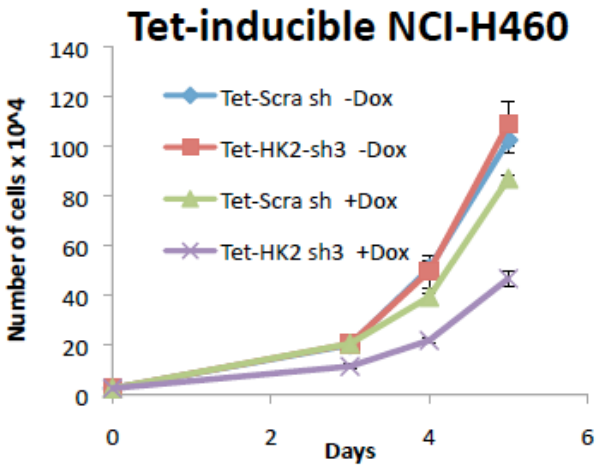
(A)


- DOX
5'LTR
TRE
TATA
TRE
HK2 shRNA
GlobinIVS
TetR
IRES
Puro
3'LTR
+DOX
5'LTR
TRE
TATA
TRE
HK2 shRNA
GlobinIVS
TetR
IRES
Puro
3'LTR

(B)


-Dox
+Dox
Scra sh
HK2 sh
Scra sh
HK2 sh
HK2
Actin
NCI-H460

(C)


Tet-inducible NCI-H460
Number of cells x 10^4
140
120
100
80
60
40
20
0
Tet-Scra sh -Dox
Tet-HK2-sh3 -Dox
Tet-Scra sh +Dox
Tet-HK2 sh3 +Dox
0
2
4
6
Days

(D)

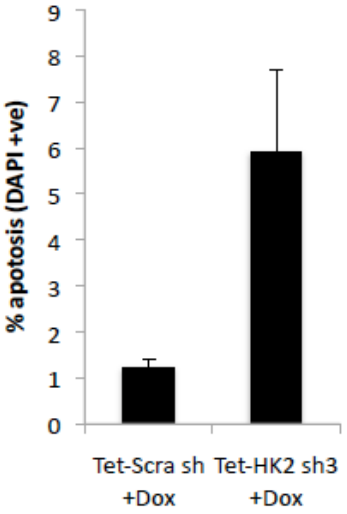
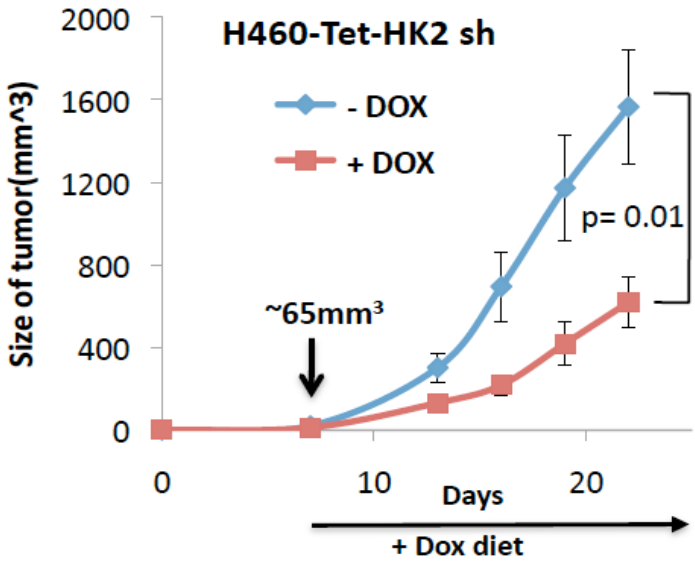
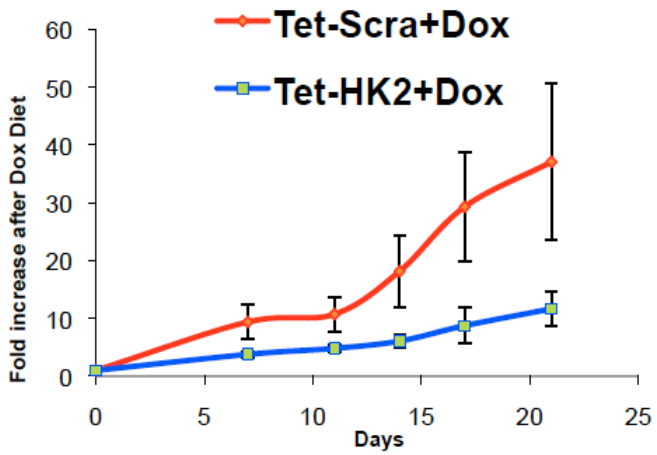

% apoptosis (DAPI +ve)
9
8
7
6
5
4
3
2
1
0
Tet-Scra sh +Dox
Tet-HK2 sh3 +Dox

Figure. 11 (continued). HK2 is required for tumor maintenance of Human NSCLC cell lines *in vivo*. (E) The induction HK2 silencing inhibits tumor growth of NCI-H460 cells *in vivo*. NCI-H460 cells ($1x10^6$) were injected subcutaneously into athymic nude mice. When tumor size reached ~65mm^3, mice were fed doxycyline containing diet (n=8) or normal chow diet (n=7), and tumor growth was followed until tumor size reached 1.5-2cm^3. (**F)** The induction HK2 silencing inhibits tumor growth of NCI-H460 cells *in vivo* and this effect is not because of doxicycline toxicity. Tet-Scramble or Tet-HK2 shRNA expressing cells (1x106) were injected subcutaneously into athymic nude mice into two opposite flanks. When the average tumor size reached ~65mm3, mice were fed doxycyline containing diet (n=5) and tumor growth was followed until tumor size reached 1.5-2cm^3. Data expressed in relative fold increase in tumor size from the day of dox diet. (G) At the end of the experiment described in E, tumor lysates were subjected to immunoblotting with anti-HK2 antibodies.

(E)


H460-Tet-HK2 sh
- DOX
+ DOX
Size of tumor(mm^3)
2000
1600
1200
800
400
0
~65mm³
p= 0.01
0
10
20
Days
+ Dox diet

(F)


Tet-Scra+Dox
Tet-HK2+Dox
Fold increase after Dox Diet
60
50
40
30
20
10
0
0
5
10
15
20
25
Days

(G)

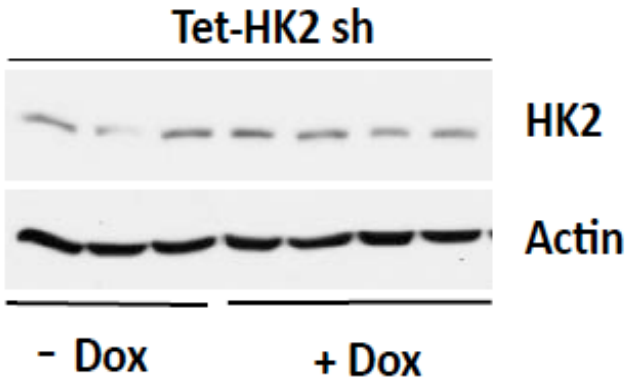
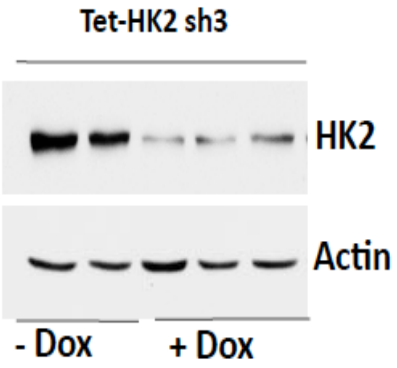
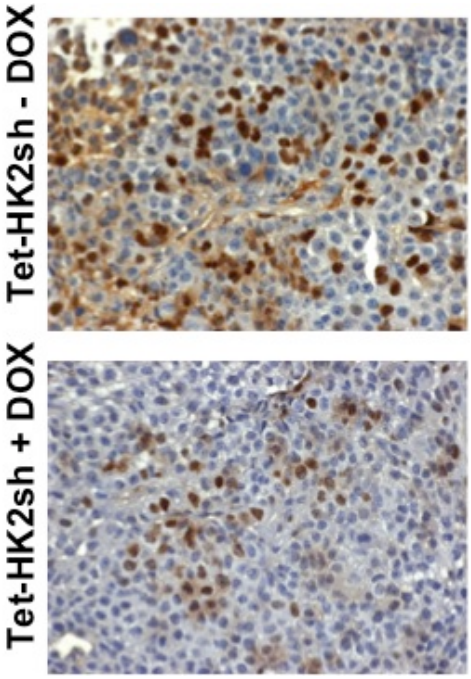

Tet-HK2 sh
HK2
Actin
- Dox
+ Dox

Figure 11 (continued) HK2 is required for tumor maintenance of Human NSCLC cell lines *in vivo*. (H) Immunoblot showing HK2 protein levels in tumor lysates harvested one week after exposure to the Dox diet. (I) Reduced BrdU incorporation in tumors one week after exposure to Dox diet. (J) and the quantification of the same from 20X field of view.

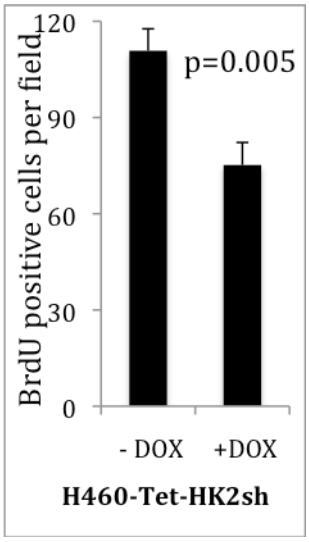
(H)


Tet-HK2 sh3
HK2
Actin
- Dox
+ Dox

(I)


Tet-HK2sh - DOX
Tet-HK2sh + DOX

(J)


BrdU positive cells per field
120
90
60
30
0
p=0.005
- DOX
+DOX
H460-Tet-HK2sh

(2) Hexokinase 2 is required for oncogenic transformation *in vitro* and tumor initiation of different mouse models of cancer *in vivo*.

A) Background

Elevated glucose consumption and metabolism is of the discernable biochemical changes observed in transformed cells. This elevated glucose metabolism supports many fundamental processes such as the ATP production by coordinated process of glycolysis and oxidative phosphorylation, which fulfils energy demand in proliferating cells. Hyperactive glucose metabolism also generates precursors for pentose phosphate (PPP), hexosamine, serine biosynthesis those required for anabolic processes(Lunt and Vander Heiden, 2011). Expressions of many enzymes of all these pathways are changed to coordinate the whole metabolic process. The high expression of HK2 isoform in cancer cells is one of the hallmarks that was demonstrated by our study and others(Mathupala et al., 2006; Wolf et al., 2011). HK1 is ubiquitously expresses at high level in most of the adult tissue. The following questions need to be addressed 1) Is HK2 is required for oncogenic transformation despite the presence of HK1? 2) What will be consequences of HK2 ablation in tumorigenic process in mouse models of tumors? 3) Most importantly what are the consequences of systemic HK2 deletion on normal body physiology and tumorigenesis process? 4) What is the functional significance of HK2 in tumors despite the presence of HK1? To address theses questions we performed a systematic study using mice with conditional knockout of HK2. We showed that HK2 is required for oncogenic transformation of mouse embryonic fibroblasts (MEFs) and is critical for tumor initiation in mouse model of NSCLC and breast cancer. In addition, cell lines derived from mammary gland tumors are dependent on HK2 for their ability to form tumors in an orthotropic transplantation model. Finally genetic ablation of HK2 reduces tumor burden in carcinogen induced liver cancer in mice.

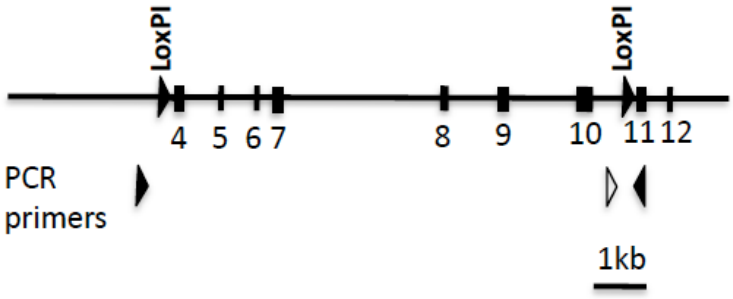
B) Results

1. HK2 conditional knockout mouse.

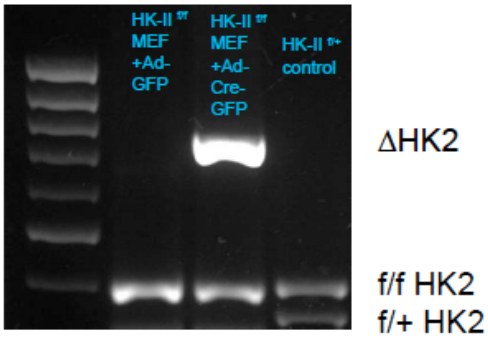
Germ line deletion of HK2 in the mouse causes early embryonic lethality (Heikkinen et al., 1999). Therefore, we employed $HK2^{flox/flox}$ ($HK2^{f/f}$) mice for our studies. These mice were generated in the laboratory of Markku Laakso (University of Eastern Finland, Kuopio, Finland). In this strain LoxP sites are integrated in intron 3 and intron 10 of the HK2 locus (Fig.12A). Therefore Cre recombinase mediated recombination would cause deletion of exons 4 to 10 causing a null allele for HK2 protein expression. We characterized the mice by isolating primary mouse embryonic fibroblasts (MEFs) from embryos generated from crosses made with homozygous $HK2^{f/f}$ mice. These primary MEFs were subjected to adenovirus expressing Cre recombinase (Ade-Cre) infection to delete HK2. Ade-LacZ infected MEFs served as control. HK2 is efficiently deleted in Ade-Cre treated cells as detected by the appearance of HK2 recombination specific PCR product through genomic PCR (Δ HK2) (Fig.12B) and an antibody specific to HK2 protein though immunoblot (Fig.12C). We utilized this HK2 conditional allele ($HK2^{f/f}$) mouse and cells generated from this mouse for subsequent studies.

Figure. 12. Characterization of HK2 conditional knockout mice. (A) Map showing the location of LoxP sites in the HK2 gene before and after excision by Cre recombinase. The loxP sites are upstream of exon 4 and upstream of exon 11. After excision by Cre the region between upstream of exon 4 and upstream of exon 11 is deleted. Primers used for PCR to distinguish between the WT, the floxed, and the deleted alleles are indicated. (B) Genomic PCR analysis of deletion of HK2 in the MEFs isolated from homozygous LoxP mice and treated with Cre or LacZ adenovirus. The detailed information of the primer and explanation is described in the experimental methods section. Note there is an appearance of recombination specific PCR product of ~800 bp in the Cre treated cells. Although an efficient HK2 deletion was achieved by Cre as assessed by immunoblot shown in panel C but still a small fraction of non-recombined allele specific PCR product appeared may be because of the saturating PCR condition. (C) Immunoblot of HK2 in the MEFs described in panel. B.

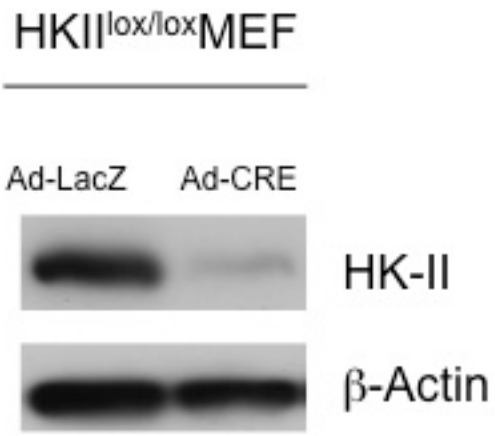
(A)


LoxPI
LoxPI
4 5 6 7
8 9
10 11 12
PCR
primers
1kb

(B)


HK-II f/f MEF +Ad-GFP
HK-II f/f MEF +Ad-Cre-GFP
HK-II f/+ control
ΔHK2
f/f HK2
f/+ HK2

(C)


HKII lox/lox MEF
Ad-LacZ
Ad-CRE
HK-II
β-Actin

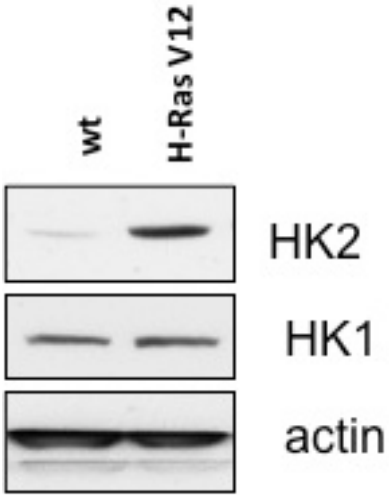
2. HK2 is required for oncogenic transformation of Mouse embryonic fibroblasts

Transformation of immortalized fibroblasts by oncogenic Ras is a classical assay to study tumorigenesis. Primary mouse embryonic fibroblasts (MEFs) generated from HK2$^{f/f}$ mice were immortalized with dominant-negative p53 (DNp53) as previously described (Skeen et al., 2006). These immortalized MEFs were subjected to transformation with oncogenic H-RasG12D as previously described (Skeen et al., 2006). Interestingly, I found that oncogenic Ras expression markedly elevated HK2 expression without any distinguishable changes in HK1 in protein level (Fig. 13A). To find out if PI3K and/or MAPK signaling downstream of activated Ras contribute to this elevated expression I treated these transformed MEFs with MEK inhibitor (U0126) or PI3K inhibitor (LY294002) and found both PI3K and MAPK inhibition inhibited the expression of HK2 (Fig. 13B). The HK2$^{f/f}$ MEFs were subjected to adenovirus expressing Cre recombinase (Ade-Cre) infection to delete HK2. Ade-LacZ infected MEFs served as control for the subsequent experiments. Here after Ade-Cre recombined cells will be referred to as HK2 KO (knockout) or HK2 Δ/Δ and Ade-LacZ treated cells will be referred as HK2 $^{f/f}$ or HK2 WT. Ade-Cre efficiently knocked out HK2 allele without affecting HK1 (Fig. 13C). When the immortalized HK2 KO MEFs expressing oncogenic H-Ras were subjected to anchorage-independent growth, the deletion of HK2 impaired the colony formation on soft agar (Fig. 13D). Furthermore, the cells also express HK1 and therefore total hexokinase activity in the Ras-transformed cells was reduced by about 70% (Fig. 13E). Interestingly glucose consumption from the culture media was reduced by about 50% in transformed HK2 KO MEFs (Fig. 13F) suggesting HK2 is a major determinant of glucose consumption in the transformed cells. However, the deletion of HK2 in transformed cells was sufficient to profoundly reverse oncogenic transformation as measured by anchorage-independent growth on soft agar (Fig. 13G). Finally, the deletion of HK2 in oncogenic Ras transformed cells markedly impaired tumor growth *in vivo* in

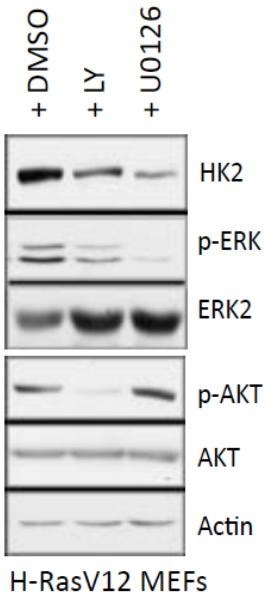
xenograft assays (Fig. 13H,I). To find out if the catalytic activity of HK2 is required for oncogenic transformation, Rat HK2-WT or a kinase dead mutant of HK2 (HK2-SA) was expressed in Ras transformed HK2 KO cells. The ectopic HK2-WT or HK2-SA mutant in HK2 KO cells expressed in equivalent amount to the endogenous HK2 protein in transformed $HK2^{f/f}$ cells (Fig. 13J). Re-expression of HK2-WT in HK2 KO MEFs restored the total HK activity, where as HK2-SA does not (Fig. 13K). Finally the re-expression of WT HK2 in HK2 KO MEFs restored their susceptibility to tumorigenesis *in vivo*, while the kinase-dead mutant HK2-SA could not (Fig. 13L). The above-described experiments suggest that HK2 activity is required for oncogenic transformation of MEFs *in vitro* and *in vivo*. To determine if HK2 ablation could reverse the tumorigenic phenotype of the murine cancer cell expressing a physiological level of mutated K-Ras , we crossed $HK2^{f/f}$ mice with $K\text{-}Ras^{LA2\text{-}G12D}$ mice (Johnson et al., 2001) . These mice develop lung tumors at early onset and develop spontaneous lung tumor because of the mutated K-Ras. These primary tumor cell lines derived from the tumors generated in mice expressed HK2 like their tumor counterparts. The lung cancer cell lines were infected with either adenovirus expressing LacZ or Cre. Efficient deletion of HK2 was observed in the cells (Fig. 14A). The deletion of HK2 in $K\text{-}Ras^{LA2\text{-}G12D}$ cells markedly reduced the proliferation of the primary cells (Fig. 14B). All the above-described experiments clearly suggest in that similar to human cancer cells, Ras transformed murine cancer cells require HK2 for their oncogenesity.

Figure 13. HK2 expression is elevated by oncogenic Ras and it is required for oncogenic transformation of mouse embryonic fibroblasts (MEFs). (A) Immunoblot of HK1 and HK2 in oncogenic H-RasV12 transformed immortalized MEFs. (B) Both PI3K and MAPK signaling pathways contribute to the elevation of HK2 expression in oncogenic Ras transformed MEFs. H-RasV12 transformed MEFs were treated with either LY294002 (20uM), U2016 (20uM) or DMSO (control) for 18 hours. Cells were than harvested for immunobloting for HK2 expression or phospho-Akt and phospho-ERK as readout for PI3K and MAPK activities respectively. (C) HK2 deletion before Ras transformation impairs anchorage-independent growth. Immortalized HK2 F/F or HK2 Δ/Δ MEFs expressing dominant-negative p53 were generated following infection by adenovirus expressing GFP alone, as a control, or coexpressing Cre and GFP to delete HK2. The MEFs were subjected to oncogenic transformation by oncogenic H-RasV12. Average number of colonies generated by WT and HK2-/- Ras transformed MEFs after growth six weeks on soft-agar is shown. Data are presented as an average of three independent experiments ± SEM. (D) MEFs derived from $HK2^{f/f}$ mice were immortalized with dominant-negative p53 (DN-p53) and then infected with Ade-Cre to delete HK2 or with Ade-LacZ as a control. The cells were then stably infected with a retrovirus expressing oncogenic $HRas^{V12}$. Cell extracts were subjected to immunoblotting with anti-HK2 or anti-HK1 antibodies. Control undeleted cells are denoted HK2F/F and $HK2^{-/-}$ cells are denoted as HK2Δ/Δ.

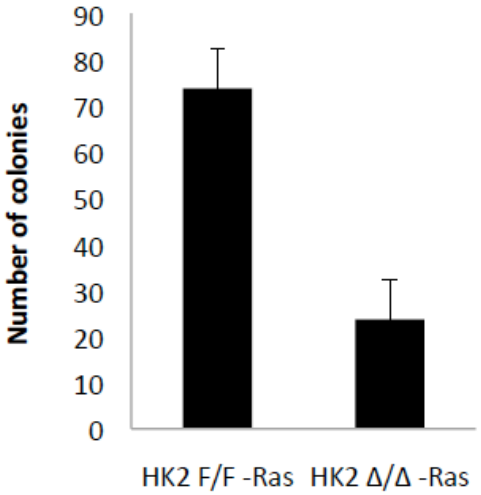
(A)


wt
H-Ras V12
HK2
HK1
actin

(B)


+ DMSO
+ LY
+ U0126
HK2
p-ERK
ERK2
p-AKT
AKT
Actin
H-RasV12 MEFs

(C)


Number of colonies
90
80
70
60
50
40
30
20
10
0
HK2 F/F -Ras
HK2 Δ/Δ -Ras

(D)

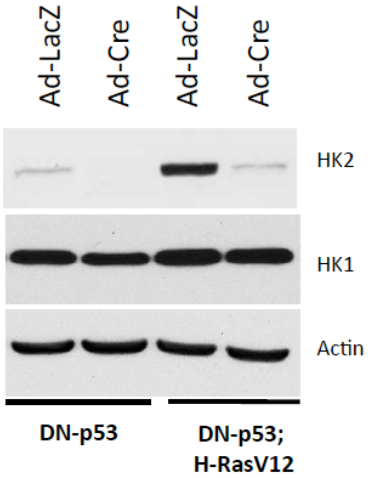
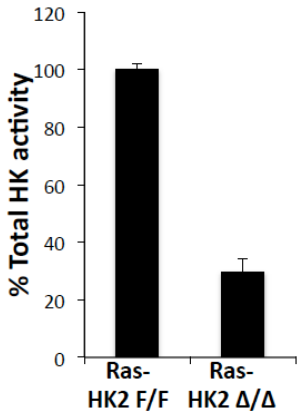
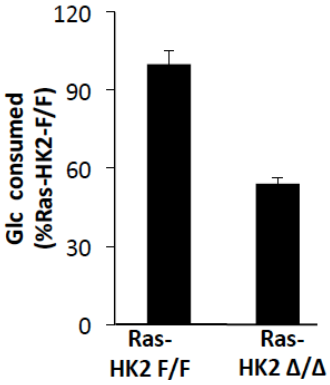

Ad-LacZ
Ad-Cre
Ad-LacZ
Ad-Cre
HK2
HK1
Actin
DN-p53
DN-p53;
H-RasV12

Figure 13. HK2 expression is elevated by oncogenic Ras and it is required for oncogenic transformation of mouse embryonic fibroblasts (MEFs)(continued). (E) Relative total hexokinase activity in WT and HK2$^{-/-}$ Ras-transformed MEFs. Hexokinase activity was measured as described in Experimental Procedures. (F) Relative glucose consumption in WT and HK2$^{-/-}$ Ras-transformed MEFs. Data represent the percentage of glucose consumed by HK2$^{-/-}$ Ras-transformed MEFs as compared to WT Ras-transformed MEFs after of culturing for 18h, and after adjusting for cell number. Glucose consumption was measured as described in Experimental Procedures. (G) HK2 deletion impairs anchorage-independent growth. Average number of colonies generated by WT and HK2$^{-/-}$ Ras transformed MEFs after six weeks growth on soft-agar is shown (for details see Experimental Procedures). Data are presented as an average of three independent experiments ± SEM. (H) Representative tumor images of WT and HK2$^{-/-}$ Ras-transformed MEFs (Scale bars: 1 cm). (I) Average tumor size formed by WT and HK2$^{-/-}$ Ras-transformed MEFs. The data represent the average size of 4 tumors per each cell type ± SEM. WT and HK2$^{-/-}$ Ras-transformed MEFs ($1x10^6$) were injected subcutaneously into athymic nude mice, when WT tumors reached 1.5-2cm^3, tumor size was calculated and mice were sacrificed (for details see Experimental Procedures).

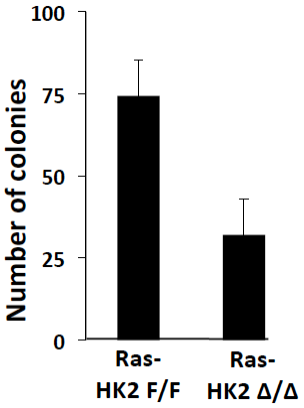
(E)


% Total HK activity
120
100
80
60
40
20
0
Ras-
HK2 F/F
Ras-
HK2 Δ/Δ

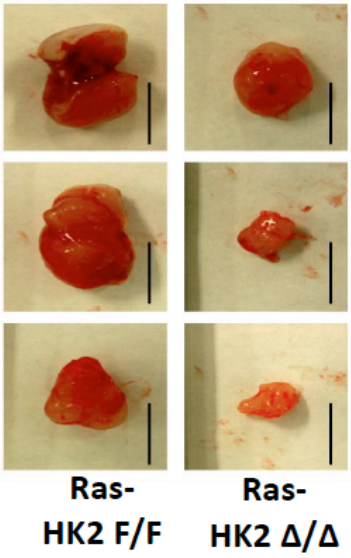
(F)


Glc consumed
(%Ras-HK2-F/F)
120
90
60
30
0
Ras-
HK2 F/F
Ras-
HK2 Δ/Δ

(G)


Number of colonies
100
75
50
25
0
Ras-
HK2 F/F
Ras-
HK2 Δ/Δ

(H)


Ras-
HK2 F/F
Ras-
HK2 Δ/Δ

(I)

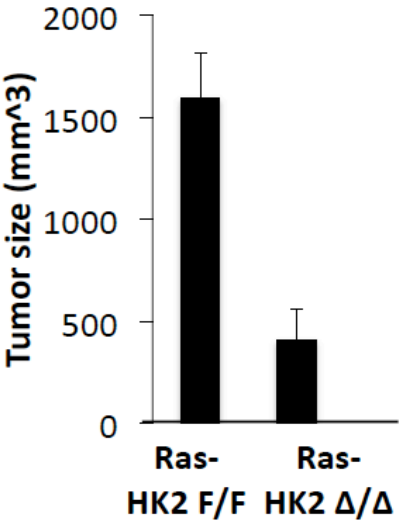
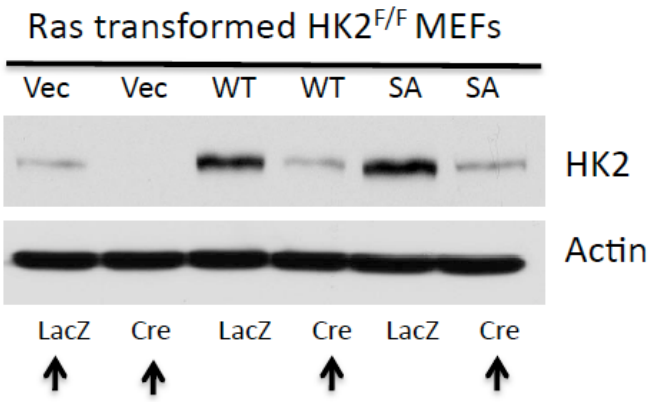
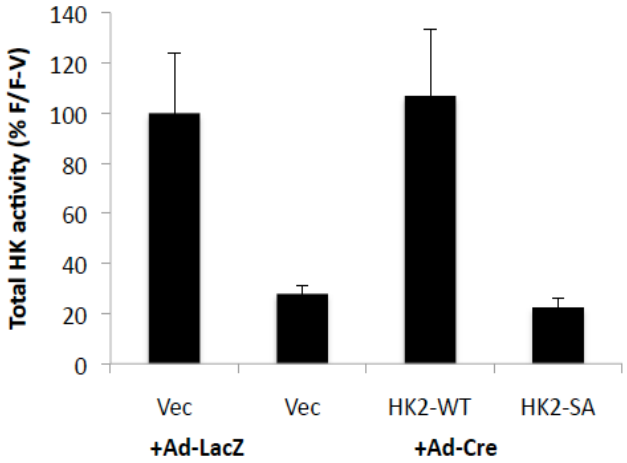

Tumor size (mm^3)
2000
1500
1000
500
0
Ras-
HK2 F/F
Ras-
HK2 Δ/Δ

Figure 13. (continued) HK2 expression is elevated by oncogenic Ras and it is required for oncogenic transformation of mouse embryonic fibroblasts (MEFs). (J)Immunoblot showing endogenous and exogenous HK2 expression before and after deletion of HK2 in Ras transformed MEFs. The Rat WT-HK2-HA or Rat HK2 kinase dead mutant (SA(S155A/S603A)-HK2-HA) were cloned into pLenti6-Blast vector to generate lentiviruses expressing WT or HK2 mutant and to establish Ras transformed MEFs expressing either control vector, WT HK2 or mutant HK2. The cells were than infected with either adenovirus expressing Cre or adenovirus expressing LacZ as a control. Protein extracts from the different cell lines were to immunoblotting using anti-HK2 antibodies. The lanes indicated by arrows show the levels of WT HK2 or mutant HK2 in the cells used for xenografts assays in Figure 1G. $HK2^{-/-}$ Ras-transformed MEFs stably expressing WT or kinase-dead HK2, and WT or $HK2^{-/-}$ control Ras-transformed MEFs were subjected to tumorigenesis *in vivo* as described in. (K) Relative total hexokinase activity in Ras-transformed HK2 F/F control (Vector) MEFs and HK2 Δ/Δ MEFs stably expressing Vector control or WT or kinase-dead HK2. Hexokinase activity was measured as described in Experimental Procedures. Data expressed in % HK2 activity of the variants as compared to control (Vector) with ± SEM. (L) HK2 activity is required for *in vivo* tumorigenesis Tumor size was calculated when tumor size of control WT Ras-transformed MEFs reached approximately $2cm^3$. The data represent the average size of 4 tumors per each cell type ± SEM.

(J)


Ras transformed HK2F/F MEFs
Vec
Vec
WT
WT
SA
SA
HK2
Actin
LacZ
Cre
LacZ
Cre
LacZ
Cre

(K)


Total HK activity (% F/F-V)
140
120
100
80
60
40
20
0
Vec
Vec
HK2-WT
HK2-SA
+Ad-LacZ
+Ad-Cre

(L)

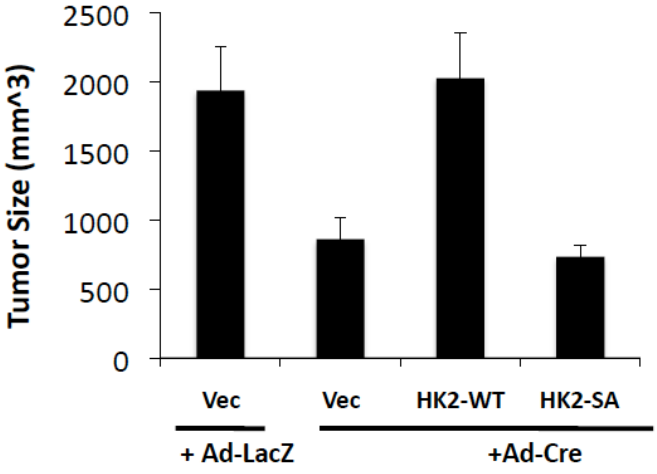
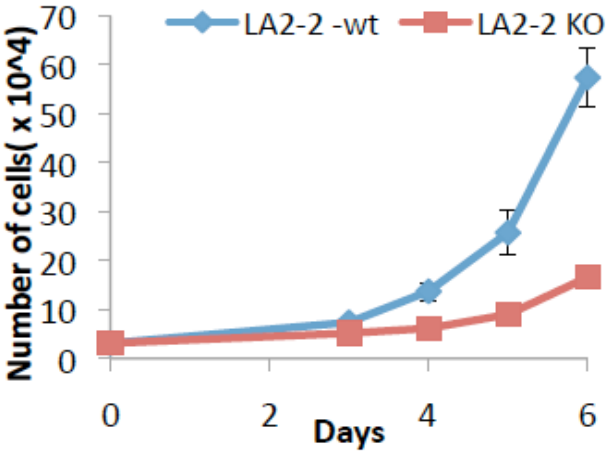

Tumor Size (mm^3)
2500
2000
1500
1000
500
0
Vec
Vec
HK2-WT
HK2-SA
+ Ad-LacZ
+Ad-Cre

Figure 14. Genetic ablation of HK2 reduces the proliferation of primary K-Ras^{G12D} mutated mouse NSCLC cells in–vitro. (A) Immunoblot of HK2 deleted primary NSCLC cell line derived from K-Ras$^{LA2-G12D}$HK2$^{f/f}$ mice. Primary NSCLC cell lines were established from K-Ras$^{LA2-G12D}$HK2$^{f/f}$ mice and HK2 was deleted from these cells by adeno-Cre. For detailed procedure please sees the experimental methods (B) The deletion of HK2 in the NSCLC cell line derived from K-Ras$^{LA2-G12D}$HK2$^{f/f}$ mice impaired cell proliferation. Proliferation rate of either cells treated with control adenovirus (LacZ) or Cre expressing virus was determined by seeding equal number of cells followed by counting cell numbers at the indicated time points.

(A)

LA2-2 HK2 F/F
LacZ
Cre
HK2
Actin

(B)


LA2-2 -wt
LA2-2 KO
Number of cells(x 10^4)
70
60
50
40
30
20
10
0
0
2
4
6
Days

3. Effect of Hexokinase 2 deletion in tumor initiation in mice models of cancer.

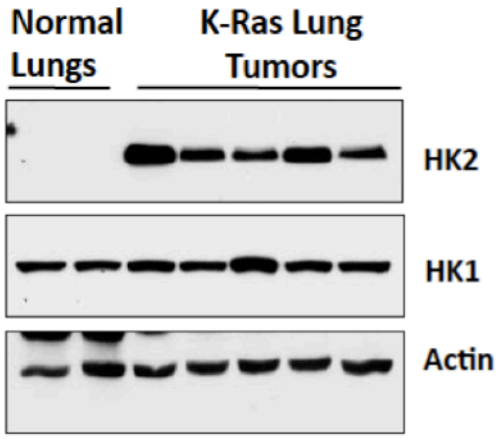
3.1 HK2 is required for tumor initiation in a mice model of NSCLC.

K-Ras is a frequently mutated in human lung, pancreas and colon cancer. Nearly 30% of human lung cancers harbor oncogenic mutations in K-Ras. Around 97% of K-Ras mutation involves the activating mutation in codon 12 or 13 in human Non-small cell lung cancer (NSCLC)(Riely et al., 2009). Genetically engineered mice having this mutation in their lungs faithfully recapitulate the events that occur in human NSCLC. Therefore we used K-Ras$^{LA2-G12D}$ and K-Ras$^{LSL-G12D}$ strains, which are well characterized mouse models for NSCLC(Johnson et al., 2001; Tuveson et al., 2004). First we isolated lungs from control WT and from K-Ras$^{LA2-G12D}$ mice, which develop lung lesions with an early onset of 2-4 weeks after birth, and lung carcinoma at 5-6 months (Johnson et al., 2001). Normal lungs do not express detectable HK2 protein. In contrast, in the individual tumors isolated from K-Ras$^{LA2-G12D}$ mice HK2 protein expression was markedly elevated, without any significant change in HK1 protein level (Fig. 15A). Therefore the oncogenic K-Ras driven lung tumors from mice have the similar pattern of expression of HK2 as seen in human NSCLC tissue samples. In K-Ras$^{LSL-G12D}$ (Lox-Stop-Lox K-RasG12D) mice activated K-RasG12D preceding a stop cassette flanked by two LoxP sites is inserted in the endogenous locus of K-Ras. Upon exposure to Cre recombinase the Stop cassette gets excised and oncogenic K-Ras expressed from its endogenous locus (Fig. 15B). To study if HK2 is required for tumor initiation in the mice, we crossed K-Ras$^{LSL-G12D}$ mice with HK2$^{f/f}$ mice to generate K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice. In these mice, exposure to adenovirus expressing Cre (Ade-Cre), through inhalation, induces activated K-Ras expression with concomitant deletion of HK2 (Fig. 15C). Control K-Ras$^{LSL-G12D}$ and K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice were subjected to adenovirus Cre through intra nasal instillation and were sacrificed 4 months after. Lung tissues were isolated to determine tumor burden, tumor size, tumor number, proliferation and apoptosis. Total tumor burden

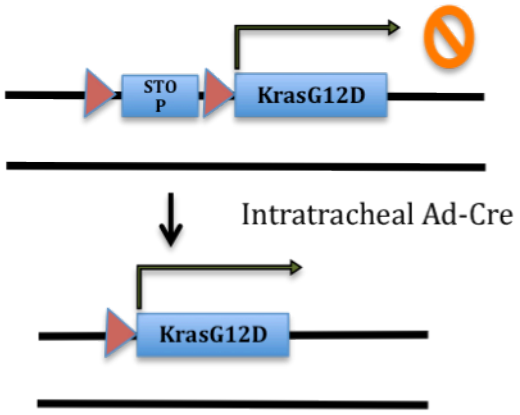
was markedly reduced in K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice, when compared with K-Ras$^{LSL-G12D}$ mice (Fig. 15D,E). Moreover, the average tumor size was significantly smaller (Fig. 15G,H), although the number of lesions was not significantly reduced (Fig. 15F). This effect is largely due to impaired proliferation as measured by BrdU incorporation (Fig. 15J,K), and since we did not observe a significant increase in cell death as measured by caspase 3 cleavage (data not shown). Tumors isolated from the K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice had residual expression of HK2, possibly due to incomplete deletion, and without any changes in HK1 expression (Fig. 15I). These results suggest that small lesions could be developed even at low level of HK2 expression, but cannot be further develop into large tumors. This was manifested by the striking effects of HK2 deletion not only on K-Ras$^{LSL-G12D}$ mouse tumor burden, but also on overall mortality. Kaplan-Meier survival curves reveal profoundly delayed mortality of K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice relative to K-Ras$^{LSL-G12D}$ mice. In addition, more than 50% of HK2-deficient K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice were still alive 250 days following Ade-Cre exposure, a time point corresponding to 100% mortality in HK2-expressing K-Ras$^{LSL-G12D}$ mice (Fig. 15L). Hence these results indicate that HK2 is a critical regulator of oncogenic K-Ras driven NSCLC.

Figure 15. HK2 is required for the development of oncogenic K-Ras driven non-small cell lung cancer (NSCLC) in mice, and its deletion extends the lifespan of these mice. (A) Cell extracts from lungs of control K-Ras$^{LSL-G12D}$ mice or from tumors in lungs of K-Ras$^{LSL-G12D}$ mice after exposure to Ade-Cre were subjected to immunoblotting with anti-HK2 or anti-HK1 antibodies. (B) Schematic diagram showing the targeted construct of K-Ras$^{LSL-G12D}$ mice. (C) Breeding and experimental outline to generate K-Ras induced lung cancer in HK2 knock out background using K-Ras$^{LSL-G12D}$ mice.

(A)


Normal Lungs
K-Ras Lung Tumors
HK2
HK1
Actin

(B)


STO P
KrasG12D
Intratracheal Ad-Cre
KrasG12D

(C)

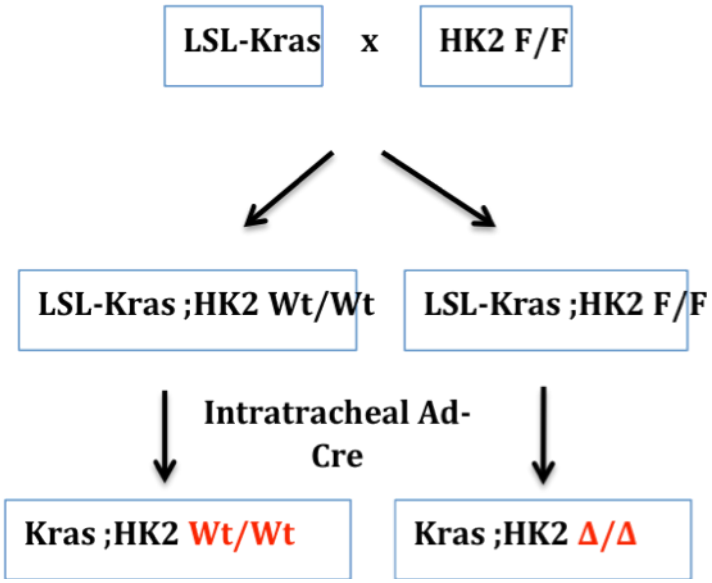
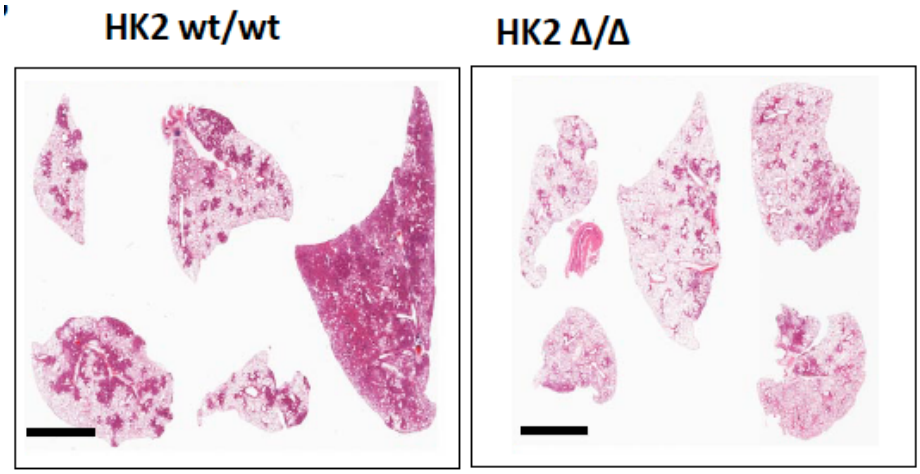
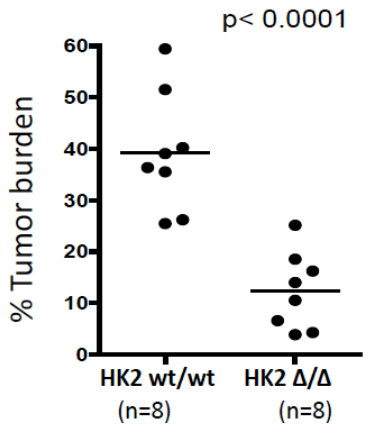

LSL-Kras
x
HK2 F/F
LSL-Kras ;HK2 Wt/Wt
LSL-Kras ;HK2 F/F
Intratracheal Ad-Cre
Kras ;HK2 Wt/Wt
Kras ;HK2 Δ/Δ

Figure 15. (continued) HK2 is required for the development of oncogenic K-Ras driven non-small cell lung cancer (NSCLC) in mice, and its deletion extends the lifespan of these mice. (D) Sixteen weeks after Cre instillation to induce lung tumors, lung sections were subjected to H&E staining (scale bar 4mm). (E) Percentage tumor burden calculated from the H&E stained sections of tumor bearing lungs from K-Ras$^{LSL-G12D}$ mice (n=8) or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice (n=8). (F) Tumors number calculated from the H&E stained sections of tumor bearing lungs from K-Ras$^{LSL-G12D}$ (n=8) or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ (n=8) mice. (G) Representative picture of average tumor size and (H) quantification of the same per lungs of K-Ras$^{LSL-G12D}$ (n=7) or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ (n=8) mice. (I) Immunoblot showing HK2 and HK1 expression in tumors obtained from K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ lungs. (J) Representative picture of BrdU staining in the indicated genotypes (K) Average number of BrdU positive cells per field as calculated from 40X magnification of tumor sections. At least ten sections of K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ lungs from three different mice were used for this analysis. For details, see Experimental Procedures. (L) Kaplan-Meier survival curves of lung tumor bearing K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice after exposure to Ade-Cre at 8 weeks of age.

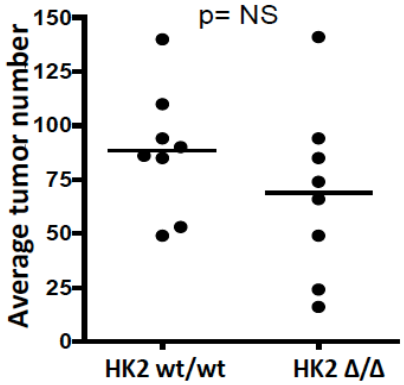
(D)


HK2 wt/wt
HK2 Δ/Δ

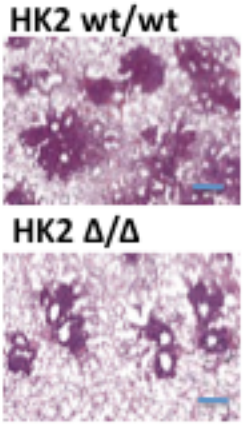
(E)


p< 0.0001
% Tumor burden
60
50
40
30
20
10
0
HK2 wt/wt
(n=8)
HK2 Δ/Δ
(n=8)

(F)


p= NS
Average tumor number
150
125
100
75
50
25
0
HK2 wt/wt
HK2 Δ/Δ

(G)


HK2 wt/wt
HK2 Δ/Δ

(H)

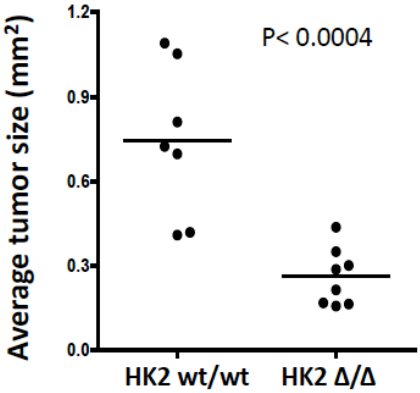
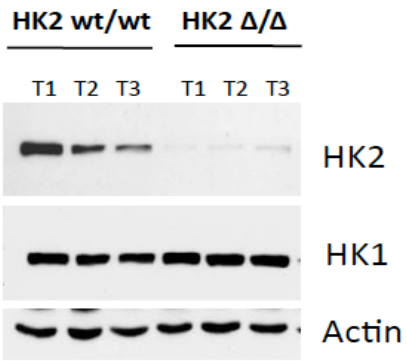
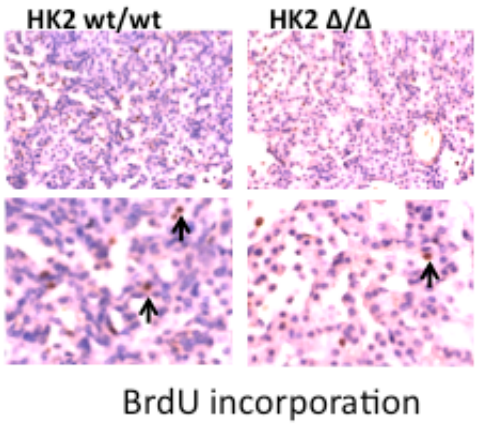

P< 0.0004
Average tumor size (mm²)
1.2
0.9
0.6
0.3
0.0
HK2 wt/wt
HK2 Δ/Δ

Figure 15. (continued) HK2 is required for the development of oncogenic K-Ras driven non-small cell lung cancer (NSCLC) in mice, and its deletion extends the lifespan of these mice. (I) Immunoblot showing HK2 and HK1 expression in tumors obtained from K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ lungs. (J) Representative picture of BrdU staining in the indicated genotypes (K) Average number of BrdU positive cells per field as calculated from 40X magnification of tumor sections. At least ten sections of K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ lungs from three different mice were used for this analysis. For details, see Experimental Procedures. (L) Kaplan-Meier survival curves of lung tumor bearing K-Ras$^{LSL-G12D}$ or K-Ras$^{LSL-G12D}$HK2$^{f/f}$ mice after exposure to Ade-Cre at 8 weeks of age.

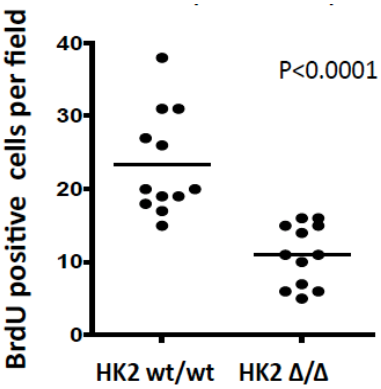
(I)


HK2 wt/wt
HK2 Δ/Δ
T1 T2 T3
T1 T2 T3
HK2
HK1
Actin

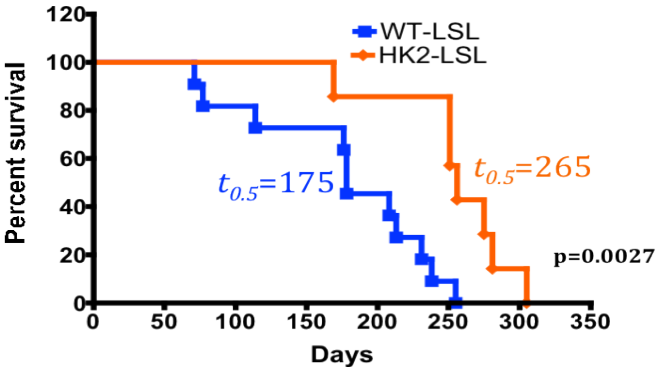
(J)


HK2 wt/wt
HK2 Δ/Δ
BrdU incorporation

(K)


BrdU positive cells per field
40
30
20
10
0
P<0.0001
HK2 wt/wt
HK2 Δ/Δ

(L)


Percent survival
120
100
80
60
40
20
0
WT-LSL
HK2-LSL
$t_{0.5}$=175
$t_{0.5}$=265
p=0.0027
0
50
100
150
200
250
300
350
Days

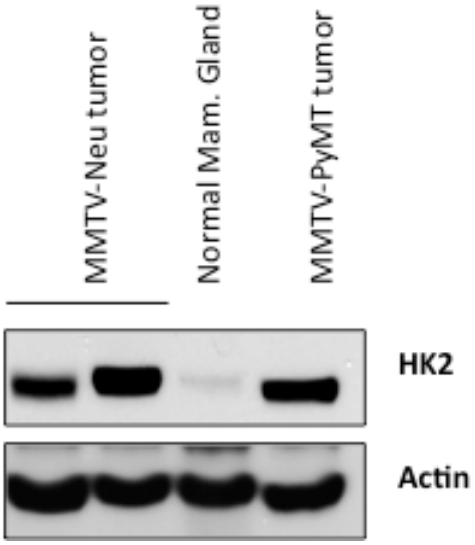
3.2 HK2 is required for tumor initiation in mice model of breast cancer.

Many genetic and epigenetic factors contribute to the initiation and progression of breast cancer. Amplification of Her2/ErbB2, Cyclin-D and c-myc oncogenes are common events seen in breast ductal carcinoma in-situ (DCIS)(Osborne et al., 2004). Because of the amplification of Her2 occurs in 20%–30% of invasive breast cancer and majority of high-grade DCISs, considerable amount of efforts has been given to study and develop therapies against this oncogene (Osborne et al., 2004). Mammary gland specific expression of activated Neu (the rat ortholouge of Human Her2) in female mice causes breast tumors sharing some of the molecular and pathological similarity to the human DCISs. For my studies I used MMTV-NeuT (c-NeuV664D), MMTV-NIC (MMTV-Neu-IRES-Cre) and MMTV-PyMT mouse models of breast cancer(Fantozzi and Christofori, 2006; Ursini-Siegel et al., 2008). Because I found that HK2 expression is elevated in human breast cancer samples, I compared the normal and tumor tissues from MMTV-NeuT and MMTV-PyMT. I observed HK2 is also markedly elevated in tumor tissues isolated from these mouse models of breast cancer (Fig. 16A). MMTV-NIC mice co-express activated Neu and Cre in the mammary gland (Fig. 16B) has similar tumor latency as MMTV-NeuT mice (ref). Therefore $HK2^{f/f}$ strain was crossed and inter-crossed to MMTV-NIC mice to generate two cohorts of mice $HK2^{wt/wt}$; MMTV-NIC (WT-NIC) and $HK2^{f/f}$; MMTV-NIC ($HK2^{f/f}$-NIC) (Fig. 16C). MMTV-NIC transgenic mice develop mammary gland tumors with almost complete penetrance (Ursini-Siegel et al., 2008). Indeed as shown in Fig. 16D all the WT-NIC mice developed tumors after 30 weeks. However, the deletion of HK2 markedly delayed the onset of tumor appearance and the incidence of tumors, and most of the mice (60%) remained tumor free after 45 weeks. When tumors isolated from $HK2^{f/f}$ -NIC mice were analyzed for HK2 expression, we found that they still express relatively high levels of HK2 (Fig. 16E). Thus, these tumors arose from cells in which HK2 was not efficiently deleted, suggesting that if HK2 would be completely

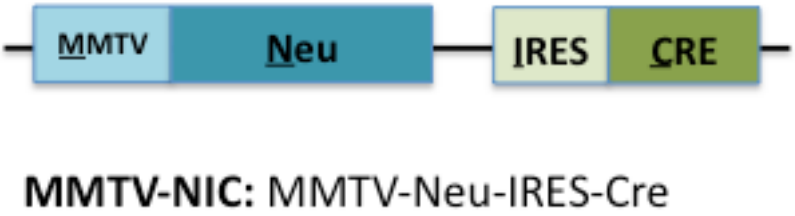
deleted in all the mammary cells, which express Neu, the outcomes would be much more substantial.

Figure. 16 HK2 is required for the development of ErbB2/Neu-driven mammary tumors in mice (A) Immunoblot showing HK2 expression in tissue lysates from normal mammary gland or mammary gland tumors isolated from MMTV-NeuT and MMTV-PyMT mice. (B) Schematic diagram showing the targeted construct of MMTV-Neu-IRES-Cre (MMTV-NIC) mice. (C) Breeding and experimental outline to generate Neu induced lung cancer in HK2 knock out background using MMTV-NIC mice.

(A)


MMTV-Neu tumor
Normal Mam. Gland
MMTV-PyMT tumor
HK2
Actin

(B)


MMTV
Neu
IRES
CRE
MMTV-NIC: MMTV-Neu-IRES-Cre

(C)

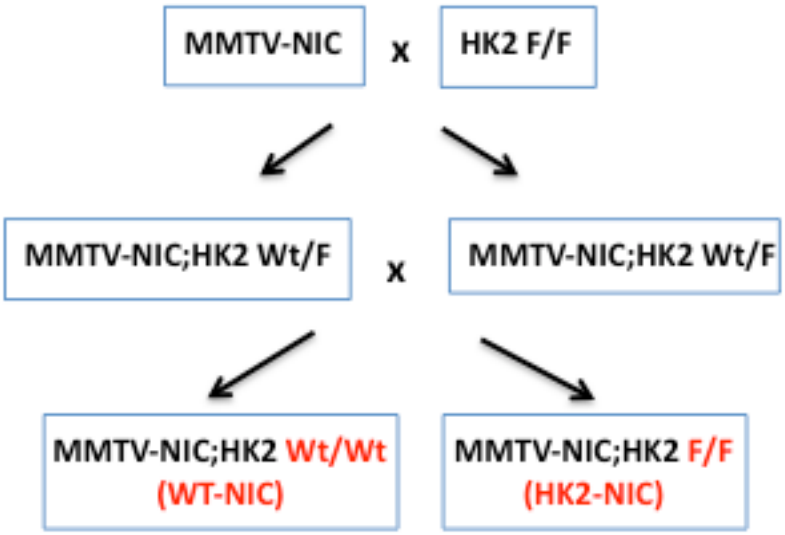
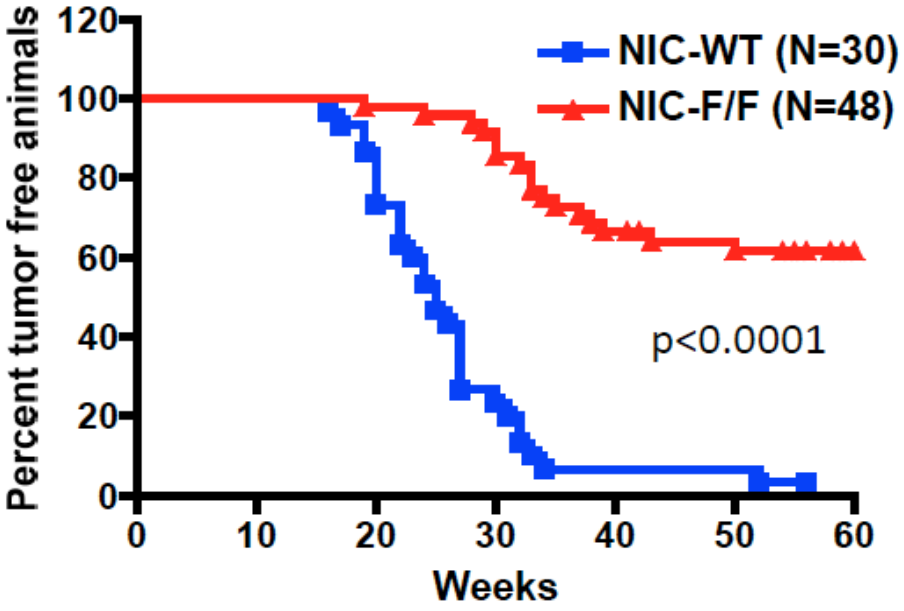
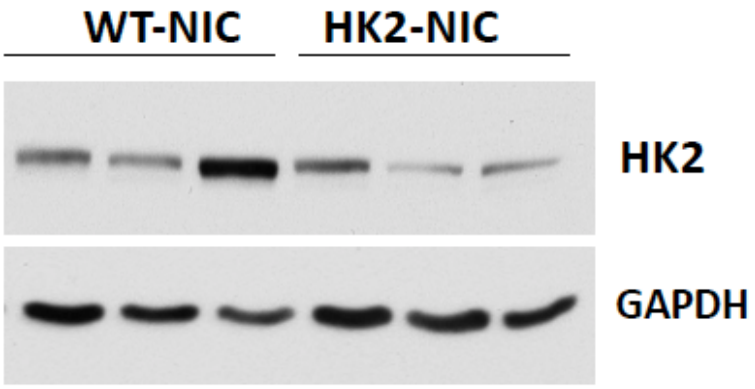

MMTV-NIC
x
HK2 F/F
MMTV-NIC;HK2 Wt/F
x
MMTV-NIC;HK2 Wt/F
MMTV-NIC;HK2 Wt/Wt
(WT-NIC)
MMTV-NIC;HK2 F/F
(HK2-NIC)

Figure 16. (continued) HK2 is required for the development of ErbB2/Neu-driven mammary tumors in mice (D) Kaplan-Meier curves of percentage tumor free survival of MMTV-NIC (WT-NIC) and MMTV-NIC-HK2$^{f/f}$ (HK2-NIC) mice. P value was calculated by log-rank test. (E) Immunoblot showing HK2 expression in tumor lysates obtained from mice with the indicated genotypes.

(D)


Percent tumor free animals
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Weeks
NIC-WT (N=30)
NIC-F/F (N=48)
p<0.0001

(E)

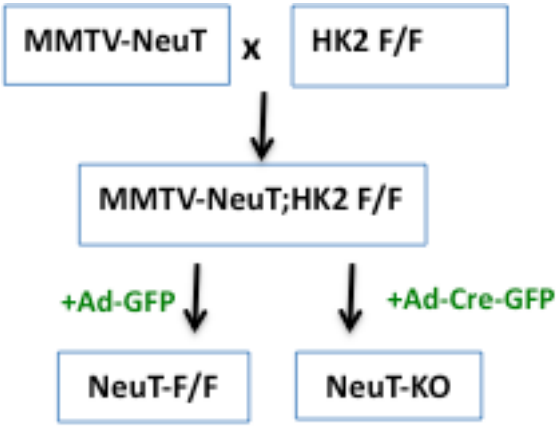

WT-NIC
HK2-NIC
HK2
GAPDH

3.3 Genetic ablation of HK2 reverses tumorigenic potential of Neu transformed breast cancer cells.

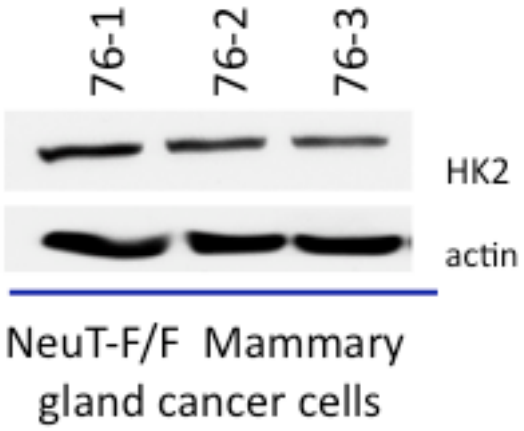
The above-described experiments suggest that HK2 is required for breast tumor initiation; therefore we verified whether the tumorigenic potential of Neu transformed murine mammary gland cells can be reversed by HK2 ablation. To test this possibility we generated $HK2^{f/f}$; MMTV-NeuT (Muller et al., 1988)compound mice by crossing $HK2^{f/f}$ and MMTV-NeuT strains (schematic diagram (Fig. 17A)). Few mammary gland cancer cell lines (named them as NeuT-F/F cells) were established from the tumors developed in this mouse and found to express HK2 in protein level (Fig. 17B). HK2 was deleted by Ad-Cre-GFP (Fig. 17C), and early passage HK2 proficient or HK2 deficient cells were subjected to orthotropic transplantation. As compared to HK2 proficient tumor cells (NeuT-F/F: Adeno-GFP treated cells) tumor formation by HK2 deficient cells (NeuT-KO: Adeno-Cre-GFP treated cells) was dramatically attenuated (Fig. 17D). HK2 deficient tumors are significantly smaller than the control tumors (Fig. 17E). The tumors isolated from HK2 showed absence of HK2 without any changes of HK1 (Fig. 17F). The impaired ability of HK2 deficient cells to form tumors is due to impaired proliferation as verified by a decrease in BrdU incorporation (Fig. 17G,H). Thus, these results clearly show that HK2 ablation could reverse the tumorigenicity of tumor cells derived from mouse mammary tumors.

Figure 17. The deletion of HK2 reverses the oncogenic potential of mouse mammary tumor cells derived from MMTV-NeuT-HK2$^{f/f}$ mice. (A) Schematic diagram of the generation of NeuT-HK2$^{f/f}$ cells. (B) Cell line derived from mammary tumor of MMTV-NeuT-HK2$^{f/f}$ mice expresses HK2. 76-1,2,3 are three cell lines derived from 3 different tumors(C) Immunoblot analysis of HK2 expression in HK2 F/F-NeuT mouse mammary gland tumor cells treated with either Adeno-GFP or Adeno-Cre-GFP to delete HK2 to generate NeuT-F/F or NeuT-KO.

(A)


MMTV-NeuT
x
HK2 F/F
MMTV-NeuT;HK2 F/F
+Ad-GFP
+Ad-Cre-GFP
NeuT-F/F
NeuT-KO

(B)


76-1
76-2
76-3
HK2
actin
NeuT-F/F Mammary gland cancer cells

(C)

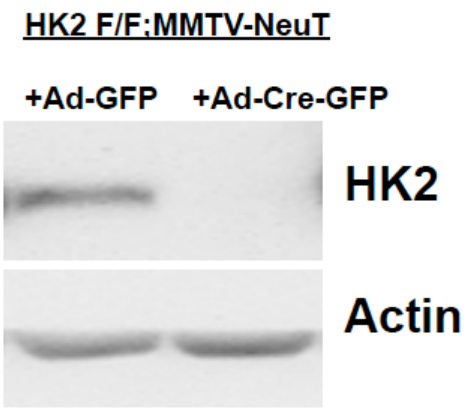
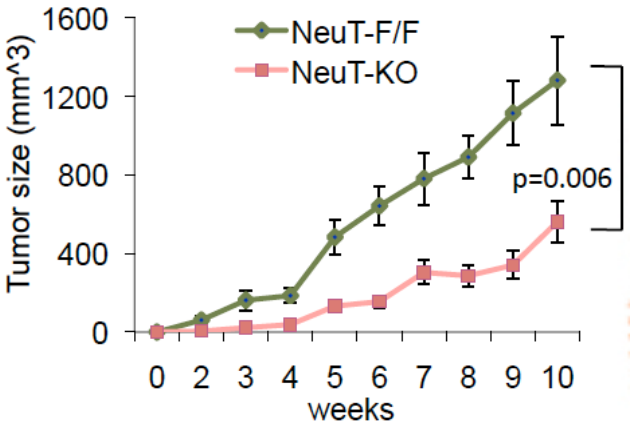

HK2 F/F;MMTV-NeuT
+Ad-GFP
+Ad-Cre-GFP
HK2
Actin

Figure 17. (continued). The deletion of HK2 reverses the oncogenic potential of mouse mammary tumor cells derived from MMTV-NeuT-HK2$^{f/f}$ mice. (D) Genetic ablation of HK2 dramatically reduces the tumorigenic potential of MMTV-NeuT cancer cells. NeuT-F/F or NeuT-KO cells were injected orthotopically (1.5 x 10^6 cells in 50% matrigel) in female athymic mice (6 mice per group) and tumor growth was followed until tumor size reached approximately 2cm^3. (E) Representative images of tumors isolated at the end point of the study. (F) Immunoblot showing HK2 protein levels in tumor protein lysates from individual tumors shown in E. (G) Representative image of BrdU staining in NeuT tumors. (H) Quantification of average BrdU incorporation in tumor 40X sections derived from the tumors described in E. BrdU incorporation, staining, and quantification are described in detail in Experimental Procedures.

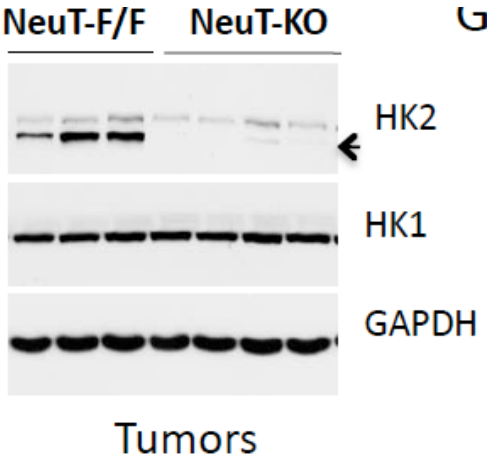
(D)


1600
1200
800
400
0
Tumor size (mm^3)
NeuT-F/F
NeuT-KO
p=0.006
0 2 3 4 5 6 7 8 9 10
weeks

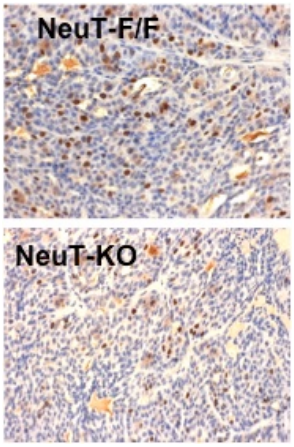
(E)

NeuT F/F
NeuT KO

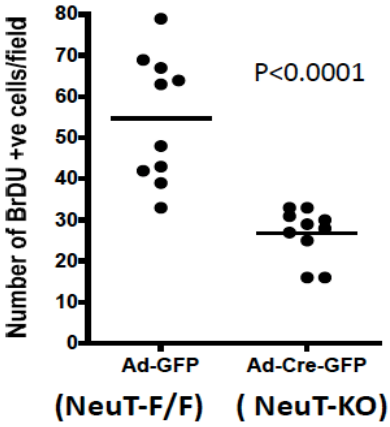
(F)


NeuT-F/F
NeuT-KO
HK2
HK1
GAPDH
Tumors

(G)


NeuT-F/F
NeuT-KO

(H)


Number of BrDU +ve cells/field
80
70
60
50
40
30
20
10
0
P<0.0001
Ad-GFP
Ad-Cre-GFP
(NeuT-F/F)
(NeuT-KO)

3.4. HK2 deletion impairs liver carcinogenesis in mice.

Glucokinase (GCK) is the only expressed hexokinase isoform in normal adult livers. In contrast HK2 expression is seen in HCC progression (Fig. 3A). Interestingly GCK expression is lost with concomitant expression of HK2 in human HCC cell lines (Fig. 7A). Because of the down regulation of HK2 expression is disadvantageous to HCC cell lines *in vitro* (Fig. 7C,D) and *in vivo* (xenograft)(Fig. 10A) we wanted to verify if HK2 is required for tumor formation in carcinogen induced liver cancer model *in vivo*. To do so HK2 $^{f/f}$ mice were crossed to albumin-cre (alb-cre) mice to generate HK2$^{f/f}$; Alb-Cre mice in which HK2 is specifically deleted in the liver (Liv HK2 KO). In Alb-Cre mice, Cre is expressed from hepatocyte-specific promoter (Albumin) and recombination of the floxed allele starts in prenatal condition, gradually increasing in the adult. As HK2 is not expressed in adult liver we could not assay the deletion efficiency in protein level. Therefore we characterized the adult HK2$^{f/f}$; Alb-Cre liver tissues by genomic PCR for the presence of recombined and non-recombined HK2 allele. Although the appearance of recombined and loss of floxed allele of HK2 is evident from genomic PCR but still we could detect the non-recombined allele in liv HK2 KO (Fig.18A) that can be explained by the fact that adult liver contains ~30% non-hepatocytes (Si-Tayeb et al., 2010).

Diethylnitrosamine (DEN) is a chemical carcinogen that induces DNA damage and oxidative stress in the liver. Two-week-old mice that receive high dose of DEN subsequently develop liver cancer when they are 8 months old(Maeda et al., 2005).

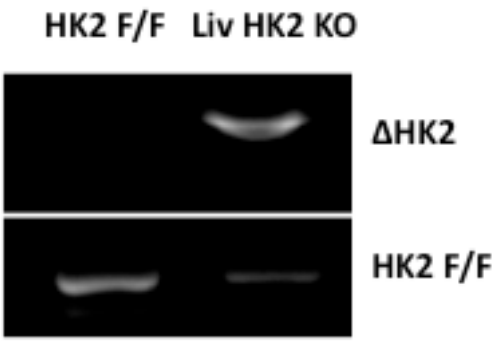
Two weeks old liv HK2 KO (Experimental) and HK2 $^{f/f}$ (Control) male mice were subjected to a single dose (25mg/Kg B.W.) of DEN to develop carcinogen induced liver cancer. The animals were sacrificed at 8-9 months age after DEN treatment and their liver was analyzed. Tumor number was significantly less in liv HK2 KO as compared to HK2 $^{f/f}$ (Fig.18C). However we could not find any statistically significant difference in tumor size.

To our surprise we found HK2 expression was not high in tumors analyzed from either HK2 $^{f/f}$ control or liv HK2 KO mice. As a very low level of HK2 was detected from tumors arised in both control and HK2 KO liver, we assume this signal is from some other tumor resident cells (i.e. immune cells). This HK2 expression pattern in DEN induced mouse tumor, contrasts our observation in human HCC samples. Therefore it is possible that a subset of cells which express HK2 in post-natal liver are transformed by DEN. Absence of HK2 from those cells may affect the transformation process.

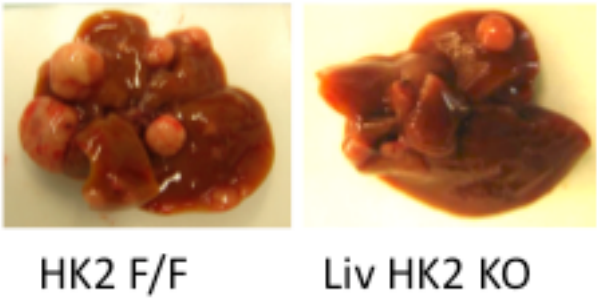
In summary, these results provided compelling evidence that HK2 is required for both oncogenic K-Ras driven lung tumorigenesis and ErbB2 driven mammary gland tumorigenesis *in vivo*. HK2 expression is essential for the neoplastic phenotype of murine breast cancer cells and that HK2 ablation reverses the tumorigenicity of these cancer cells *in vivo*. HK2 ablation from the tumors causes a proliferative disadvantage *in vivo*. Thus the importance of HK2 that was observed in human NSCLC and breast cancer cells was recapitulated in mouse models of NSCLC and breast cancer. Using a DEN induced chemical carcinogenesis method of liver tumorigenesis in mice we showed that HK2 is required for tumor initiation although these tumors do not express HK2 as observed in human HCC tissues and cell lines.

Figure 18. Liver specific knockout of HK2 reduces tumor burden in chemical carcinogen induced HCC in mice. (A) Genomic PCR showing the appearance of recombination specific and reduction of unrecombined PCR product in HK2 deleted liver. (B) HK2 deletion reduces the tumor burden in DEN induced tumor. Picture of tumors generated in $HK2^{f/f}$ and liver specific knockout of HK2 (Liv HK2 KO). Two weeks old mice of indicated genotypes were injected with a high dose of DEN (25mg/Kg B.W). Nine month after tumor generation animals were opened and tumor number and size was quantified. (C) Average tumor number (greater than 1.0mm) in HK2 F/F (N=24) and Liv HK2 KO (N=20) mice. (D) Immunoblot for HK2 in the tumor and non-tumor tissues from the indicated genotype shows there is no change of HK2 in protein level in either genotype after nine months. MEFs were used as a positive control for HK2. Tubulin was used as a loading control. Note: To achieve the HK2 signal in the tumors, the HK2 blot was exposed for a very long time.

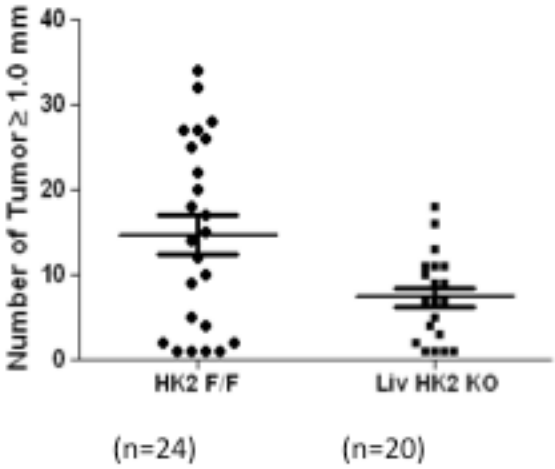
(A)


HK2 F/F
Liv HK2 KO
ΔHK2
HK2 F/F

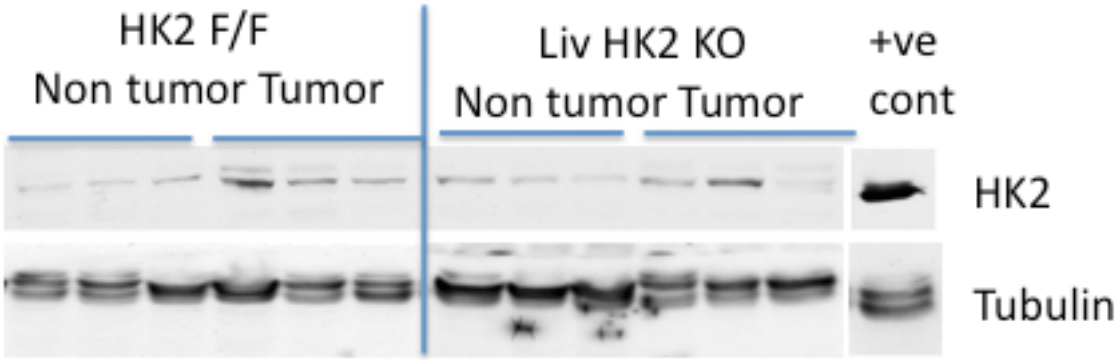
(B)


HK2 F/F
Liv HK2 KO

(C)


Number of Tumor ≥ 1.0 mm
40
30
20
10
0
HK2 F/F
Liv HK2 KO
(n=24)
(n=20)

(D)


HK2 F/F
Non tumor Tumor
Liv HK2 KO
Non tumor Tumor
+ve
cont
HK2
Tubulin

3. Systemic HK2 deletion in adult mice does not cause an overt phenotype but reduces tumor burden of lung cancer

A) Background

HK2 is highly expressed in many cancers (Shinohara, 1994), and although it is abundantly expressed in embryonic tissues, it constitutes the predominant hexokinase isoform in only a limited number of adult tissues. In contrast, the HK1 isoform is ubiquitously expressed and constitutes the major hexokinase in most normal adult tissues (Wilson, 1985; Wilson, 2003). As was shown in the previous sections, HK2 is expressed at high levels in cancer cells but is hardly detectable in the corresponding normal tissues from which they were derived. HK2 is the predominant expressed isoform in only limited number of adult tissues such as fat, muscles and heart, and mice with 50% deletion of HK2 do not show an overt phenotype except of mildly impaired glucose homeostasis in response to exercise and high fat diet (Fueger et al., 2003). The next question that needed to be addressed is whether systemic whole body ablation of HK2 can be tolerated and whether it can reverse tumorigenesis without eliciting adverse physiological consequences. Therefore we systemically deleted HK2 in adult mice and found there is no overt phenotype. Also we showed that tumor burden was significantly reduced after systemic deletion of HK2 following tumor onset in K-Ras induced NSCLC mouse model.

B) Results

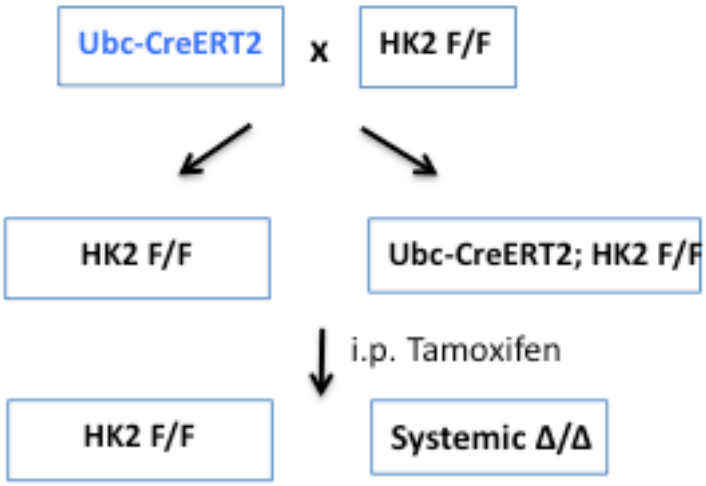
1. Systemic deletion of HK2 is tolerated in mice without any overt physiological consequences.

In order to systemically delete HK2 in all adult tissues we used ($UBC^{CreERT2}$) (Ruzankina et al., 2007), which ubiquitously express tamoxifen inducible Cre. Following exposure to tamoxifen, Cre mediated recombination occurs in systemic manner with

relatively high efficiency. These mice were crossed to HK2$^{f/f}$ to generate HK2$^{f/f}$UBC^{CreERT2} mice. The mice were treated with tamoxifen when they were two month old (Fig. 19A). As shown in Fig. 19B, HK2 was effectively deleted in adult tissues expressing high levels of HK2, although the deletion was incomplete in some of the tissues. Importantly, HK2 deletion was almost complete in skeletal muscle, where this is the major isoform and HK2 deletion did not result in any compensatory expression of HK1 isoform in skeletal muscle (Fig. 19B). Surprisingly, global HK2 ablation in adult mice was well tolerated, and HK2-deficient mice were indistinguishable from control mice both morphologically and in terms of growth and body weight (Fig. 19C). We then examined if these mice display impaired glucose homeostasis. Interestingly, mice globally deficient for HK2 had normal levels of insulin and under both fed or fasting conditions glucose level was similar to WT mice (Fig. 19D-E). In glucose tolerance test (GTT) both the WT and HK2 deficient mice showed similar response kinetics (Fig. 19F). These mice were continuously monitored for over two years without development of any overt phenotype that distinguishes them from control mice. These important results indicate that systemic HK2 inhibition is feasible and can be exploited as a safe target for cancer therapy, without any major metabolic consequences.

Figure 19. Systemic ablation of HK2 in adult mice does not cause any overt phenotype. (A) Schematic diagram of breeding strategy and generation of systemic HK2 knockout mice. $HK2^{f/f}$ or $HK2^{f/f}UBC^{CreERT2}$ mice treated with tamoxifen at 8 weeks. For details see Experimental Procedures. (B) Immunoblot showing HK2 and HK1 protein expression in tissues isolated from either $HK2^{f/f}$ or $HK2^{f/f}UBC^{CreERT2}$ mice two weeks after final tamoxifen injection. Systemic HK2 ablation does not cause any effect on glucose homeostasis in mice. The animals were treated with tamoxifen at 8 weeks (C) body weight (D) Fed glucose, (E) Insulin level D) glucose tolerant test (GTT) were measured at 6 months of age. 5-6 mice per group were used for the experiment.

(A)


Ubc-CreERT2
x
HK2 F/F
HK2 F/F
Ubc-CreERT2; HK2 F/F
i.p. Tamoxifen
HK2 F/F
Systemic Δ/Δ

(B)

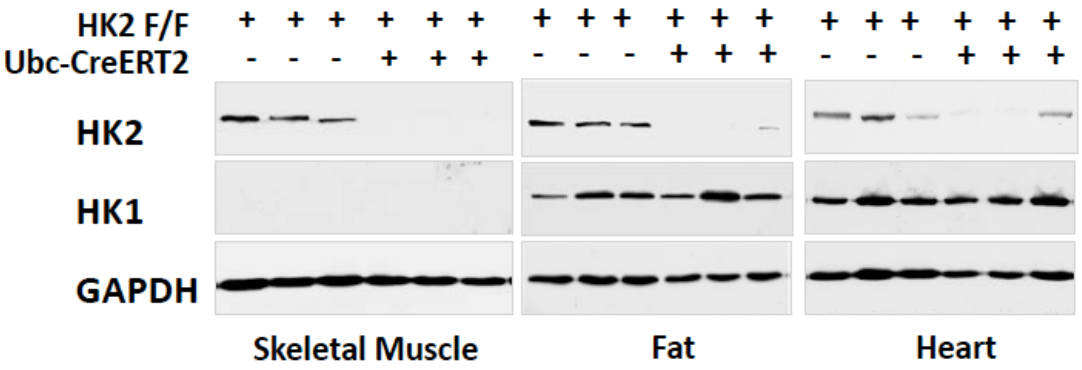
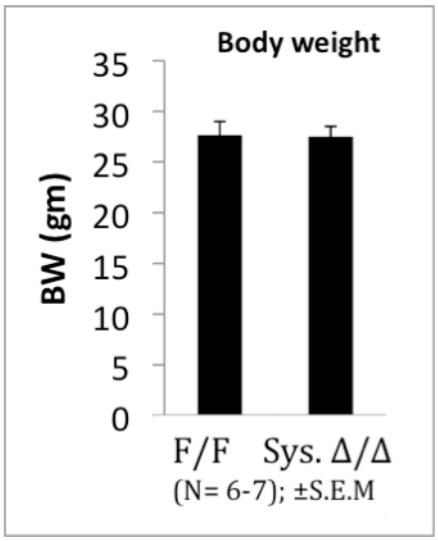
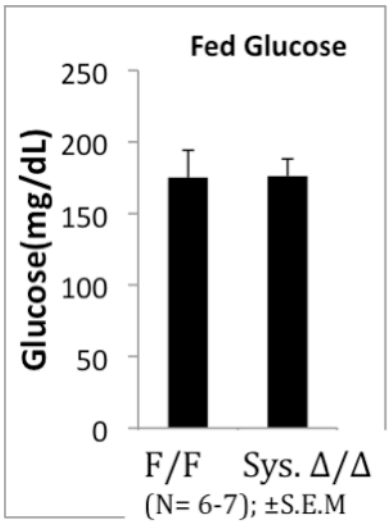

HK2 F/F
Ubc-CreERT2
HK2
HK1
GAPDH
Skeletal Muscle
Fat
Heart

Figure 19. (continued) Systemic ablation of HK2 in adult mice does not cause any overt phenotype. Systemic HK2 ablation does not cause any effect on glucose homeostasis in mice. The animals were treated with tamoxifen at 8 weeks (C) body weight (D) Fed glucose, (E) Insulin level D) glucose tolerant test (GTT) were measured at 6 months of age. 5-6 mice per group were used for the experiment.

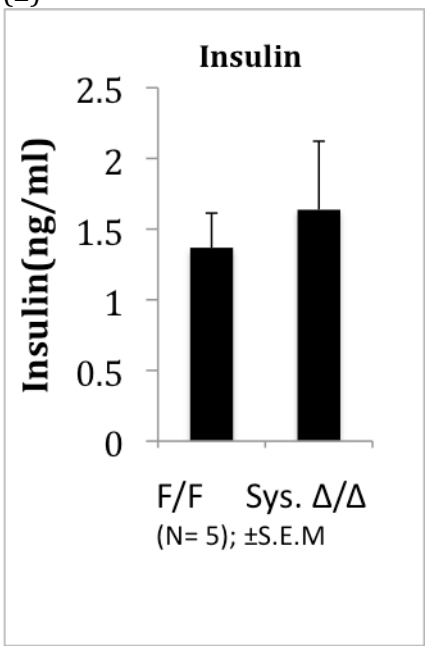
(C)


Body weight
BW (gm)
35
30
25
20
15
10
5
0
F/F
Sys. Δ/Δ
(N= 6-7); ±S.E.M

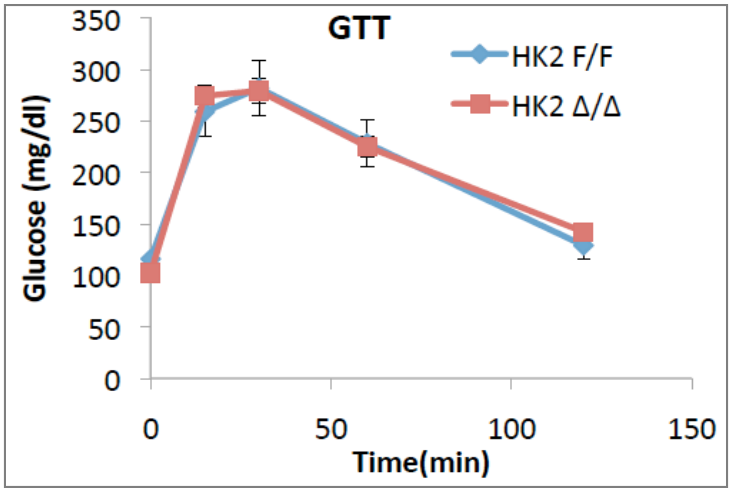
(D)


Fed Glucose
Glucose(mg/dL)
250
200
150
100
50
0
F/F
Sys. Δ/Δ
(N= 6-7); ±S.E.M

(E)


Insulin
Insulin(ng/ml)
2.5
2
1.5
1
0.5
0
F/F
Sys. Δ/Δ
(N= 5); ±S.E.M

(F)


GTT
HK2 F/F
HK2 Δ/Δ
Glucose (mg/dl)
350
300
250
200
150
100
50
0
0
50
100
150
Time(min)

2. Systemic ablation of HK2 is therapeutic in a mouse model of lung cancer.

As was shown in the previous sections, HK2 is required for the initiation of K-Ras induced lung tumor and systemic ablation of HK2 is well tolerated in mice. Therefore we asked the next important question of therapeutic relevance, i.e. if systemic ablation of HK2 can reverse tumorigenesis *in vivo*. We employed the K-Ras$^{LA2-G12D}$ NSCLC mouse model, which develops lung tumors with an early onset and complete penetrance (Johnson et al., 2001) along with tumors in many other organs. As shown in Fig. 20A small tumor lesions are visible at 10 weeks and progresses to a high lung tumor load around 20 weeks. We intercrossed HK2$^{f/f}$UBC^{CreERT2} and K-Ras$^{LA2-G12D}$ mice to generate K-Ras$^{LA2-G12D}$UBC^{CreERT2}, K-Ras$^{LA2-G12D}$HK2$^{f/f}$, and K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice. These mice were injected with tamoxifen after tumor lesions onset, at 2 months of age, to systemically delete HK2. Mice were sacrificed 4 months later and lungs were analyzed for tumor burden (see schematic diagram in Fig. 20B). There was no significant difference in tumor burden among tamoxifen treated control groups K-Ras$^{LA2-G12D}$HK2$^{f/f}$ and K-Ras$^{LA2-G12D}$UBC^{CreERT2} treated with tamoxifen (Fig. 20D). However, tumor burden was substantially decreased after HK2 deletion in K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice as compared to K-Ras$^{LA2-G12D}$HK2$^{f/f}$ mice (Fig. 20C-D). Likewise, tumor numbers per mouse were significantly decreased after HK2 deletion (Fig. 20E). Because of the stochastic nature the inducible Cre system, HK2 expression was still detectable in some tumors arising in tamoxifen-treated K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice (Fig. 20F). Therefore, it is likely that a sub-group of the tumors that appeared in tamoxifen–injected K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice arose from cell lineages that escaped complete HK2 deletion.

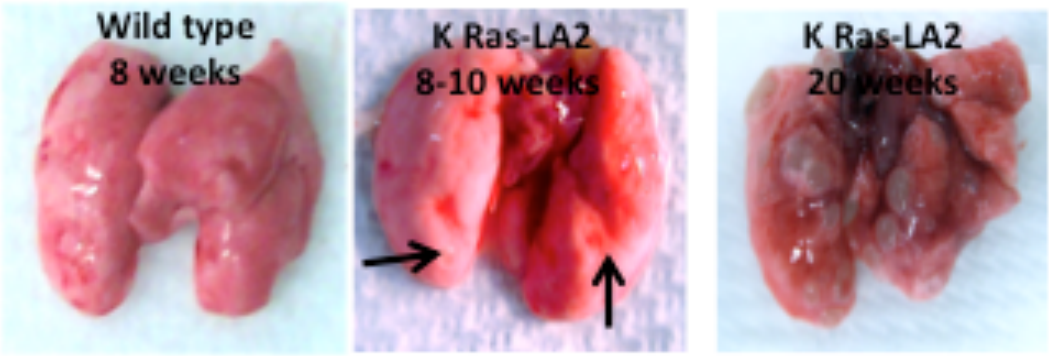
In order to characterize the tumors immediately after HK2 loss, we injected tamoxifen to 16-18 weeks old mice, at a stage in which most of the tumors express very high levels of HK2. One week after tamoxifen injection, we analyzed the tumors for BrdU

incorporation. We found that the tumors derived from the tamoxifen-injected K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice incorporated substantially less BrdU when compared to control mice (Fig. 20G), indicating that HK2 is required for the proliferation of established lung tumor cells *in vivo*.

Importantly, taken together, these observations provided compelling genetic evidence that systemic deletion of HK2 in adult is well tolerated and genetic ablation of HK2 in established lung tumor dramatically decreases tumor burden in mice. Therefore HK2 could be an attractive target for lung cancer therapy, without adverse physiological consequences.

Figure 20. Systemic deletion of HK2 in the mouse is therapeutic for oncogenic K-Ras driven NSCLC. (A). The picture of the lungs of K-Ras$^{LA2\text{-}G12D}$ mouse showing appearance of small adenomas at 8-10 weeks which results in a massive tumor load around 20 weeks. WT mouse lungs were presented for a comparison. (B) Schematic illustration of the experiment described in (C-F).

(A)


Wild type
8 weeks
K Ras-LA2
8-10 weeks
K Ras-LA2
20 weeks

(B)

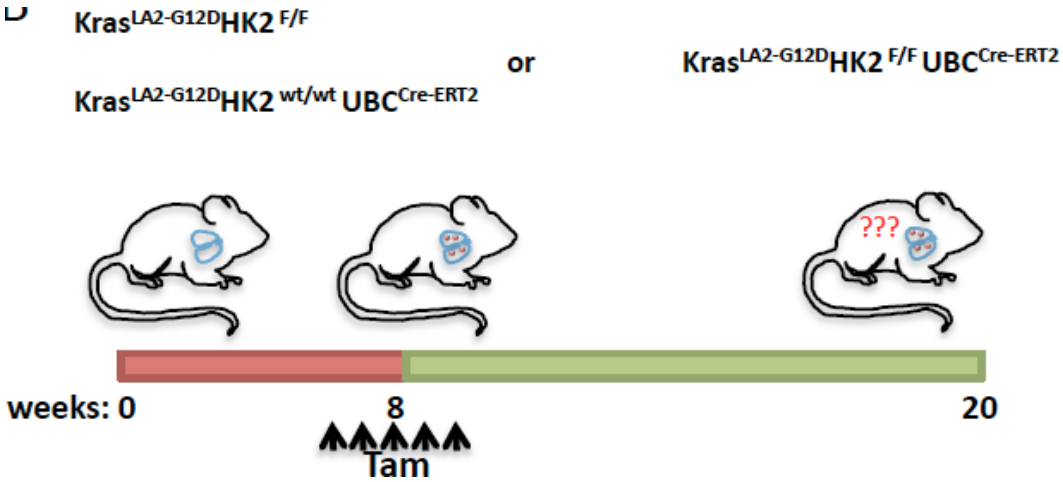
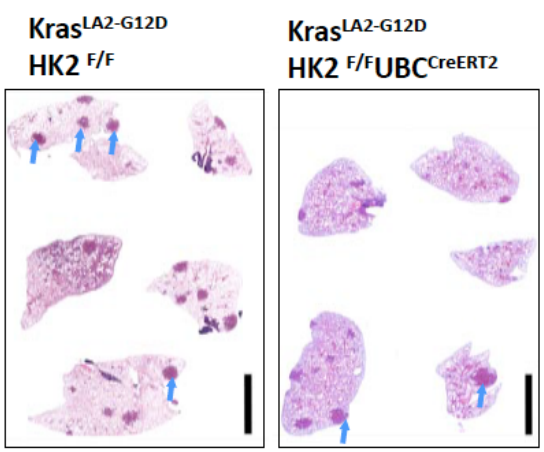
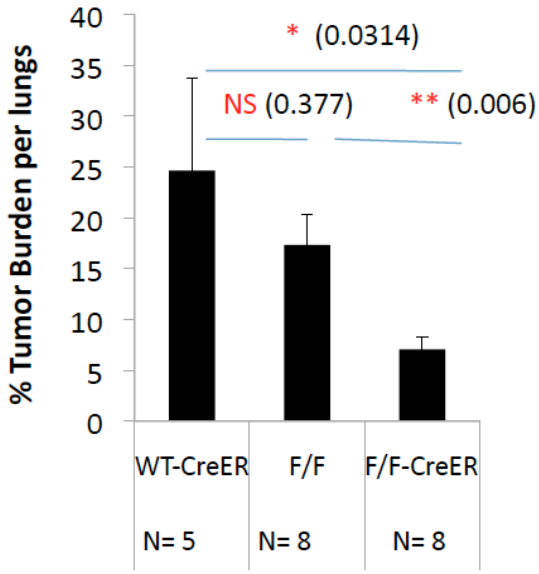

Kras$^{LA2-G12D}$HK2$^{F/F}$
Kras$^{LA2-G12D}$HK2$^{wt/wt}$ UBC$^{Cre-ERT2}$
or
Kras$^{LA2-G12D}$HK2$^{F/F}$ UBC$^{Cre-ERT2}$
???
weeks: 0
8
20
Tam

Figure 20. (continued) Systemic deletion of HK2 in the mouse is therapeutic for oncogenic K-Ras driven NSCLC (C) Representative images of H&E staining of whole-mount lung sections prepared from either K-Ras$^{LA2-G12D}$HK2$^{f/f}$ or K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} lungs mice, 12 weeks after injection of tamoxifen at 8 weeks of age. Individual tumors are indicated by arrows (scale bare 4mm). (D) Percentage tumor burden as calculated from the H&E sections of tumor bearing lungs from either K-Ras$^{LA2-G12D}$UBC^{CreERT2} (N=5) or K-Ras$^{LA2-G12D}$HK2$^{f/f}$ (N=8) or K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} (N=8) at 20 weeks of age after injection of tamoxifen at 8 weeks of age. P-values were given on the top figure in red asterisks (numbers) (E) Average tumor number per lung in whole-mount lung sections prepared from either K-Ras$^{LA2-G12D}$HK2$^{f/f}$ or K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice (F). Immunoblot showing HK2 and HK1 protein levels in tumor lysates extracted at 20 weeks of age, and after tamoxifen injection at 8 weeks of age. (G) Average number of BrdU positive cells/per field calculated from (40X magnification) of tumor sections. K-Ras$^{LA2-G12D}$HK2$^{f/f}$ or K-Ras$^{LA2-G12D}$HK2$^{f/f}$UBC^{CreERT2} mice were injected tamoxifen for 5 doses at 16-18 weeks, culled 1 week after last tamoxifen treatment and tumor tissue sections were analyzed for BrdU positive cells.

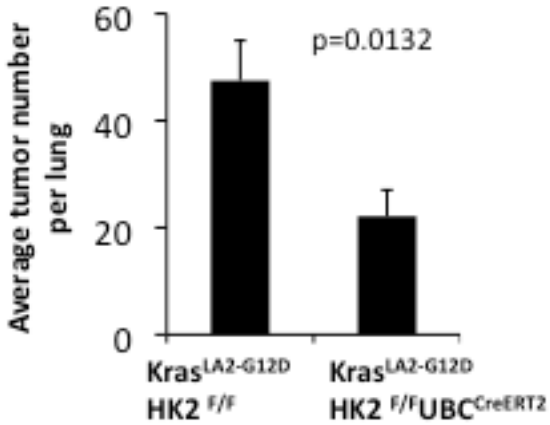
(C)


KrasLA2-G12D
HK2 F/F
KrasLA2-G12D
HK2 F/FUBCCreERT2

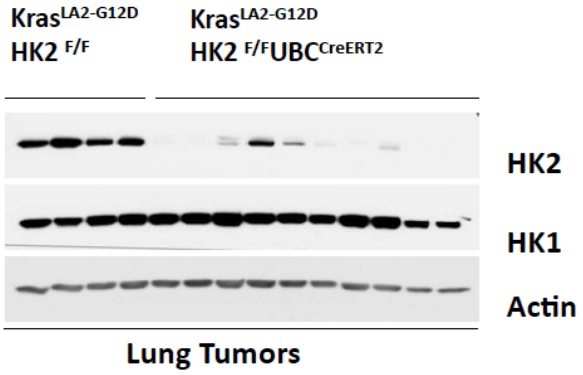
(D)


% Tumor Burden per lungs
40
35
30
25
20
15
10
5
0
* (0.0314)
NS (0.377)
** (0.006)
WT-CreER
F/F
F/F-CreER
N= 5
N= 8
N= 8

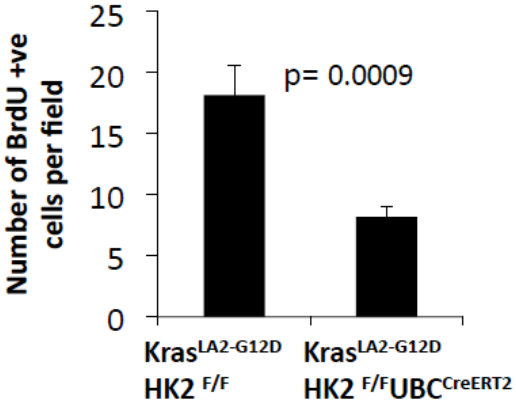
(E)


Average tumor number per lung
60
40
20
0
p=0.0132
KrasLA2-G12D
HK2 F/F
KrasLA2-G12D
HK2 F/FUBCCreERT2

(F)


KrasLA2-G12D
HK2 F/F
KrasLA2-G12D
HK2 F/FUBCCreERT2
HK2
HK1
Actin
Lung Tumors

(G)


Number of BrdU +ve cells per field
25
20
15
10
5
0
p= 0.0009
KrasLA2-G12D
HK2 F/F
KrasLA2-G12D
HK2 F/FUBCCreERT2

4.Molecular mechanisms underlying how HK2 affects tumorigenesis.

A) Background

HK2 is one of the hexokinase, which possesses glucose phosphorylating activity and bind to mitochondria. Both HK1 and HK2 bind mitochondria and couple oxidative phosphorylation to glucose metabolism. The binding to mitochondria was also shown to maintain the integrity of mitochondria to promote cell survival(Robey and Hay, 2006). Our observation in MEFs showing that a catalytically inactive mutant cannot rescue the impaired tumorigenesis, suggests that the catalytic activity of HK2 plays a major role in tumorigenesis process. Both HK1 and HK2 are required for the first committed step in glucose metabolism that generates glucose-6-phopsphate (G6P). G6P is then utilized and required in multiple fundamental metabolic processes, which are essential for many downstream pathways in rapidly dividing cells(Lunt and Vander Heiden, 2011). These include 1) NADPH (which can balance reactive oxygen species) and ribonucleotides synthesis through the PPP, 2) the generation of hexosamines, which are required for over all glycosylation of the proteins 3) intermediates for de novo Serine/Glycine amino acids biosynthesis pathways. It is, therefore, possible that the induction of HK2 expression in cancer cells, which already express HK1, is required for the high glycolytic flux for accelerated anabolic processes such as DNA, RNA and lipid synthesis in cancer cells. Therefore we conducted metabolic profile analysis of HK2 proficient and deficient NSCLC cells through mass spectrometry. HK2 deletion in K-Ras^{G12D}-NSCLC cells diminished glucose-derived ribonucleotides, phosphoserine, and fatty acids synthesis, and unexpectedly suppressed glutamine-derived carbon utilization in anaplerosis. Thus, HK2 is required for pivotal metabolic pathways in cancer cells.

B) Results

1. HK2 efficiency in K-Ras^{G12D}-NSCLC cells impairs the downstream metabolites

1.1 Nucleic acid biosynthesis pathway.

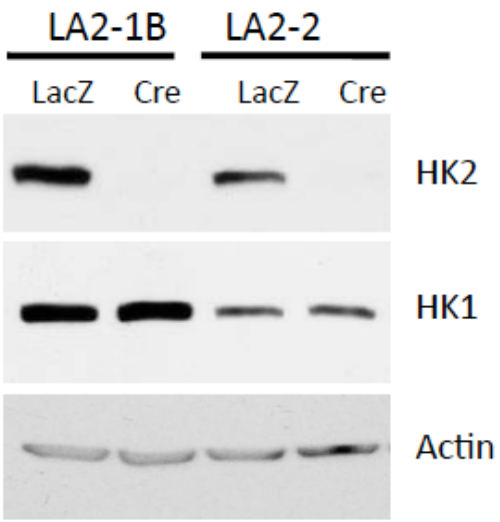
To study the metabolic changes occur by HK2 deletion we first established tumor cell lines from lung tumors of K-Ras$^{LA2-G12D}$ HK2$^{f/f}$ mice. For long-term studies the cell lines were first infected with retrovirus expressing dominant-negative p53. Two isogenic pairs of cell lines were chosen for further analyses, and were subjected to adenovirus infection with either Ade-LacZ or Ade-Cre to delete HK2. The deletion of HK2 (Fig. 21A) impaired the proliferation of the cells (Fig. 21B). We then conducted metabolomics analyses of the cells using liquid chromatography tandem mass spectrometry (LC/MS/MS). Although the steady levels of many metabolites were decreased by the deletion of HK2 in both cell lines (Fig. 22A), the most statistically significant decrease was found in the levels of fructose 1, 6 bisphosphate (F1, 6BP) and phosphoserine. To further corroborate these results and to determine that the decrease in the metabolites is due to impaired glucose flux, we conducted flow injection-time-of-flight mass spectrometry as previously described (Fuhrer et al., 2011; Kung et al., 2012). Analysis of a large pool of metabolites by this method we found that level of several nucleic acid and fatty acids are significantly down regulated by HK2 deletion (Fig. 22B). Confirming the LC/MS analyses we found again that phosphoserine levels are markedly reduced by the deletion of HK2 (Fig. 22C), suggesting that HK2 is required to fully support the serine biosynthesis pathway. To follow the glucose metabolism in HK2 knockout cells with labeled isotopomer of glucose (U6-^{13}C glucose, 1,2-^{13}C glucose, 3,4-^{13}C glucose). We found a significant decrease in phosphohexoses level, which was further confirmed by the reduced incorporation of ^{13}C labeled glucose into phosphohexoses (Fig. 22D). The analyses also showed that the steady state level of intracellular levels of ribonucleotides as well as the incorporation of the ^{13}C labeled glucose

into ribonucleotides were severely impaired by the deletion of HK2 (Fig. 22E). However, glucose derived ribulose-5-phosphate (Ru5P), which is the end product of the oxidative PPP was not affected (Fig. 22F). The glucose derived S7P, in the non-oxidative branch of the PPP was also not significantly reduced (Fig. 22F). Nonetheless, glyceraldehyde 3-phosphate (G3P) derived from glucose, which is required for the generation of ribose-5-phosphate (R5P) in the non-oxidative branch of the PPP is significantly reduced (Fig. 22G). In a pervious study in a mouse model of K-Ras-dependent pancreatic ductal adenocarcinoma it was shown that oncogenic K-Ras induces nucleotides biosynthesis largely through the enhancement of the non-oxidative branch of the PPP (Ying et al., 2012). Therefore, it is likely that the effect of HK2 deletion on nucleotides biosynthesis is due, at least in part, to the reduced G3P as well as dihydroxyacetone-phosphate (DHAP) derived from glucose. We therefore speculate that in HK2 deleted cells the PPP is in a mode that does not support high generation of ribonucleotides through the non-oxidative branch of the PPP but rather maintains G6P/F6P equilibrium and NADPH derived from the oxidative PPP (Fig. 24). Indeed, as shown in Fig. 22A, NADP and NADPH levels were not significantly changed after HK2 deletion. Maintaining NADPH levels is required for redox balance and for lipid biosynthesis(Vander Heiden et al., 2009). Therefore, it is possible that in the absence of HK2 more Ru5P and R5P are shunted to regenerate F6P instead of being used to generate ribonucleotides. Regeneration of F6P consumes G3P although not to the same extent as for the generation of R5P (Fig. 24). Thus, it is possible that because of the utilization of G3P in glycolysis and other pathways its level is not maintained to fully support oncogenic K-Ras dependent nucleotide biosynthesis. Consistent with the reduced diversion of glucose into ribonucleotides synthesis in the absence of HK2, we found a significant reduction in the incorporation of both U6-^{14}C labeled glucose and 1-^{14}C labeled glucose, which labels only nucleotides derived from the non-oxidative branch of the PPP, into DNA and RNA in the

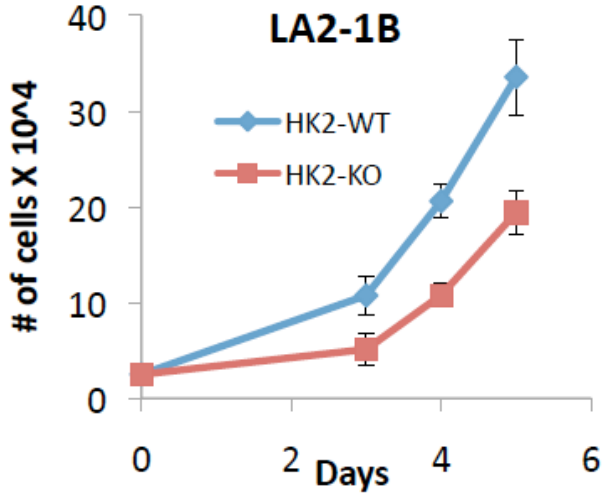
absence of HK2 (Fig. 22H). Hence glucose flux through HK2 is important for maintaining phosphoserine and ribonucleotide pool in cancer cells.

Figure 21. Characterization of the K-Ras^{G12D} NSCLC cell lines used in the metabolomic analysis. (A) Immunoblot of HK1 and HK2 in two cell lines LA2-1B and LA2-2 cells. (LA2-1B and LA2-2), derived from tumors generated in HK2$^{f/f}$K-Ras$^{LA2-G12D}$ mice were infected with dominant-negative p53 (DN-p53) followed by infection with Ade-Cre to delete HK2 or with Ade-LacZ as a control. Cell extracts were subjected to immunoblotting with anti-HK2 or anti-HK1 antibodies. (B) Cell proliferation curves of control LA2-1B (WT) and LA2-2 (WT) or HK2- deleted LA2-1B (KO) and LA2-2 (KO) cells. Cells ($2.5x10^4$) were seeded on 6 cm plates. Cells were counted at the indicated time points in triplicates.

(A)


LA2-1B
LA2-2
LacZ
Cre
LacZ
Cre
HK2
HK1
Actin

(B)


LA2-1B
HK2-WT
HK2-KO
# of cells X 10^4
0
10
20
30
40
0
2
4
6
Days

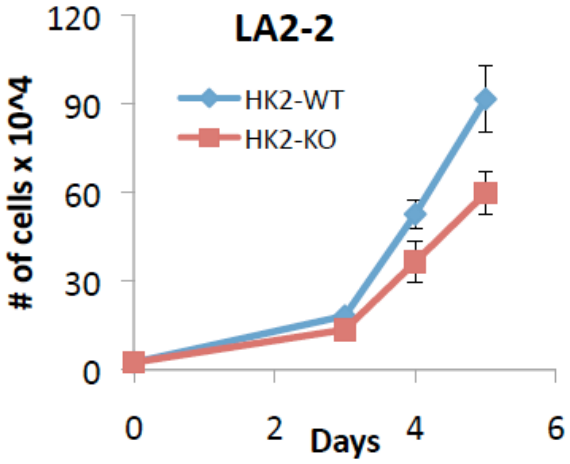
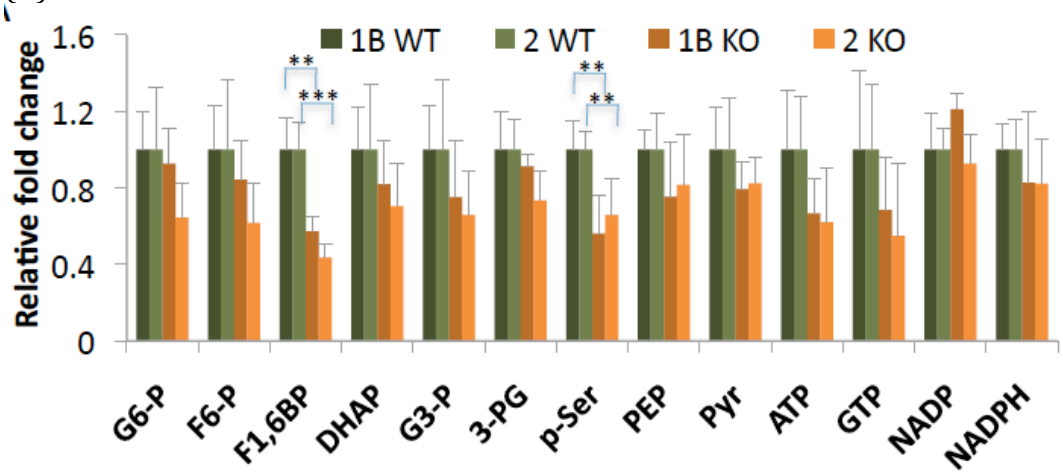
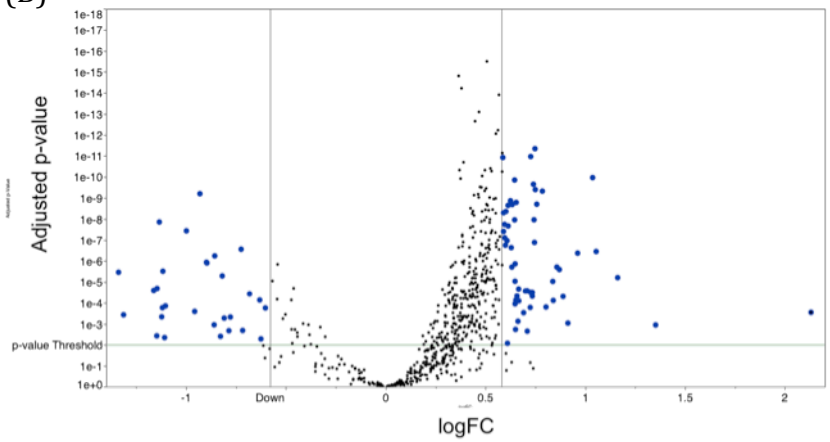

LA2-2
HK2-WT
HK2-KO
# of cells x 10^4
0
30
60
90
120
0
2
4
6
Days

Figure 22. The effect of HK2 deletion in K-Ras^{G12D} NSCLC cells on the intracellular levels of metabolites derived from glucose or glutamine. A. Steady state levels of glycolytic metabolites in LA2-1B and LA2-2 cells in the presence or absence of HK2. Data are presented as fold changes of WT versus KO. **$p < 0.005$, ***$p < 0.0005$. (B) Volcano plot of relative distribution of intracellular metabolites in HK2 knockout as compared to control LA2-1B cells analyzed in flow injection-time-of-flight mass spectrometry. Note the blue dots in the left side of the plot (which represents the level of metabolites down) are either nucleic acid or fatty acids. (C) The effect of HK2 deletion on phosphohexoses. (D) HK2 deletion decreased intracellular level of phosphoserine. Cells were grown in regular culture medium. Medium was changed to medium containing 1,2-^{13}C-glucose. At 30 minutes after medium replacement, ^{13}C isotopomers were quantified. Y-axes in B-D indicate level of signal.

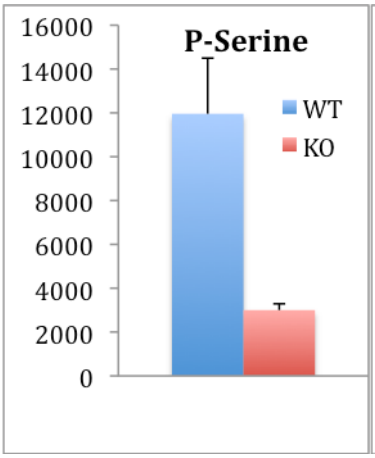
(A)


1B WT
2 WT
1B KO
2 KO
Relative fold change
1.6
1.2
0.8
0.4
0
**
***
**
**
G6-P
F6-P
F1,6BP
DHAP
G3-P
3-PG
p-Ser
PEP
Pyr
ATP
GTP
NADP
NADPH

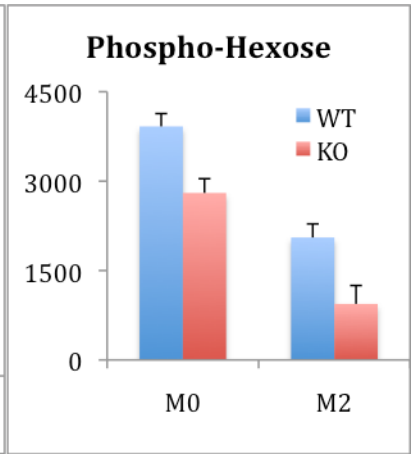
(B)


Adjusted p-value
p-value Threshold
Down
logFC

(C)


P-Serine
WT
KO
16000
14000
12000
10000
8000
6000
4000
2000
0

(D)


Phospho-Hexose
WT
KO
4500
3000
1500
0
M0
M2

1.2 Precursors of fatty acid biosynthesis.

Analysis of palmitate derived from glucose suggests that HK2 is important for the diversion of glucose into fatty acid synthesis (Fig. 22I). Reduced lipogenic channeling of glucose in HK2-deficient cells can be attributed, in part, to reduced availability of glucose-derived citrate (Fig. 22J). Cytosolic citrate is the precursor of Acetyl-CoA, which is the backbone of major fatty acids such as palmitate. Notably, the steady state level of citrate was also markedly reduced (Fig. 22J). Citrate is generated from both glucose and glutamine, and indeed glutamine-derived citrate was also decreased (Fig. 23B).

2. Glutamine-derived carbon incorporation into TCA cycle intermediates is reduced in K-Ras^{G12D}-NSCLC cells following HK2 deletion

Oncogenic Ras-transformed cells are highly dependent on the availability of glutamine, which is heavily incorporated into TCA cycle intermediates in these cells (Gaglio et al., 2011; Weinberg et al., 2010). We therefore examined HK2 deletion for the ability to alter glutamine requirements in oncogenic Ras-expressing cells. Interestingly, we found that exogenous glutamine restriction markedly reduced proliferation of both K-Ras^{G12D}-NSCLC cells and MEFs transformed by oncogenic Ras. In contrast, proliferation of these cells following HK2 deletion was not further affected by glutamine restriction (Fig. 23C).

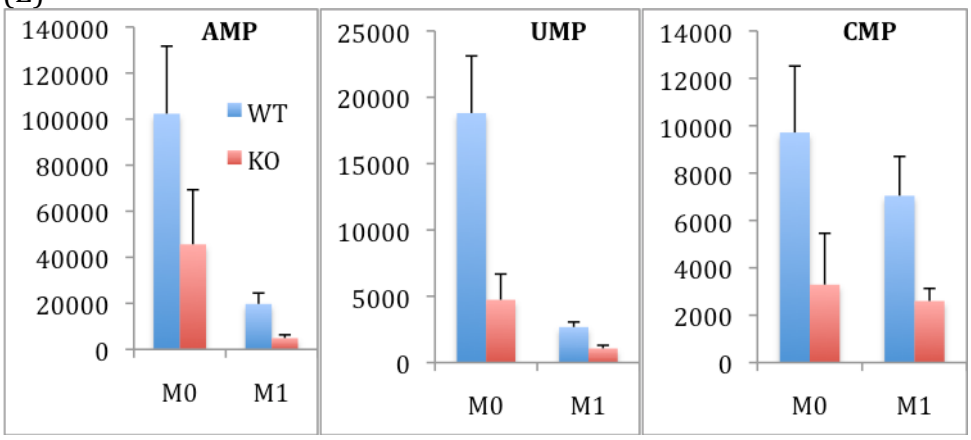
The steady state levels of glutamate, malate, and aspartate were significantly reduced by HK2 deletion (Fig. 23A). Consistently, the incorporation of U5-^{13}C glutamine into glutamate, aspartate, and citrate were markedly reduced in conditional HK2-deficient cells (Fig. 23B). A greater relative reduction in glutamate than TCA cycle intermediates is compatible with alternate glutaminolytic fates. Corresponding reductions in citrate abundance – derived from both glucose and glutamine carbon (Figs. 22J & 23B) – could be both a cause and a consequence of reduced glutamine incorporation, as anaplerosis and

cataplerosis are always tightly matched to maintain TCA cycle carbon balance (Owen, 2002). Regardless of the explanation, these results suggest that the high HK2 expression induced by oncogenic Ras is an indirect determinant of glutamine utilization. Therefore, it is possible that proliferation of oncogenic Ras-transformed cells in which HK2 is deleted is not further inhibited by glutamine restriction due to a reduced anaplerotic role for glutamine-derived carbon in these cells.

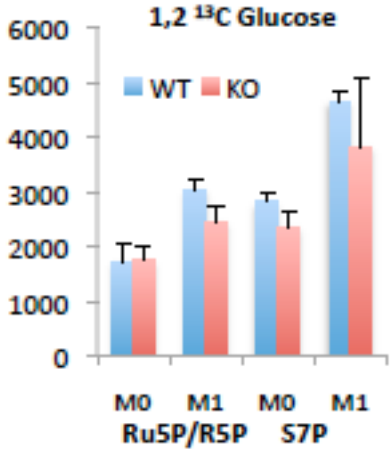
In summary, the metabolomic analysis showed that HK2 is required for high glucose flux into the ribonucleotide biosynthesis pathway in cancer cells. Reduction of phosphoserine and fatty acid precursors by HK2 deletion signifies the importance of HK2 in serine biosynthesis pathway. HK2 is required for the glutamine derived TCA cycle metabolites accumulation in cancer cell. We conclude that the high expression of Hk2 in cancer cells is required for essential metabolic processes that cannot be fully supported only by HK1, in the rapidly dividing cancer cells.

Figure 22. (continued). The effect of HK2 deletion in K-Ras^{G12D} NSCLC cells on the intracellular levels of metabolites derived from glucose (E) HK2 deletion decreased glucose-derived ribonucleotides. ^{13}C isotopomers were quantified 30min after labeling with 1,2-^{13}C-glucose. (F) Incorporation of ^{14}C labeled glucose into DNA of LA2-1B or LA2-2 cells in the presence or absence of HK2. Cells were grown in 5mM glucose pulsed with 4 micro curies U-^{14}C or 1-^{14}C glucose for 24 hr. The DNA was extracted and radioactivity measured by liquid scintillation counting (G) HK2 deletion decreased glucose derived G3P and DHAP. ^{13}C isotopomers were quantified 30min after labeling with 1,2- ^{13}C-glucose. (H) Glucose-derived Ru5P/R5P and S7P after labeling with 1,2- ^{13}C-glucose for 30 min. Y-axes in E, G, and H indicate level of signal.

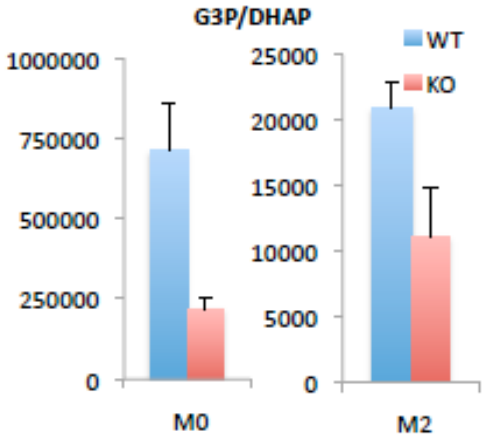
(E)


AMP
UMP
CMP
WT
KO
M0
M1

(F)


1,2 13C Glucose
WT
KO
M0
M1
M0
M1
Ru5P/R5P
S7P

(G)


G3P/DHAP
WT
KO
M0
M2

(H)

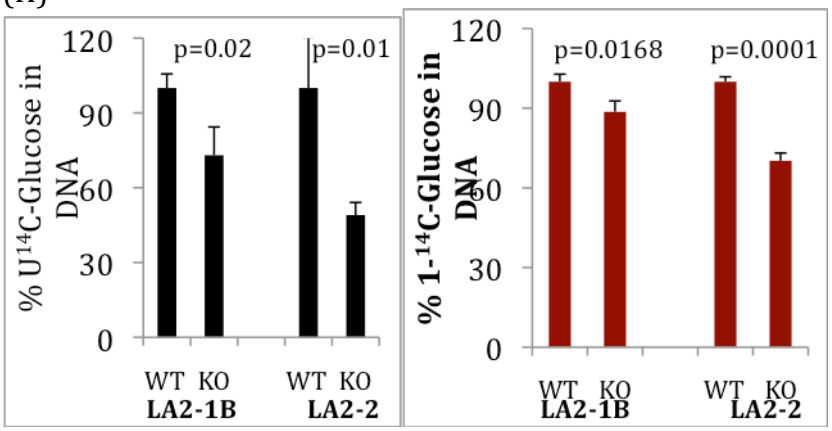
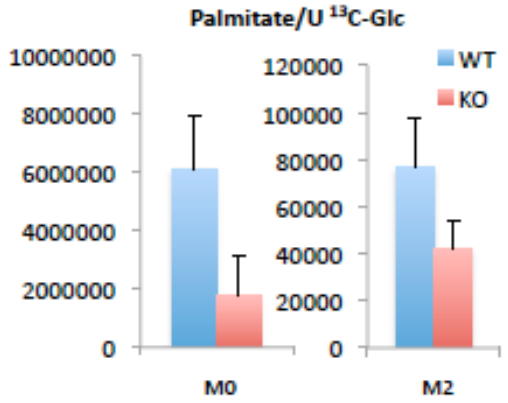

% U14C-Glucose in DNA
p=0.02
p=0.01
WT KO
LA2-1B
WT KO
LA2-2
% 1-14C-Glucose in DNA
p=0.0168
p=0.0001
WT KO
LA2-1B
WT KO
LA2-2

Figure 22. (continued). The effect of HK2 deletion in K-Ras^{G12D} NSCLC cells on the intracellular levels of metabolites derived from glucose (I) HK2 deletion decreased glucose-derived palmitate. ^{13}C isotopomers were quantified 6 hr after labeling with U-^{13}glucose. (J) HK2 deletion decreased glucose-derived citrate. ^{13}C isotopomers were quantified 6 hr after labeling with U-^{13}C glucose. Y-axes in indicate level of signal.

(I)


Palmitate/U 13C-Glc
10000000
8000000
6000000
4000000
2000000
0
120000
100000
80000
60000
40000
20000
0
WT
KO
M0
M2

(J)

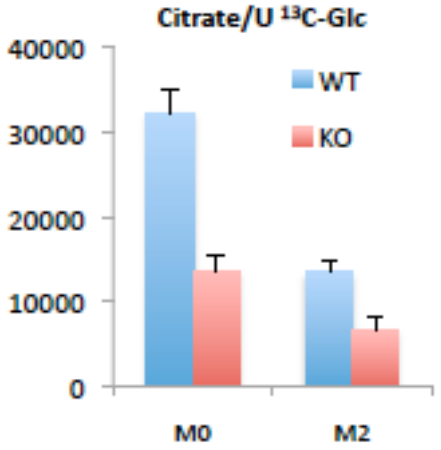
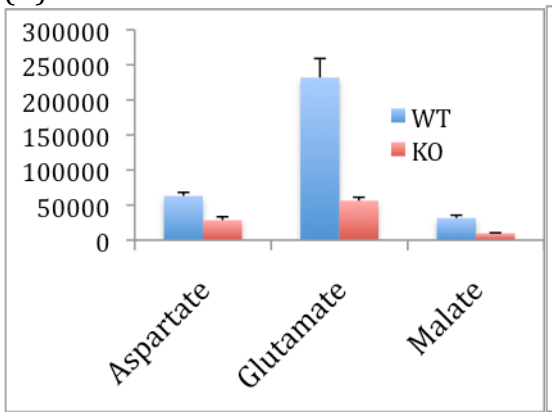
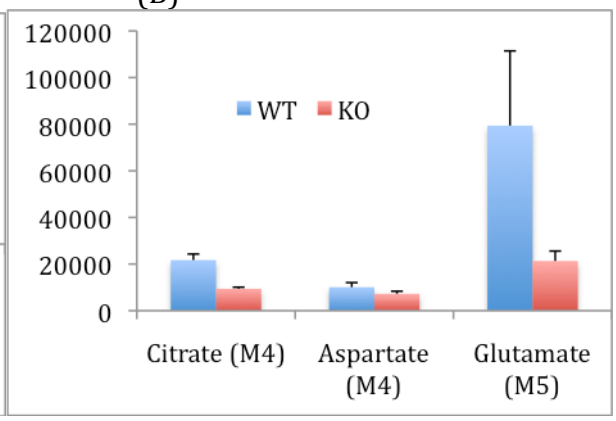

Citrate/U 13C-Glc
40000
30000
20000
10000
0
WT
KO
M0
M2

Figure 23. The effect of HK2 deletion on glutamine metabolism in ras transformed cells (A) HK2 deletion inhibits glutamine incorporation in K-Ras^{G12D} NSCLC cells. Quantification of the indicated metabolites 30 min after labeling with U-^{13}C-glutamine (B) HK2 deletion reduces the steady state metabolite level of aspartate, glutamate and malate. Y-axes in indicate level of signal. (C) Growth curves of K-RasG12D-NSCLC cell lines (D) Ras transformed MEFs (WT or HK2-KO) at the indicated concentrations of glutamine. Glutamine concentrations were indicated in the graph legends.

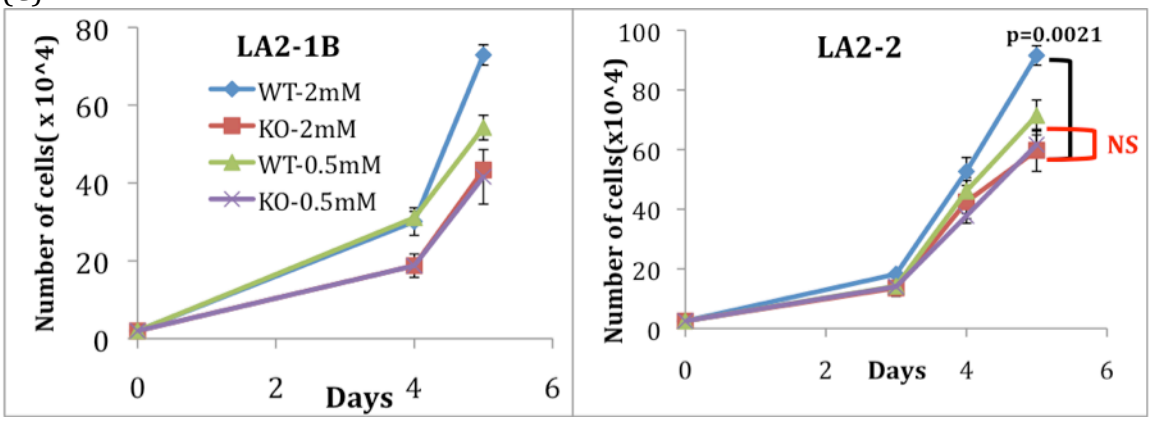
(A)


300000
250000
200000
150000
100000
50000
0
WT
KO
Aspartate
Glutamate
Malate

(B)


120000
100000
80000
60000
40000
20000
0
WT
KO
Citrate (M4)
Aspartate (M4)
Glutamate (M5)

(C)


LA2-1B
Number of cells(x 10^4)
80
60
40
20
0
WT-2mM
KO-2mM
WT-0.5mM
KO-0.5mM
0
2
Days
4
6
LA2-2
p=0.0021
NS
Number of cells(x10^4)
100
80
60
40
20
0
0
2
Days
4
6

(D)

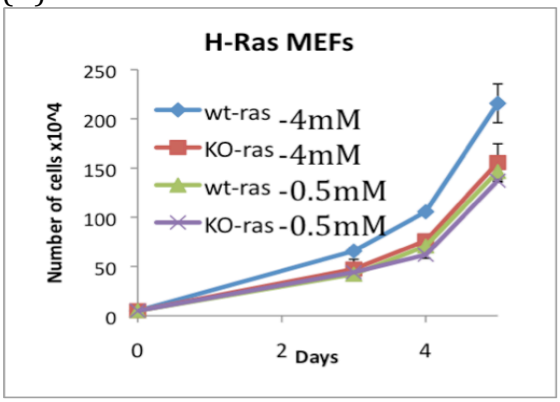
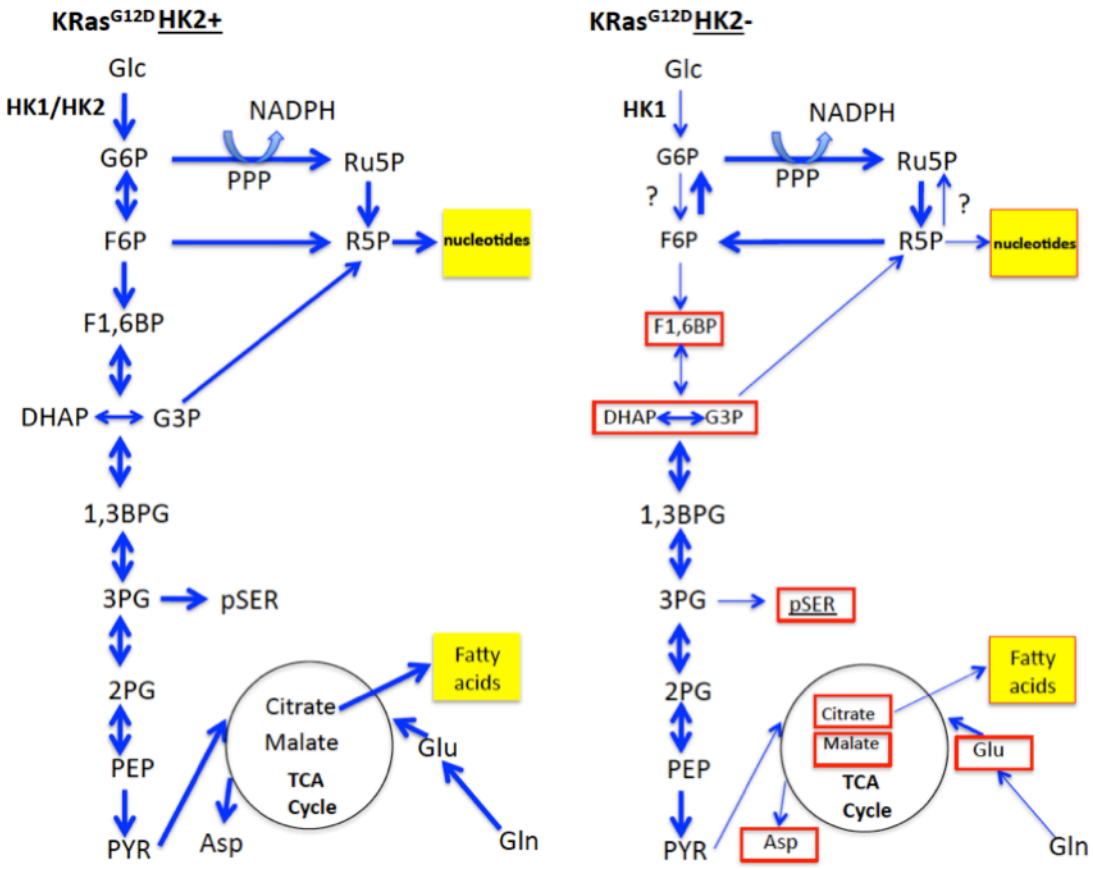

H-Ras MEFs
Number of cells x10^4
250
200
150
100
50
0
wt-ras -4mM
KO-ras -4mM
wt-ras -0.5mM
KO-ras -0.5mM
0
2
Days
4

Figure 24. Schematic illustration depicting metabolomics changes induced by the deletion of HK2 in K-Ras^{G12D}NSCLC cells. Metabolites with a decreased abundance are framed by red line. Potential reduced fluxes are indicated by reduced arrows' width. For details see text.


KRas^{G12D} HK2+
Glc
HK1/HK2
NADPH
G6P
PPP
Ru5P
F6P
R5P
nucleotides
F1,6BP
DHAP
G3P
1,3BPG
3PG
pSER
2PG
Citrate
Malate
TCA Cycle
Fatty acids
Glu
PEP
PYR
Asp
Gln
KRas^{G12D} HK2-
Glc
HK1
NADPH
G6P
PPP
Ru5P
?
?
F6P
R5P
nucleotides
F1,6BP
DHAP
G3P
1,3BPG
3PG
pSER
2PG
Citrate
Malate
TCA Cycle
Fatty acids
Glu
PEP
PYR
Asp
Gln

CHAPTER-IV

DISCUSSION

Signature alterations in the metabolism of cancer cells have been recognized for over three-quarters of a century (Robey and Hay, 2009; Vander Heiden et al., 2009). The growing interest in cancer glucose metabolism is providing a plethora of knowledge on rewired biochemical pathways, which control important nodes in cancer initiation and progression. Since the high flux of glucose utilization distinguishes many cancer cells from their normal counterparts, many of these studies provide rationales for targeting certain glycolytic enzymes for cancer therapy. However, because of the fundamental biological importance of glycolysis in normal metabolic homeostasis, concerns arise regarding potential adverse homeostatic consequences of targeting specific glycolytic enzymes in a systemic manner (Cheong et al., 2012). Selective cancer-associated expression of specific isoforms of certain glycolytic enzymes could render these enzymes potential selective targets for cancer therapy, if their expression is critical for the neoplastic phenotype. Among all these potential targets, the hexokinase isoform, HK2, could be the ideal target. HK2 is highly expressed in many cancers (Shinohara et al., 1994), and although it is abundantly expressed in embryonic tissues, it constitutes the predominant hexokinase isoform in only a limited number of adult tissues. In contrast, the HK1 isoform is ubiquitously expressed and constitutes the major hexokinase in most normal adult tissues (Wilson, 1995; Wilson, 2003).

Previous studies have demonstrated the ability of RNAi-mediated HK2 silencing to inhibit tumor growth in a xenograft model of glioblastoma (Wolf et al., 2011). However it is not known whether these results can be extrapolated to other forms of cancer, and the specific contributions of – and requirements for – HK2 and the other major hexokinase

isoform, HK1, in both tumor initiation and progression are incompletely understood. In addition, it is not known whether systemic targeting of HK2 is a viable approach to treating cancer. The comprehensive studies described herein employ global conditional HK2 deletion in established mouse models of cancer to emulate isoform-specific drug therapy and address all these issues. To our knowledge, these studies provide the first successful demonstration of systemic glycolytic enzyme targeting for cancer therapy without independently affecting either metabolic homeostasis or lifespan in intact animals.

In our study we found out that HK2 expression is selectively expressed in tumor tissues of human patients where as the corresponding normal tissues do not express this isoform. Including our analysis lung, liver and breast cancer this expression pattern of HK2 can be generalized across many cancer types including glioblastoma as shown by Wolf et al. In clinic, the fidelity of cancer detection though ^{18}-FDG/PET shown well correlated with HK2 expression in many liver, lung and breast cancer patients(Brown et al., 2002; Mamede et al., 2005; Paudyal et al., 2008). One of the interesting observations we had in the liver cancer is that HK2 expression is elevated in dysplasia, which is a pre-neoplastic condition. Hepatic steatosis, a pre-malignant condition is associated with a pronounce changes with the expression and serum profile of metabolic enzymes in humans(Notarnicola et al., 2012). Therefore HK2 expression may be causing a permissive environment starting from a premalignant condition for the development of HCC.

In human breast cancer patient samples HK2 expression is observed in hyperplastic and advanced tumor lesions. Although the tumor tissue samples used in our study, were not categorized to different subtypes but it is interesting to examine if higher HK2 expression is associated with particular molecular subtypes. A gene expression dataset involving a large number of breast cancer patients we showed that high HK2 expression is correlated with poor survival of the individuals (van de Vijver et al., 2002). This study is consistent with

another observation that breast tumors, which metastasize to brain, express a higher HK2 expression than the non-metastatic ones and the patient survival is correlated with HK2 expression. In NSCLC patient samples, although there is a higher expression of HK2 was observed in tumor samples than the normal lung, but there are few tumor samples we could not observe a very high expression. Therefore it is possible that a sub-set of lung tumors express a very high HK2 in human patients. Oncogenic K-Ras activation (this study) and LKB1 deletion(Shackelford et al., 2009), both elevate HK2 expression and co-mutation of these two genes is associated with high- grade metastatic lung tumors. Therefore it is possible that HK2 expression may be dependent on the genetic context. In line with our observation in human tissue samples, we found that mouse embryonic fibroblasts express a very high level of HK2 when transformed by oncogenic Ras *in vitro* in a MAPK or PI3K dependent manner. c-Myc and HIF1 alpha, which directly activate HK2 transcription, are downstream effectors of MAPK or PI3K pathway. As MAPK or PI3K signaling is implicated in lung and breast cancer, therefore the high HK2 expression may be consequences of the activation of these pathways.

To see the effect of HK2 attenuation on tumorigenic potential of human cancer cells, we silenced HK2 by shRNA in a panel of lung, liver and breast cancer cell line. The absence of HK2 affected the oncogenic capacity of these cells *in vitro* and *in vivo*, despite the presence of HK1. Moreover, established tumors cannot grow into larger size if HK2 is conditionally silenced during tumor progression. H460 NSCLC cell line harbor both K-Ras and LKB1 mutation, a molecular subtype that is irresponsive to current chemotherapies used in the clinic(Chen et al., 2012). We found that, the cells with higher level of knockdown of HK2 were lost during tumor progression suggesting, HK2 inhibition will be more effective for advanced grade tumors. In our analysis we found silencing HK2 predominantly causes a cytostatic effect. Hence combining other stresses such as chemotherapeutic drugs

can induce apoptosis in HK2 attenuated cells. This assumption is supported by the observation that, HK2 attenuation combined with ionizing radiation cause apoptosis in glioblastoma cells (Wolf et al., 2011).

The requirement of HK2 in oncogenesis was recapitulated in conditional HK2 knockout MEFs. HK2 knockout MEFs are impaired in their transformation ability *in vitro* and *in vivo*. Most importantly the glucose phosphorylating ability of HK2 is required for ability to form tumors *in vivo*. This suggests that Glucose-6-phosphate produced by HK2 undergo various metabolic pathways to support anabolic growth that is required for oncogenic growth. HK2 couples glycolysis to oxidative phosphorylation by binding to the mitochondria. Therefore loss of kinase function can both affect downstream glycolysis and mitochondrial oxidative phosphorylation. Hence, any small molecule inhibitor affecting glucose-phosphorylating function of HK2 can be effective against cancer.

K-Ras induced NSCLC in mice faithfully recapitulate the pathophysiology of NSCLC in human (Jackson et al., 2001). In our study, HK2 expression was only observed in lung tumors compared with non-tumor tissue in K-Ras^{LA2} mice where as the expression of HK1 was found in both normal and tumor tissues in a comparable level. Genetic ablation of HK2 with concomitant K-Ras activation significantly reduces the tumor burden in K-Ras^{LSL} mice underscores the importance of HK2 in the tumor initiation process. As the tumor numbers are not changed but the tumor size was largely reduced in HK2 null tumors, therefore we concluded although tumors can be formed in the absence of HK2 but they can not still grow to larger size. The lower BrdU incorporation in HK2 null tumors, without any difference in cleaved-caspase staining also supports the role of HK2 in proliferation rather than in apoptosis in this setting.

Along with mutations in PI3K/Akt pathway, Her2 amplification is a common etiological feature of human breast cancer(Osborne et al., 2004). We found that HK2 is not

expressed in normal mammary gland and significantly up regulated in tumor tissues derived from genetically engineered mice expressing Her2/Neu and PyMT. Genetic ablation of HK2 substantially delayed the tumor onset in mice, expressing activated Neu in their mammary gland. The tumors analyzed form HK2 knockout mice in the late stage of tumorigenesis still express HK2, which can be explained by incomplete recombination of HK2. Therefore the inhibition of tumor progression would have been more dramatic if we could have achieved a complete deletion of HK2. Using Her2/Neu transformed HK2 conditional knock out cells from mice, where HK2 can be deleted efficiently *in vitro*, we showed that loss of HK2 reverses the tumorigenic phenotype of breast cancer cells. Therefore HK2 inhibition may be effective in Her2 amplified tumors in humans.

Tumor specific expression of some isoforms of metabolic enzymes can be exploited as potential targets for cancer therapy, if their expression is critical for the neoplastic phenotype. Among all these potential targets, HK2 could be an ideal target. HK2 is highly expressed in many cancers (Shinohara et al., 1994) and in specific adult tissues like heart, fat and skeletal muscle. We did not observe any overt phenotype after systemic deletion of HK2 in adult mouse. Except skeletal muscles all other adult mouse tissues that abundantly express HK2 also express relatively high levels of HK1 (Fig. 19A), which might play a compensatory or redundant role in the absence of HK2. In addition, the ability of tissues such as myocardium and skeletal muscle to utilize fatty acids as an alternate energy substrate could also buffer the metabolic impact of HK2 deletion in these tissues (Felig and Wahren, 1975). Therefore, HK2 might not be required for skeletal muscle glucose homeostasis under resting conditions but could be important during exercise-induced glucose metabolism. Since the deletion of HK2 by the inducible Cre is not complete, it is possible that the residual HK2 activity is sufficient to maintain normal glucose homeostasis under normal chow diet and in the absence of exercise. However, since our analyses of

glucose homeostasis was not extensive and did not include clamp studies it cannot be excluded that the mice would display impaired glucose homeostasis under certain conditions, and reduced endurance during exercise. Notably, it was reported that mice with 50% germ line deletion of HK2 do not have a marked change in fed glucose levels under normal chow diet (Heikkinen et al., 1999). These mice do display hyperinsulinemia during hyperinsulinemic-euglycemic clamp especially if combined with a high fat diet, and also display reduced endurance during exercise (Fueger et al., 2003). Nonetheless, the possibility that the phenotype observed in mice with 50% germ line deletion is exerted through a developmental defect cannot be completely excluded. Thus, adult mice with a conditional deletion of HK2 may not exert these phenotypes.

To emulate drug therapy we performed systemic ablation of HK2 by inducible Cre in lung tumors developed in K-Ras^{LA2} mice. Importantly the lung tumor burden was significantly reduced in upon HK2 deletion albeit some tumors were detected in HK2 knockout mice, may be because of the incomplete recombination. As systemic knockout of HK2 in adult does not cause any overt physiological phenotype and have a profound effect on tumor progression in established lung tumor, these results provide a proof of principle that HK2 may be a safe metabolic target for NSCLC.

Accumulating data suggest that the metabolites generated by the branched pathways are much more critical for cancer cells than glycolysis per-se (Vander Heiden et al., 2009). These metabolites are critical to support anabolic processes such as nucleic acids and lipid synthesis. Previous results showed that oncogenic Ras elevates ribonucleotides synthesis largely through the non-oxidative branch of the PPP (Ying et al., 2012). Our results show that in the absence of HK2 the synthesis of glucose-derived ribonucleotides in K-Ras^{G12D}-NSCLC cells is substantially reduced (Fig. S6G). Thus, the induction of HK2 expression by oncogenic Ras is critical for the accelerated ribonucleotides synthesis.

Another branched pathway that was shown to be important for cancer cells is the serine biosynthesis pathway. Here again the deletion of HK2 profoundly reduced the level of phosphoserine, which is a major intermediate in this pathway. Metabolic serine flux can be quite high in cancer cells where increased serine biosynthesis has been appreciated for almost three decades (Kalhan and Hanson, 2012). The levels and activities of enzymes in this pathway are elevated in cancer cells, and found to be critical for cancer cells' maintenance for reasons that are not completely understood (Locasale and Cantley, 2011; Possemato et al., 2011).

In the study by Ying et al shows that oncogenic K-Ras increases O-glycosylation though increased glucose metabolism. We failed to demonstrate any effect on glycosylation on HK2 ablation in K-Ras^{G12D}-NSCLC cells. Oncogenic K-Ras controls expression of many enzymes involved in glycosylation pathway, along with HK1 and HK2. Therefore, it is unlikely that only HK2 deletion is sufficient to affect global glycosylation, if the amount of phosphohexoses generated by HK1 is adequate for providing the precursors for glycosylation pathway. However it may be possible that a specific glycosylation modification may be affected by HK2 loss that we could not detect with our experimental method. As mentioned before, G-6P is metabolized in the oxidative pentose phosphate pathway (PPP) to generate NADPH, which helps in detoxification of reactive oxygen species (ROS). As HK2 is expression is elevated in cancer cells, we were expecting a decrease in the amount of NADPH and subsequent increase in ROS upon HK2 deletion. How ever we did not observe any significant difference in NADPH or ROS in the HK2 knockout K-Ras^{G12D}-NSCLC cells. It may be explained by the fact that oncogenic K-Ras does not affect the oxidative branch of PPP rather it affect non-oxidative branch (Ying et al., 2012).

Finally, it appears that HK2 expression in K-Ras^{G12D}-NSCLC cells is important for the operation of the TCA cycle; and the reduced levels of glucose-derived citrate in the absence

of HK2 might be responsible for the reduced levels of glucose-derived fatty acids (Fig. 22 I, J). In cancer cells and oncogenic Ras transformed cells TCA cycle intermediates, except citrate, are predominately derived from auxotrophic glutamine (DeBerardinis et al., 2007; Gaglio et al., 2011; Marin-Valencia et al., 2012). Surprisingly, the deletion of HK2 significantly inhibits glutamine-derived carbon utilization in anaplerosis (Fig. 23B) for a reason that is not immediately clear to us. However, it is possible that reduced glucose-derived citrate somehow contributes to the attenuation of the flux from glutamine to other TCA cycle intermediates.

In summary, HK2 plays a critical role in the diversion of glucose into the branched pathways, which are required for the anabolic activities in cancer cells and does not appear to affect metabolites in the canonical glycolysis pathway. Interestingly, it was shown and proposed previously that in cancer cells downstream glycolytic activities are attenuated in order to divert glucose into the branched pathways. Our results suggest that an increase in the most upstream step in glucose metabolism catalyzed by HK2 is also critical for diverting glucose into these pathways in cancer cells. Therefore, this could explain why cancer cells, in contrast to their normal counterparts, express high levels of HK2.

Future implications for cancer therapy.

Our studies clearly demonstrate that global HK2 ablation in the mouse is well tolerated, while it is therapeutic for cancer. Thus, placing HK2 as a therapeutic target for NSCLC and breast cancer that its ablation selectively affects the cancer cells and not their normal counterparts. Moreover, it is possible that HK2 ablation would inhibit metastasis as it was shown that breast cancer metastasis to the brain significantly associated with high HK2 expression with poor patient survival (Palmieri et al., 2009).

It was previously shown that that mitochondrial hexokinases, HK1 and HK2, binding to mitochondria promotes cell survival (Gottlob et al., 2001; Majewski et al., 2004a;

Majewski et al., 2004b; Robey and Hay, 2005a; Robey and Hay, 2005b). Thus, it is expected that the ablation of HK2 would increase cell death. The results presented here, however, show that the deficiency of HK2 exerts cytostatic effect *in vitro* and *in vivo*. There are several possible reasons for why we did not observe an increase in cell death after HK2 ablation. First, the cells also express HK1, which could be sufficient to protect from cell death. Second, the experiments were done with established cell lines in which HK2 was either deleted or was stably knocked down, and thus it is possible that during the establishment of the cell lines, cells in which HK2 was ablated beyond a certain threshold level were eliminated. Supporting this possibility is the observation that upon a conditional knockdown of HK2, there was a significant increase in cell death although at low magnitude (Fig. 11D). Finally, although we did not observe a significant cell death *in vivo*, we cannot completely rule out an increase in cell death because it might be technically difficult to observe low levels of cell death *in vivo*, especially if the dead cells are phagocytosized. Nevertheless, it is possible that the deficiency of HK2 will render cancer cells more susceptible to cell death by pro-apoptotic drugs. Notably, investigations on drug resistance phenotypes of breast cancer cells showed that up regulated glucose metabolism, and especially HK2 expression are associated with the chemoresistance phenotypes in breast cancer cells (Kaplan et al., 1990; Komurov et al., 2012; Lyon et al., 1988). Therefore, HK2 may be a genetic modifier of drug resistance that can be therapeutically exploited.

Taken together our results provided strong rationales for developing small molecules inhibitors of HK2 for cancer therapy. However, perhaps the biggest challenge is to develop inhibitors that could preferentially target HK2 and not HK1. Although HK1 and HK2 are very similar to each other, they exhibit some specific properties that could be exploited for isoform specific inhibition. For instance, both HK1 and HK2 are inhibited to the same extent by their own product, G6P. However, inorganic phosphate antagonizes the

inhibition of HK1 by G6P, while it increases the inhibition of HK2 by G6P (Wilson, 2003). Since an increase in intracellular inorganic phosphate is usually observed in high glycolytic cells, such as cancer cells, this property of HK2 could be exploited for the preferential inhibition of HK2 in cancer therapy.

CHAPTER-V

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VI. VITAE

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CURRENT POSITION: Graduate Research Fellow

INSTITUTION AND LOCATION	DEGREE	MM/YY	FIELD OF STUDY
Univ. of Illinois at Chicago, Chicago, USA	PhD-continuing	2005-present	Studying Cancer metabolism in mouse models of cancer
Indian Institute of Technology, Kharagpur, India	M.Tech	2004	Enzymology/ Biotechnology
Sambalpur University, Sambalpur, India	M.Sc	2001	Life Sciences
Utkal University, Bhubaneswar, India	B.Sc	1999	Biology

EDUCATION/TRAINING:

POSITIONS:

2005-present: Graduate Research Fellow
Advisor: Dr. Nissim Hay
Dept. of Biochemistry and Molecular genetics
University of Illinois at Chicago, Chicago, USA

2004-2005: Junior Research Fellow
Advisor: Dr. Rintu Banerjee
Indian institute of Technology, Kharagpur, India

AWARDS AND HONORS:

2011-Current: Department of Defense Pre-doctoral Fellowship, USA.

2005-2010: University Fellowship, Graduate College, University of Illinois at Chicago.

2003-2004: Council of Science and Industrial Research NET/JRF Award, Govt. of India .

2002-2003: All India Graduate Aptitude Test of Engineering Fellowship, Govt. of India.

2013: Selected speaker for oral presentation at "Tumor metabolism/ PI 3-Kinase and Interplay with Other Signaling Pathways". Keystone Meetings, 2013.

2012: Selected speaker for a short talk at "Mechanisms and Models of cancer". Cold spring Harbor Laboratory Meeting, 2012.

PUBLICATIONS:

1) **Patra KC** et al. Hexokinase 2 is required for oncogenesis and its systemic deletion is therapeutic in mouse models of cancer. (Under revision in "**Cancer Cell**").

2) Bhaskar PT, Nogueira V, **Patra KC**, Jeon SM, Park Y, Robey RB, Hay N.mTORC1 hyperactivity inhibits serum deprivation-induced apoptosis via increased hexokinase II and GLUT1expression, sustained Mcl-1 expression, and glycogen synthase kinase-3 beta inhibition. Mol Cell Biol. 2009 Sep;29(18): 5136-47

3) Banerjee R, Mukherjee G, **Patra KC**. Microbial transformation of tannin-rich substrate to gallic acid through co-culture method. Bioresour Technol. 2005 May; 96(8): 949-53

Abstracts and Presentations:

1) *Oral presentation*: Hexokinase 2 is required for oncogenesis and its systemic deletion is therapeutic in mouse models of cancer. **Krushna Patra** and Nissim Hay. Keystone meetings. "Tumor metabolism/ PI 3-Kinase and Interplay with Other Signaling Pathways". Feb 24-Mar 1, 2013

2) *Short talk:* Requirement of Hexokinase 2 in the process of tumorigenesis. **Krushna Patra** and Nissim Hay. Cold spring Harbor meeting. Mechanisms and Models of Cancer. Aug 14-19, 2012

3) *Abstract:* Requirement of HK2 in the process of tumorigenesis. **Krushna Patra** and Nissim Hay, COM research forum, UIC. Nov 11, 2011

4) *Abstract:* Hexokinase-2 is required for *Ras* mediated tumorigenesis. **Krushna Patra** and Nissim Hay, AACR special conference: Metabolism and cancer. Oct 16-19, 2011