

**p53-REGULATED HEXOKINASE 1b IS A NOVEL TARGET
FOR METABOLIC THERAPY AGAINST NON-SMALL-
CELL LUNG CANCER**

by
YASEMİN YOZGAT

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METABOLIC THERAPY AGAINST NON-SMALL-CELL LUNG CANCER

APPROVED BY:

Prof. Dr Hüveyda Başağa.....
(Thesis Supervisor)

Prof. Dr. Devrim Gözüaçık.....

Asst. Prof. Dr. Murat Alper Cevher

Prof. Dr. Ülkan Kılıç

Asst. Prof. Dr. Bilal Ersen Kerman

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YASEMİN YOZGAT

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(p53), glycolysis, cisplatin, drug targets, apoptosis, autophagy

ABSTRACT

Deregulation of glycolysis is common in non-small cell lung cancer (NSCLC). p53 controls the cellular metabolism pathway by activating hexokinases (HKs) to increase glycolysis. HK enzymes catalyze the phosphoryl-group-transfer in glucose metabolism. Unlike HK2, HK1 has several transcript variants. However, the functional differential roles of HK1 isoforms in glucose metabolism and the malignant tumor progression are still elusive. Here, we show that primary NSCLC patient tumor cells metabolically differ from the normal lung epithelium in that they display predominant expression of one of the HK1 isoforms, hexokinase1b (HK1b). Interestingly, we show that p53 positively regulates HK1b expression using CRISPR-Cas9 system. We utilized CRISPR-Cas9 system to selectively target specific HK1b isoform in NSCLC and show that silencing HK1b in NSCLC cells inhibits tumorigenesis through diminishing glycolysis and proliferation. Finally, HK1b deletion sensitizes NCLC cells to cisplatin treatment and the combination therapy synergistically increases both the p53- mediated apoptotic cell death by cisplatin

and autophagic cell death by increased formation of LC3-II associated autophagic vesicles and myelinoid bodies in an AMPK-independent manner. Our findings reveal that targeting HK1b isoform alone or in combination with cisplatin may represent a novel strategy for NSCLC patients.



KÜÇÜK HÜCRELİ DIŞI AKCİĞER KANSERİNDE p53 TARAFINDAN REGULE EDİLEN HK1b METABOLİK TEDAVİ İÇİN YENİ BİR HEDEFTİR.

YASEMİN YOZGAT

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Anahtar kelimeler: Küçük hücreli dışı akciğer kanseri (KHDAK), heksokinaz 1b, p53, glikoliz, cisplatin, ilaç hedefleri apoptoz, otofaji

ÖZET

Küçük hücreli dışı akciğer kanserinde (KHDAK) glikolizin deregülasyonu yağın olarak görünen kanser karakteristiklerinden biridir. p53 glikolizi arttırmak için hexokinaz (HK) enzimlerini aktive eder ve böylelikle glukoz metabolizma yolağını kontrol eder. HK enzimleri glukoz metabolizmasında fosforil-grup transferini katalize etmek için görevlidir. HK2 den farklı olarak HK1 geni birden fazla transcript varyantlarına sahiptir fakat bu HK1 izoformlarının glukoz metabolizmasında ve malign tümör gelişiminde fonksiyonel diferansiyel rolleri halen belirsizdir. Bu çalışmamızda ilk olarak KHDAK hasta tümör hücrelerinin HK1 izoformlarından biri olan HK1b izoform genin yüksek baskın ifadesi sebebi ile metabolik olarak normal akciğer epitel hücrelerinden farklı olduğunu gösterdik. Ayrıca, ilginçtirki p53 genin HK1b izoform ifadesini pozitif olarak regüle ettiğini gösterdik. KHDAK’de spesifik HK1b izoformunu seçici olarak hedeflemek ve KHDAK hücrelerinde gen ifadesi baskılanmış HK1b’nin glikolizi ve proliferasyonu azaltmasına sebep verdiğini ve böylece tümörjenezini inhibe edebildiğini göstermek amacı ile CRISPR-Cas9 sistemi kullandık. Son olarak, HK1b izoform susuturulması KHDAK hücrelerini cisplatin

tedavisine karşı hassaslaştırdığını gösterdik. Kombinasyon terapisi sinerjistik olarak p53- aracılı apoptotik hücre ölümünü sisplatin aracılığı ile arttırırken diğer yandan artan LC3-II ile ilişkili otofajik kesecikler ve miyelinoid cisimlerin artması sebebi ile AMP- activated protein kinaz (AMPK)'den bağımsız olarak otofojik hücre ölümüne sebep verdiğini gösterdik. Bulgularımız, HK1b izoformunun tek başına veya sisplatin ile kombinasyon halinde KHDAK hastaları için özgün ve yeni bir ilaç tedavi strateji olabileceğini ortaya koymuştur.



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Dedication

To my parents Hasan Yozgat and Nuriye Yozgat. I love you so much

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LIST OF SYMBOLS AND ABBREVIATIONS

18FDG	Fluoro-deoxy Glucose (18)
3D	3 Dimensions
A549	Non- small-cell Lung Carcinoma Cell Line (p53 WT)
AMPK 5'-	AMP activated kinase
ATP	Adenosine-5'-triphosphate
BAX	BCL2-Associated X Protein
BCL-2	B-cell lymphoma 2
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas	CRISPR associated
Caspases	Cysteine Proteases with Aspartate Specificity
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylene-Diamine-Tetra-Acetic acid
ERK	Extracellular Receptor Kinase
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
G6P	Glucose 6-phosphate
GFP	Green fluorescent protein
Glc	Glucose
H1299	Non- small-cell Lung Carcinoma Cell Line (p53 null)
IHC	Immunohistochemistry
MAPK	Mitogen Activated Protein Kinase
mRNA	messenger Ribonucleic acid
mTOR	Mammalian Target of Rapamycin

mtDNA	Mitochondrial DNA
NSCLC	Non–small-cell lung cancer
NADP	Nicotianamide adeninine diphosphate-oxidized
NADPH	Nicotianamide adeninine diphosphate-reduced
O ₂	Oxygen
PET	Positron Emission Tomography
PI3K	PhosphatidylinositolV3Vkinase
PMSF	Phenylmethylsulfonyl fluoride PPP Pentose phosphate pathway
pSer	Phosphoserine
PVDF	Polyvinylidene fluoride
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
RNA-Seq	RNA Sequencing
rpm	Rotation per minute
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STAT3	Signal Transducer and Activator of Transcription 3
TCA	Tri-carboxylic acid
TCGA	The Cancer Genome Atlas Program
TEM	Transmission electron microscopy
WT	Wild type

1 BACKGROUND

1.1 Lung Cancer

Lung cancer is the most common malignancy and leading cause of cancer deaths worldwide. A total of 1.8 million new cases of lung cancer and 1.6 million related deaths were reported in 2012 [1]. The processes of lung tumor initiation and progression may be modulated by multistep carcinogenesis factors that contribute to induction of DNA damage and mutations, which occur naturally or via environmental factors [2, 3]. Although tobacco use causes 80% to 90% of all lung cancers, many other environmental exposure causes of lung cancer are identified such as arsenic, asbestos, radon, nickel [4]. The diagnostic evaluation for suspected lung cancer has three simultaneous steps includes tissue diagnosis, staging, and determination of functional capacity. Management of lung cancer patients is based on individual tumor histology, molecular diagnosis, tumor staging, and performance status [2, 5, 6]. Patient selection in lung cancer as of today is based on the recognition of both different histological subtypes and driver mutations that determine the biology of these malignancies [7, 8]. There are two main types of primary lung cancer: small-cell lung cancer (SCLC) represents about 15 to 20% of cases and non-small-cell lung cancer (NSCLC) accounts 80 to 85% of cases. Even though both cancers affect the lungs but they have several key differences, including how they are treated and their average progression time [9, 10].

1.2 Non-small cell lung cancer

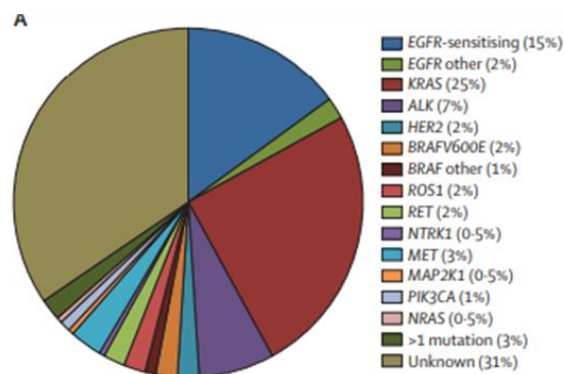
Non-small-cell lung cancers (NSCLCs) is the most common type of all lung cancer, are known to have diverse pathological features. NSCLC is sub-classified based on the histological features into three types: squamous-cell lung carcinoma, lung adenocarcinoma, and large-cell lung carcinoma [8, 11].

Squamous-cell lung carcinoma (lung SCC) is made up 25–30% of all lung cancer cases. Squamous cell lung cancer begins in the squamous cells in the airway epithelial. Most squamous cell carcinomas of the lungs are located in the bronchial tubes in the center of the lungs. Lung SCC subtype is strongly correlated with cigarette smoking, accounting for approximately 95% of cases [11-13].

The most common type of lung cancer is lung adenocarcinoma (lung ADC), represents about 40% of all lung cancers. It evolves from small mucosal glands, type II alveolar cells, in the lung periphery [14]. Although most cases of lung ADC subtype is usually associated with smoking, is the most common form of lung cancer to occur in patients who have never smoked [11].

NSCLC is histologically and molecularly heterogeneous, which reflects changes in the biology and clinical implication of the disease [15]. Immunohistochemistry (IHC) is currently one the main tool to distinguish between lung SCC and lung ADC along with the observation of morphological changes. Expression of cytokeratin- 5 and cytokeratin-6 and/or the transcription factors SRY-box 2 (SOX2) and p63 are indicators of lung SCC whereas expression of thyroid transcription factor (TTF)-1 is the main indicator of lung ADC subtype [10, 16, 17]. It is generally accepted that there are different cells-of –origin, which drive the distinct phenotypes and genotypes of NSCLCs [10]. Recent advances in genomic studies of NSCLC, particularly in lung ADC, have discovered several driver mutations that potentially can be used for targeted therapy or disease progression. The most common driver genetic alterations in lung ADC and driver mutation candidates in lung SCC are shown in Figure 1.2.1 [18, 19].

**Frequency of driver mutations
in lung ADC**



**Frequency of driver mutation
candidates in lung SCC**

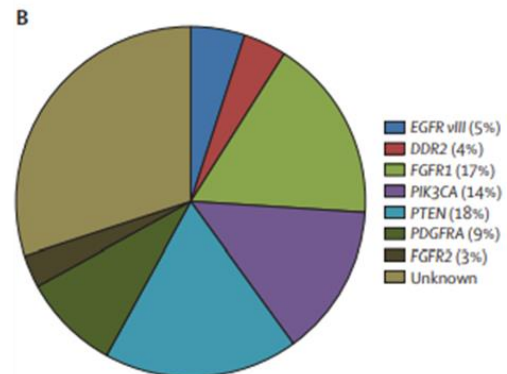


Figure 1.2.1 A) Frequency of driver mutations in lung ADC and B) driver mutation candidates in lung SCC

1.2.1 The most common mutation seen in NSCLC patients

KRAS and *EGFR* activating mutations are commonly mutated genes in lung ADC patients [8]. An anaplastic lymphoma kinase (ALK) rearrangement is only seen in lung ADC and only accounting for 7 to 8% of all lung ADC patients. *EGFR* (epidermal growth factor receptor) is mutated, in about 10 to 15% of patients with lung ADC and 5% with lung SCC. About 25% of *KRAS* -activating mutations is commonly seen in patients with lung ADC, and more than 97% of *KRAS*-mutant cases affect exon 2 and 3 (G12, G13, and Q61) (Figure 1.2.2) [8, 19, 20]. Mutations in *TP53* gene is the most common alterations seen in NSCLC and 80% of these alterations are located in the DNA binding domain as a missense mutations. The frequency TP53 mutation is 70% to 80% in lung SCC and 50% in lung ADC (Figure 1.2.3) [21, 22]. Most clinical studies suggest that TP53 alterations in patients with NSCLC displays worse prognosis and may be relatively more resistant to chemotherapy and radiation [22].

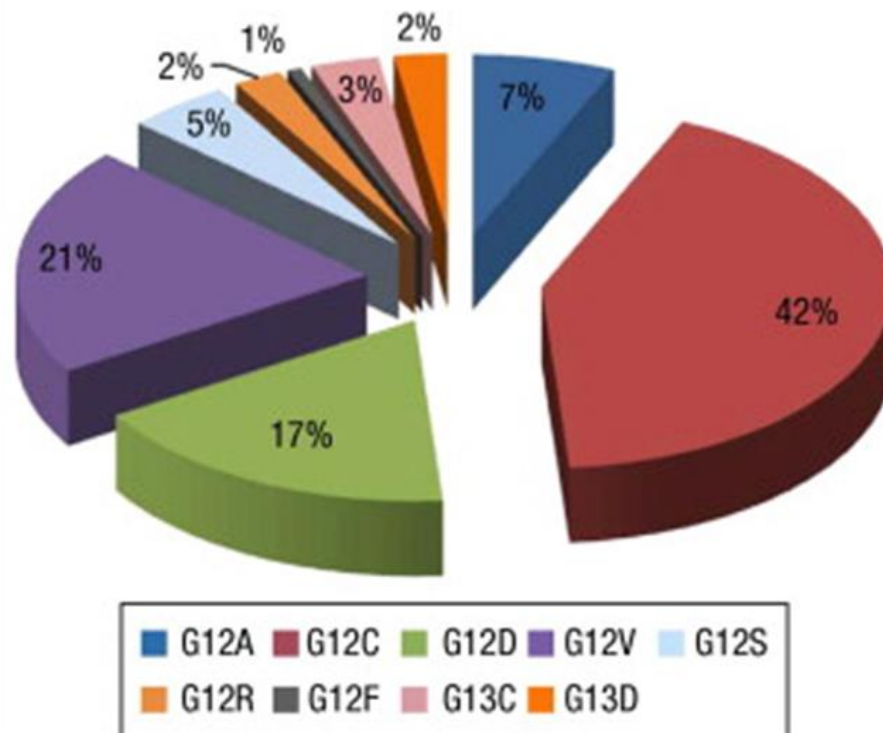


Figure 1.2.2 Distribution of Kras mutations in NSCLC.

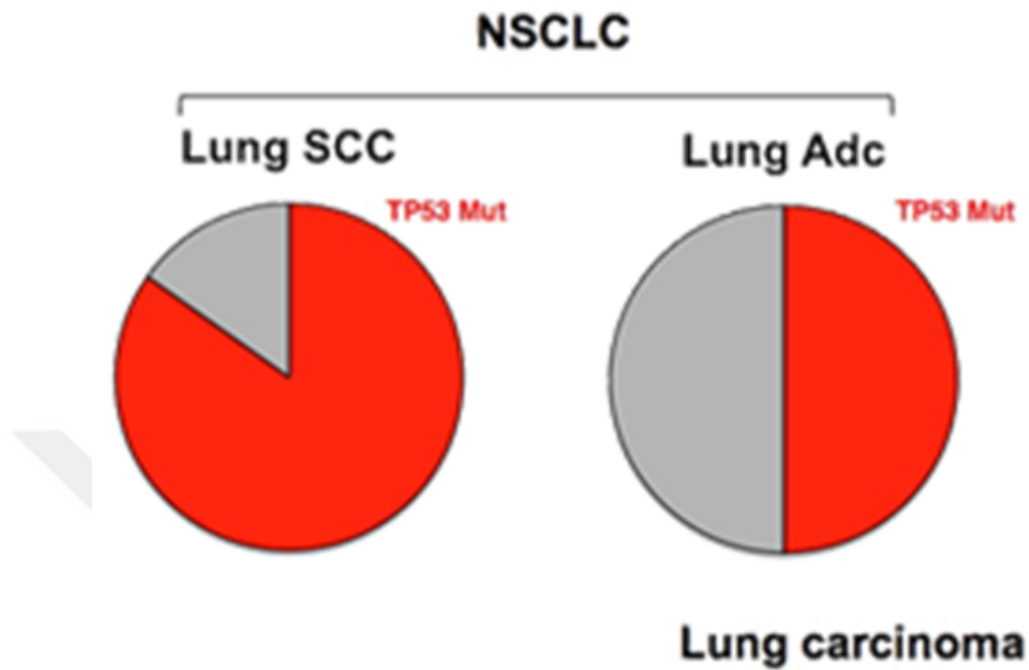


Figure 1.2.3 Distribution of TP53 mutations in NSCLC subtypes

1.2.2 Role of p53 pathway in NSCLC

Potent activator of p53 function serves as a DNA damage checkpoint [23]. In response to oncogenic cellular stresses, such as DNA damage, p53 protein acts as a transcription factor that regulates diversity of biochemical activities including transient cell cycle arrest, cellular senescence and apoptosis [24]. However, recent studies showed that p53 has also other important regulatory role in other cellular processes such as cellular metabolism [25, 26]. Upon stress signals, p53 pathway in cellular response is simplified in figure 1.2.4 [27].

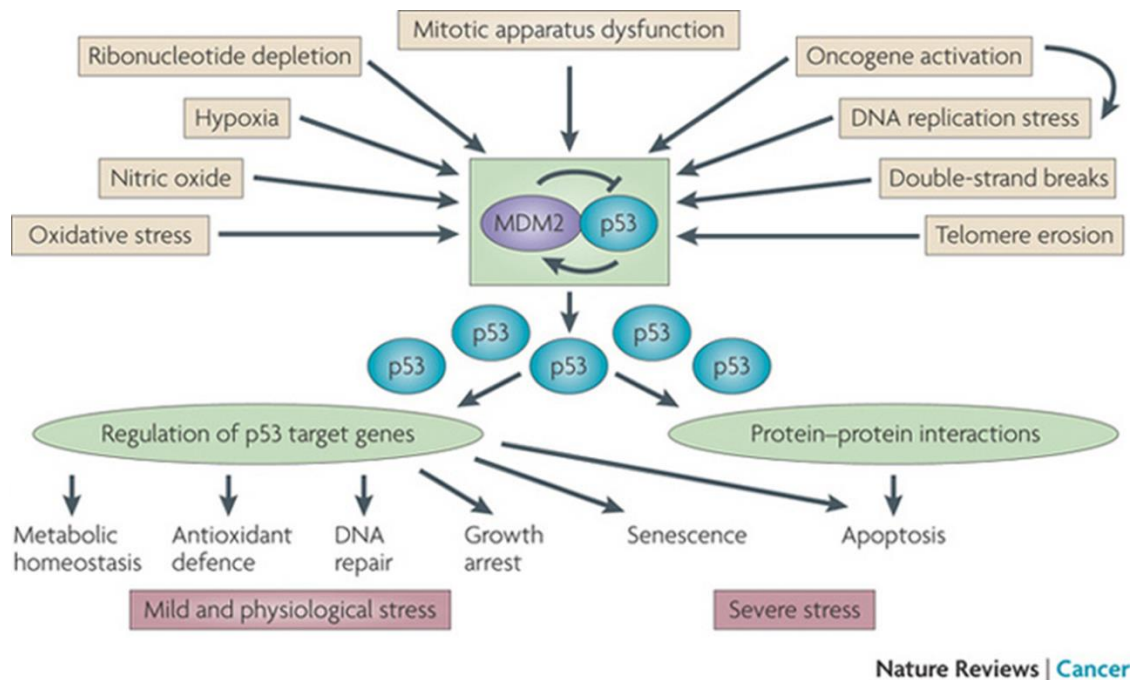
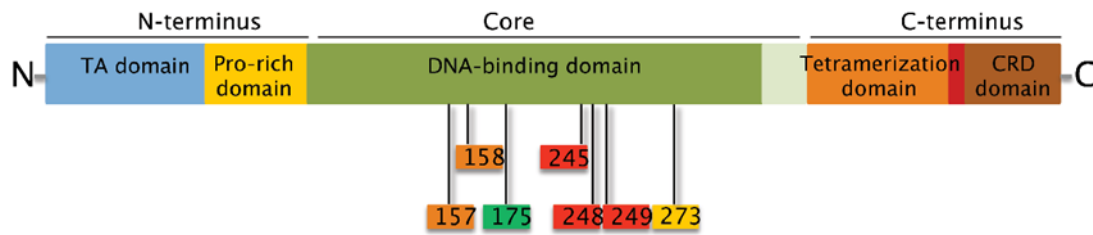


Figure 1.2.4 Scheme of p53 signaling pathway

Allelic dilution of WT p53 by chromosomal deletion, along with point mutation in the remaining copy, is frequently observed in diverse human tumor types as a mechanism to bypass this critical checkpoint [28].

Interestingly, besides classic tumor suppressor functions that are lost upon mutation of the protein, both classes of mutants have also been shown to produce gain-of-function (GOF) phenotypes. p53 with GOF mutation acquires several novel oncogenic properties that promote invasion, proliferation, chemo-resistance, metastasis, and metabolic reprogramming [29-31]. The Cancer Genome Atlas (TCGA) projects on lung ADC and lung SCC revealed the spectrum of p53 mutations data. Although mutations have been found at almost all amino acid positions in the protein, several hot spot regions have been identified (V157, R158, R175, G245, R248, R249, and R273), which highlight the critical function of the core DNA-binding domain of p53 (Figure 1.2.5) [28, 32].

B Mutational hotspots in lung cancer



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Figure 1.2.5 Mutaional hotspots residues of p53 in lung cancer

In the course of evolution of a normal cell toward a cancerous one is a complex process, accompanied by multiple steps of genetic and epigenetic alterations that confer selective advantages upon the altered cells [33]. During tumorigenesis process, mutation in TP53 exerts its function on promoting tumor development. In a heterozygous situation, where both wildtype (WT) and mutant alleles exist, mutant p53 can antagonize WT p53 tumor suppressor functions in a dominant negative manner for varying time periods. At the stage of carcinogenesis/metastasis, WT p53 allele is generally lost through loss-of-heterozygosity (LOH), resulting in the sole existence of only the mutant p53 (Figure 1.2.6) [33, 34].

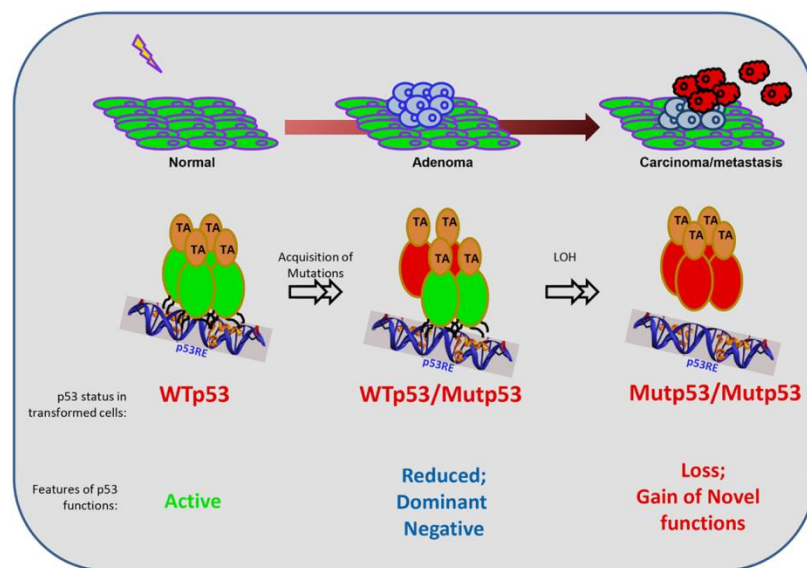


Figure 1.2.6 Functions of mutant p53 during the evolution of a cancer cell transformation.

Most clinical studies suggest that TP53 alterations in patients with NSCLC displays worse prognosis and may be relatively more resistant to chemotherapy and radiation [35]. Several studies indicated that NSCLC patients with mutations of TP53 survived for a shorter period of time [36].

Like all cancer types, NSCLC patients with p53 mutations have poor clinical outcomes and most of the patients develop eventually chemo-resistance [22, 37]. Although targeting p53 is attractive therapeutic strategy for this disease, attempts to develop drugs that target mutant p53 proteins have, so far, been unsuccessful [38, 39]. Currently, much attention is focused on p53 downstream pathways such as cancer metabolism.

1.3 Cancer metabolism

Living organisms require a constant supply of energy to maintain cell and organ function. Sufficient balance between the production of energy (catabolism) by combustion of fuel sources and the utilization of energy by synthesizing macromolecules (anabolism) is essential to maintain cellular homeostasis. These nutrients provide energy and support essential anabolic processes such as nucleic acid, protein, and lipid synthesis. In rapidly proliferating cells there is a higher demand of glucose and glutamine for generation of biosynthetic precursors to synthesize the building blocks that support anabolic processes [40-42].

In normal conditions, most mammalian tissues utilize glucose as the main source of energy. However, in tissues with high energy requirement, such as in heart and resting skeletal muscle, up to 50% of energy, in the form of ATP production generated from fatty acid beta-oxidation [43]. Glucose is oxidized in the presence of oxygen (O_2) to generate 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 [41].

In the presence of oxygen, normal cells predominantly utilize mitochondrial oxidative phosphorylation. In contrast, cancer cells rely mainly on the glycolysis pathway-converting

glucose to lactate-to fulfil their respective energetic requirements even if oxygen is available [41]. Otto Warburg, first reported that cancer cells displays a hyperactive glycolysis even in the presence of oxygen and is popularly known as the “Warburg effect” [44]. Warburg initially hypothesized 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 [45]. Therefore, altered energy metabolism has been accepted as hallmarks of cancer [46]

As numerous connections between signal transduction pathways and metabolic activities contribute to metabolic reprogramming of transformed cells [40]. Distinct hallmarks of tumorigenesis-associated metabolic reprogramming and their functional contribution to the establishment and maintenance of the tumorigenic state have been determined, these hallmarks are: (1) deregulated uptake of glucose and amino acids, (2) use of opportunistic systems of nutrient obtainment, (3) use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, (4) increased demand for nitrogen, (5) alterations in metabolite-driven gene regulation, and (6) metabolic interactions with the microenvironment [47]. Schema describing the changes in metabolic reprogramming during tumorigenesis is presented in Figure 1.3.1 [48].

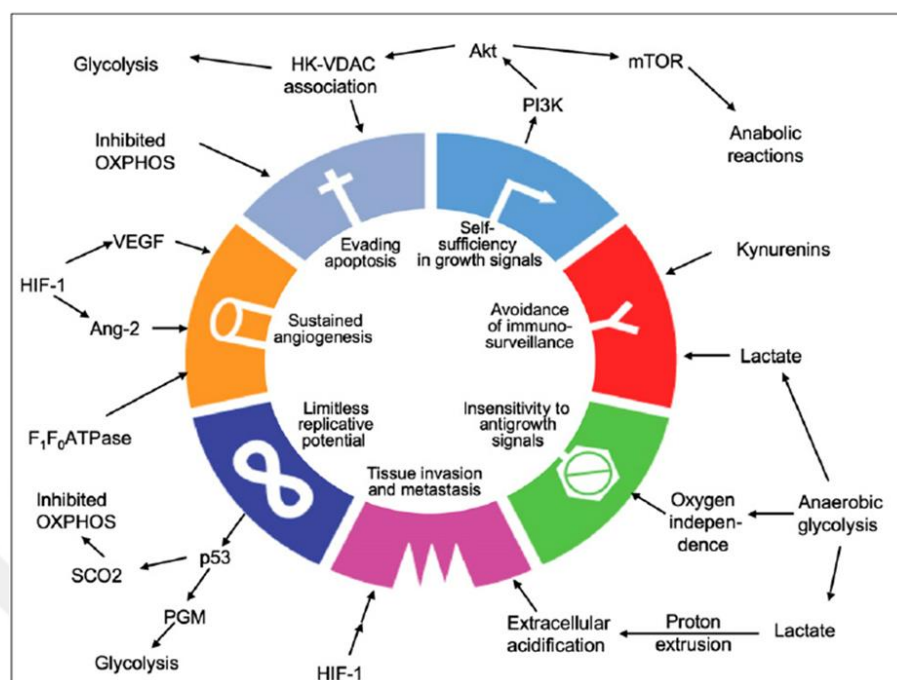


Figure 1.3.2 Connection of metabolic reprogramming with other seven hallmarks of cancer.

Extensive studies on molecular mechanism of cancer-specific metabolic reprogramming revealed that several signaling pathways, such as phosphatidylinositol-3-kinase/serine/threonine-specific protein kinase (PI3K/Akt), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk), and p53, are critical for maintaining the metabolism towards anabolism, thereby supporting cancer cell growth, proliferation, and survival [42, 48]. These signaling pathways regulate cancer metabolism either by inducing the expression and/or increasing the catalytic activity of several enzyme that are responsible of glycolysis [49].

1.3.1 Role of PI3K/AKT signaling in cancer metabolism

Phosphoinositide 3'-kinases (PI3K) are a family of lipid kinases. There are three classes of PI3K based on sequence homologies, lipid substrate specificity and structure including PI3K class I, PI3K class II, and PI3K class III. PI3K class I is a heterodimeric complex that is composed of a regulatory subunit (p85) and a catalytic subunit (p110). The two regulatory subunits p85 α and p85 β isoforms both contain two src-homology 2 (SH2) domains, inter-SH2 domain, SH3 domain, two proline-rich domain, and Bcr/Rac GTPase-

activating protein (GAP) homology domain. SH2 domains activate the catalytic subunit (p110) by coupling p85 isoforms to phosphorylated tyrosine of the receptor [50-52]. PI3K is activated via binding its p85 subunit to the phosphorylated receptor tyrosine kinase (RTK). There are several key downstream targets of PI3K pathway that are activated upon activation of PI3K including serine/threonine-specific protein kinase AKT, protein kinase C (PKC), mammalian target of rapamycin (mTOR), p70 S6 kinase, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), and glycogen synthase kinase 3 beta (GSK3 β) that play a key role in multiple cellular processes [53-55].

AKT is a serine/threonine-specific protein kinase. PI3K activation leads to co-localization of activated PDK1 and AKT. PDK1 phosphorylates AKT on threonine 308, leading to partial activation of AKT. AKT becomes fully activated upon phosphorylation site serine 473 by the TORC2 complex of the mTOR protein kinase. Activated AKT phosphorylate numerous substrates that control a variety of downstream processes, such as metabolism, apoptosis, cell proliferation, cell survival, and cell migration [56-58]. AKT regulates cancer metabolism via increasing glycolytic flux and upregulating glycolytic enzymes. AKT increases glycolysis in cancer cells through (1) increasing the catalytic activity of hexokinases (HK1 and HK2), (2) increasing the expression of Glucose transporters (GLUT1, GLUT2, GLUT3, and GLUT4), (3) activating phosphofructokinase-1 and 2 (PFK1 and PFK2) [49, 59].

mTOR is a serine/threonine protein kinase that is part of the PI3K –related kinase protein family. mTOR exists in two distinct multi-complexes with different protein components and downstream substrates: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The cellular balance between anabolism and catabolism is maintained by mTORC1 in response to the environmental signals such as growth factors, amino acids, and stress [60, 61]. Activation of mTORC1 induces protein synthesis by promoting ribosome biogenesis and mRNA translation by activating ribosomal S6 kinase (S6K) and the inhibitory eIF4E-binding proteins (4E-BPs) [61-63]. mTOR is downstream of AKT in PI3K pathway and mTOR is phosphorylated at Ser-2448 by AKT kinase [64]. mTOR regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription [65]. Moreover, mTORC1 phosphorylates ULK1 and inhibits the activation

of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) under nutrient sufficient condition, thereby blocking autophagy induction [66].

Glycogen synthase kinase 3 (GSK3) is a proline-directed serine-threonine kinase that has two isoforms: GSK-3 α and GSK-3 β . GSK3 β is downstream of AKT in PI3K pathway and it regulates energy metabolism, cell proliferation, apoptosis, neuronal cell development, and body pattern formation [67, 68]. GSK3 activity is inhibited through phosphorylation of serine 21 in GSK-3 α and serine 9 in GSK3 β and is found to be phosphorylated and inactivated in multiple cancer type [69, 70]. Several studies have showed that GSK3 phosphorylation is regulated by AKT, cAMP-dependent protein kinase A, S6K1, and ERK1/2 [67, 71-73]. Glycolytic metabolism promotes proliferation of cancer cells, but it also protects them from cell death. The dynamic balance between increased glycolysis and cell death is also regulated by GSK3 β . Association of HK1 and HK2 to the outer mitochondrial membrane (OMM) is enhanced when GSK-3 is inactivated through phosphorylation at Ser9 by the survival kinase AKT (Figure 1.3.3) [74].

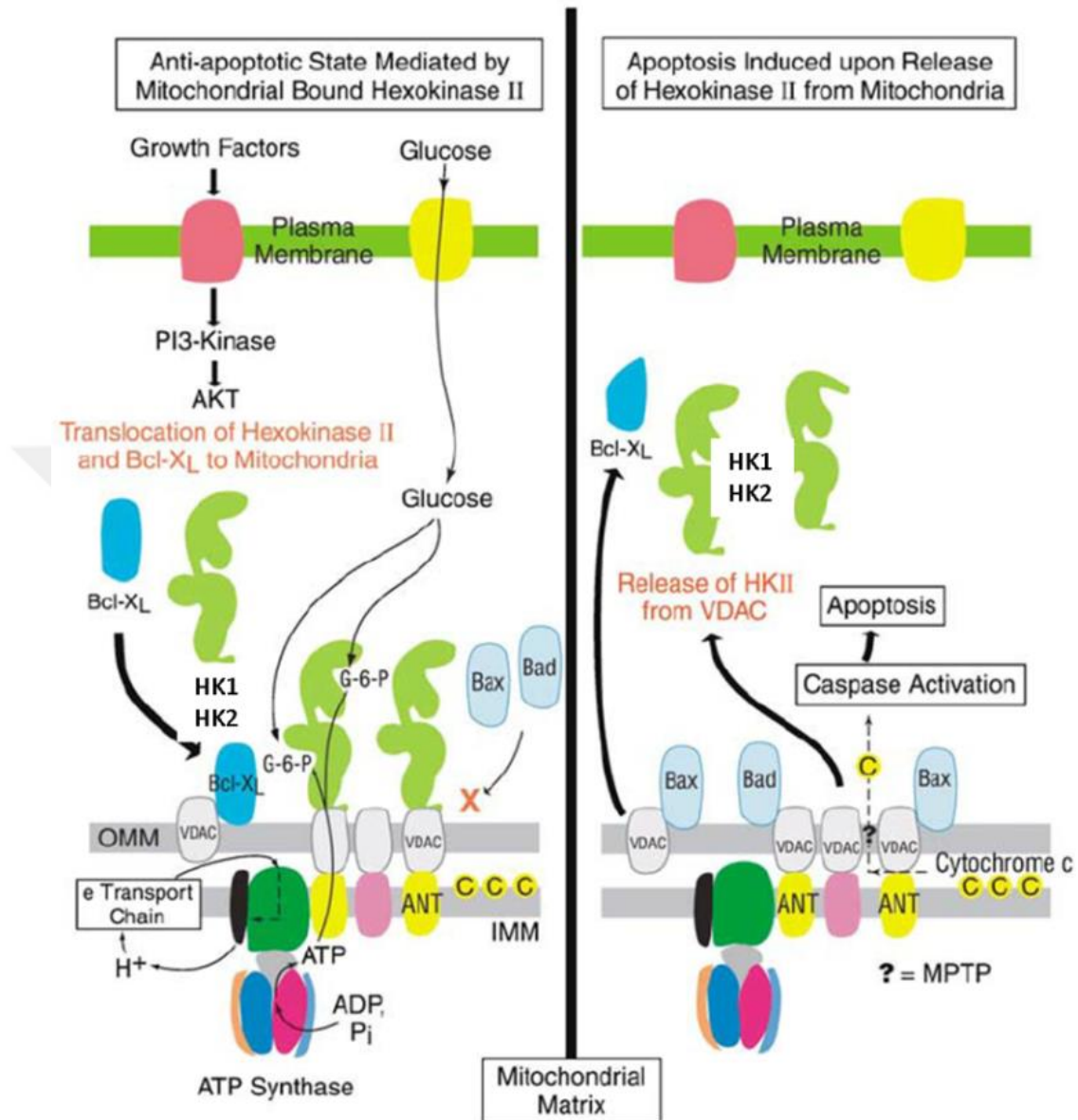


Figure 1.3.3 GSK3β role in regulation of Mitochondrial-bound HKs (HK1 and HK2).

1.3.2 Role of MAPK/ERK signaling in cancer metabolism

Mitogen-activated protein kinase (MAPK) cascades are a key signaling pathway that has important role in cell division, cell survival, cell adhesion, transcription and metabolism [75]. MAPK/ERK activation starts with RAS activation. RAS is a GTP-binding protein that is activated when it is bound to GTP. RAS has two conformations: an active guanine triphosphate (GTP) bound form and an inactive guanine diphosphate (GDP) bound form, and activation is regulated by guanine nucleotide exchange factors, or GEFs

[76, 77]. Once RAS is activated, it then activates the serine/threonine kinase RAF-1 by interacting with it. Activated RAF-1 in turn activates dual-specificity kinases MEK 1 and 2 by phosphorylating them, which in turn activate the mitogen-activated protein kinases ERK1 and ERK2 through phosphorylation of Threonine202/Tyrosine204 residues.

These two isoforms (ERK1 and ERK2) are co-expressed in almost all tissues but with a variable quantity [78-80]. Phosphorylated ERKs translocate to the nucleus, where it regulates gene expression by activating transcription factors [80-82].

Several studies have showed that activation of ERK1/2 pathway promotes proliferation and aerobic glycolysis via induction of transcriptional regulators of glycolysis, the TCA cycle, and macromolecular biosynthesis [83]. ERK1/2 regulates expression levels of various glycolytic genes, including GLUT1, GLUT3, and HK1, 2 [84-86] and affects metabolic enzymes, such as PGK1, pyruvate dehydrogenase kinase (PDK) [87].

1.3.3 Role of p53 signaling in cancer metabolism

Until recently, p53 tumor suppressor gene has been known to play key roles in promoting cell cycle arrest, apoptosis and senescence upon diverse stress signals, including DNA damage, hypoxia, and oncogenic activation. However, now p53 has added other important regulatory role in other cellular processes such as cellular metabolism [25, 88].

Several studies have revealed that p53 functions in balancing the use of glycolysis and oxidative phosphorylation, through transcriptional regulation of target genes. The complexity of p53 role in regulation of both glycolysis and oxidative phosphorylation is depending on the level of nutrient and oxygen levels [25, 88].

p53 has several functions in regulating oxidative phosphorylation that include: (1) suppresses the glucose uptake by inhibiting the transcription of glucose transporter GLUT 1, GLUT 3 and GLUT 4, thereby opposing the Warburg effect. (2) promotes oxidative phosphorylation by regulating IKK, I κ B kinase; NF- κ B, nuclear factor- κ B; SCO2, synthesis of cytochrome c oxidase 2; TCA, tricarboxylic acid [25, 88].

p53 increase glycolysis under some circumstances, including (1) P53 activates phosphoglycerate mutase (PGM), (2) regulates genes that control autophagy (promote or

inhibit autophagy) depending on metabolic stress that is caused by level of nutrient, (3) another important regulatory roles of p53 is to control the cellular metabolism pathway, by activating Hexokinases (HKs) to increase glycolysis depending on level of glucose and oxygen. P53 regulation of glycolysis and oxidative phosphorylation is illustrated in Figure 1.3.4 [25, 88].

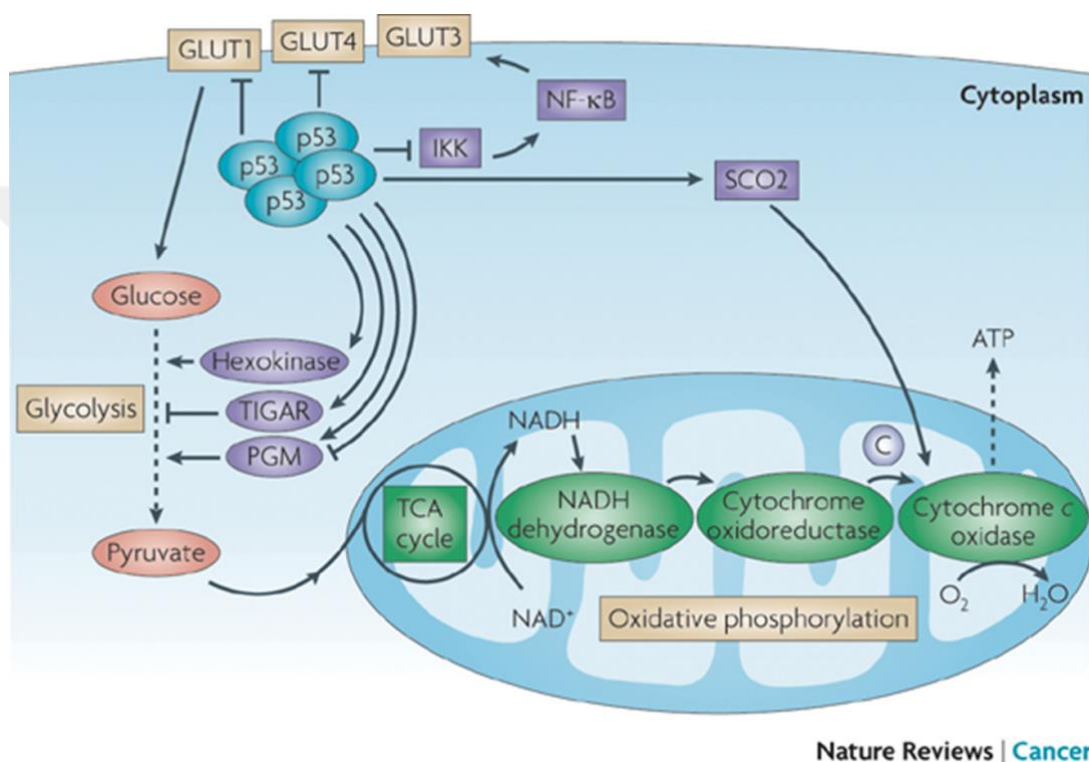


Figure 1.3.4 p53 role in regulation of metabolic pathway.

Therefore, p53 with mutation not only increases cell proliferation but also promotes glycolysis and enhance anabolic pathway, key processes that promote and sustain tumor growth. Thus, identification of the enzyme(s) that catalyze the glycolysis pathway in cancer cells could be important not only to distinguish cancer cells from normal cells, but also to preferentially target cancer cells to die.

1.4 HEXOKINASES

Hexokinases (HKs) catalyze the essentially irreversible first step of glucose metabolism in cells by phosphorylating glucose to glucose-6-phosphate (G-6-P). Hexokinases play a

major role in the cellular uptake and utilization of glucose thereby promoting and sustaining a concentration gradient that facilitates glucose entry into cells and the initiation of all major pathways of glucose utilization [89-91]. There are 4 isozymes of hexokinase encoded by separate genes, HK1, HK2, HK3, and HK4. HK1 is ubiquitously expressed in almost all mammalian tissues and HK2 is normally expressed in insulin-sensitive tissues such as adipose, skeletal, and cardiac muscles. HK3 is usually expressed at low levels and HK4 expression is restricted to the pancreas and liver. Among these, HK1, HK2 and HK3 have a much lower K_m value for glucose, suggesting higher affinity for glucose compared to HK4. However, HK1 is the only isozyme that shows reduced inhibition by its product G6P in the presence of inorganic phosphate. Therefore, during periods of high energy demand, in which the intracellular concentration of P_i would typically increase while that of G6P decrease, the HK1 activity would increase, causing more glucose being phosphorylated by HK1 and entering downstream metabolism primarily for energy production [74, 91-93]. Among them, both HK1 and HK2 share the most similar structure that they both have N and C- terminus domain and they both bind to the outer mitochondrial membrane via a mitochondrial binding domain (Figure 1.4.1) [94].

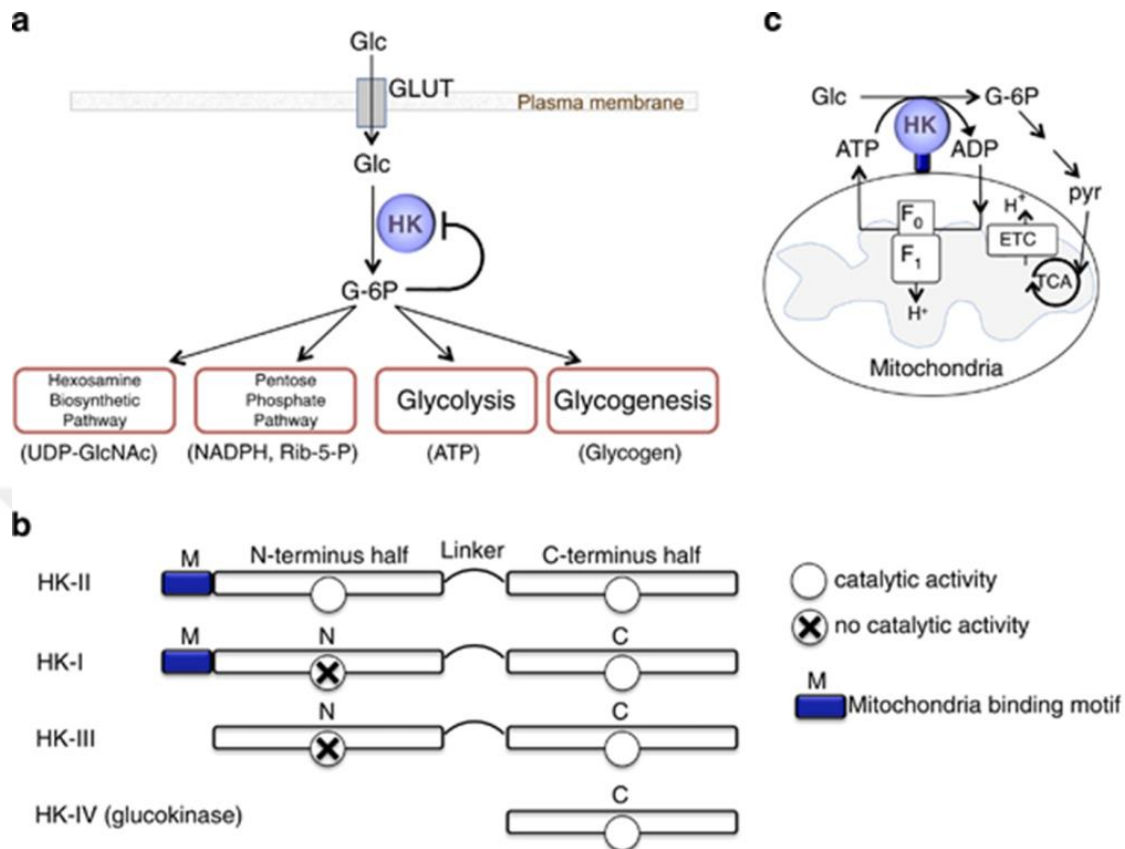


Figure 1.4.1 HK isozymes a) metabolic roles of HK b) structure of HK subtypes c) Tight coupling of both mtHK1 and mtHK2 to ATP generated by mitochondria.

Although both HK1 and HK2 isozymes have overlapping tissue expression but they have different subcellular distribution. HK1 associated mainly with mitochondria and HK2 associated with both mitochondria and cytoplasmic compartments. John et al. found that in response to changes in glucose, subcellular translocation of HK2 dynamically directs the metabolic fate of glucose between catabolic and anabolic uses, while HK1 remains associated with mitochondria to promote glycolysis [95].

Elevated HK expression is associated with many cancer types. In recent years, both HK1 and HK2 elevated expression have been observed in malignant tumors from patients with lung, gastrointestinal and breast cancer. In the latter group, further increases in HK activity were associated with metastatic disease. In tumor cells, expression of high level of HK1 and HK2 distinguishes them from the normal differentiated cells. [95-98]. Kudryavtseva et al. showed that both HK1 and HK2 are key enzymes for glucose metabolism thereby

survival of tumor cells in colorectal and melanoma cancer cells [99]. Moreover, the high level of HK expression and activity in glycolytic cancers is determined by the use of positron emission tomography (PET) to visualize the tumor growth. PET scan works using [18F] fluoro-2-deoxyglucose (FDG), fluorinated glucose analog, this is then taken up by glycolytic cancer cells and phosphorylated by hexokinase to form FDG-phosphate [100].

HK's role in cancer cell growth and proliferation via high glycolysis metabolic activity is essential and reprogramming of metabolic pathway is described as one of the hallmark of cancer [46]. It has been known that association of both mtHK1 and mtHK2 with mitochondrial outer membrane (MOM) prevents mitochondrial outer membrane permeabilization (MOMP) to block cytochrome c release, which is an essential step, for mitochondrial dependent apoptosis [101, 102]. Therefore, mtHK1 and mtHK2 binding to mitochondria induce cell death resistance, which alter cell survival signaling pathway in NSCLC.

Compared to *HK2* gene which is expressed as a single transcript, there are several transcripts of the *HK1* gene due to alternative splicing. According to the RefSeq gene annotations, which is one of the largest resources for transcript definitions, there are 10 different isoforms of the *HK1* gene. Half of these isoforms are specific to testis tissue, and are not expressed in other tissues. *HK1* gene expression is highly elevated in lung cancer, is shown in *HK1* mRNA expression profiles of multiple cancer types from TCGA and human protein atlas database (Figure 1.4.2) [103].

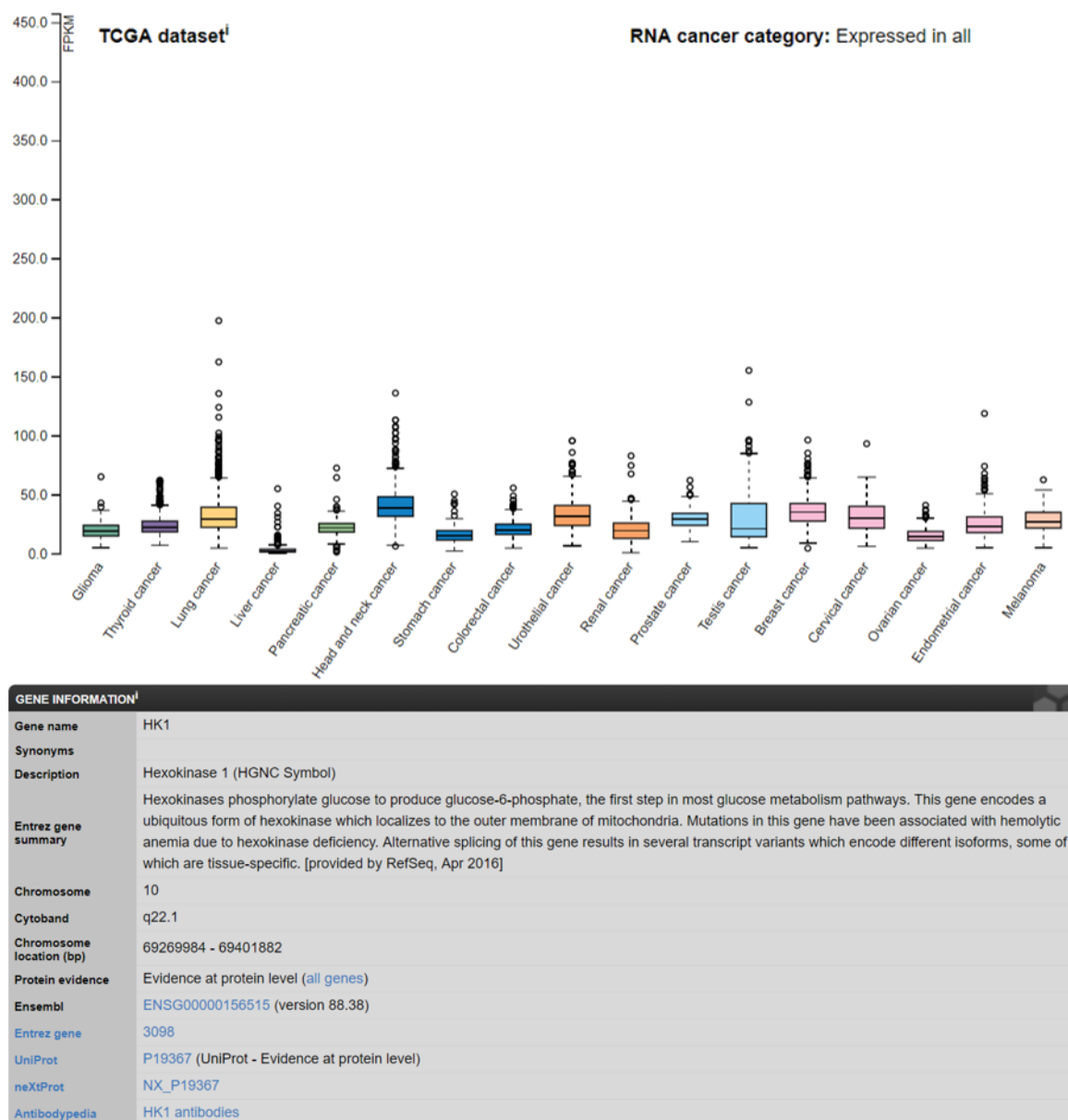


Figure 1.4.2 HK1 mRNA gene expression profiles.

2 INTRODUCTION

Patients with non-small-cell lung cancer (NSCLC) is often diagnosed with aggressive/metastatic disease which accounts 85% of all lung cancer, and has limited treatment options [104]. NSCLC patients with Kras-activating mutations (10–30 %) or loss of function point mutations in p53 (70 %) have poor clinical outcomes and most of the patients eventually relapse following the chemotherapies [105, 106]. Although targeting Kras or p53 is attractive therapeutic strategy for this disease, attempts to develop drugs that target mutant Kras and p53 proteins have, so far, been unsuccessful. Currently, much attention is instead focused on blocking Kras and p53 downstream elements to inhibit NSCLC progression [107, 108]. Metabolic reprogramming is one of the distinct hallmarks of cancer [46]. Unlike normal cells, cancer cells rely on increased glycolytic flux for their growth and proliferation which renders them more vulnerable to the disruption of glucose metabolism [41]. Although development of new therapies for targeting altered glucose metabolism of cancer has found some success in preclinical studies, its application in clinical settings has been limited due to induced adverse side effects [109].

Deregulation of glycolysis is common in NSCLC [110, 111]. One of the important regulatory roles of p53 is to control the cellular metabolism pathway by activating Hexokinases (HKs) to increase glycolysis depending on level of glucose and oxygen [25, 88]. Hexokinases (HKs) catalyze the essentially irreversible first step of glucose metabolism in cells by phosphorylating glucose to glucose-6-phosphate (G-6-P). The HK enzymes are encoded by four genes, HK1, HK2, HK3, and HK4. While HK1 is ubiquitously expressed in almost all mammalian tissues, HK2 is normally expressed in insulin-sensitive tissues such as adipose, skeletal, and cardiac muscles. HK3 is usually expressed at low levels while HK4 expression is restricted to pancreas and liver [74]. Among these, HK1, HK2 and HK3 have a much lower K_m value for glucose, suggesting higher affinity for glucose compared to HK4. However, HK1 is the only isoform that shows reduced inhibition by its product G6P in the presence of inorganic phosphate. Therefore, during periods of high energy demand, in which the intracellular concentration of P_i would typically increase while that of G6P decrease, the HK1 activity would increase, causing more glucose being phosphorylated by HK1 and entering downstream

metabolism primarily for energy production [91-93]. Moreover, both HK1 and HK2 share the most similar structure as they both have N and C- terminal domains, and they both bind to the outer mitochondrial membrane *via* a mitochondrial binding domain. Although both HK1 and HK2 have overlapping tissue expression profiles they have different subcellular distributions. While HK1 is associated mainly with mitochondria, HK2 is associated with both mitochondria and other cytoplasmic compartments [95]. The association of both HK1 and HK2 with mitochondria is regulated by Protein Kinase B (AKT) and Glycogen synthase kinase 2 β (GSK3 β), rendering cells resistant to apoptosis, and is therefore recognized as a key factor for survival of NSCLC cells [102].

Compared to *HK2* gene which is expressed as a single transcript, there are several transcripts of the *HK1* gene due to alternative splicing. According to the RefSeq gene annotations, which is one of the largest resources for transcript definitions, there are 10 different isoforms of the *HK1* gene. Half of these isoforms are specific to testis tissue, and are not expressed in other tissues [112, 113]. Here we focused on 3 isoforms with high sequence and structural homology: *HK1a*, *HK1b* and *HK1c*. Although these transcripts share most of their exons, some of the transcripts have certain exons that can differentiate them from the other transcripts. However, differential expression patterns of *HK1* isoforms (*HK1a*, *HK1b*, and *HK1c*) have not been studied, and their role in NSCLC tumorigenesis, cell survival, and drug response is still elusive. The identification of isoform-specific contributors to cancer cell glucose metabolism that could be selectively targeted to eliminate cancer cells without compromising systemic homeostasis or corresponding metabolic functions in normal cells could be a very attractive approach for cancer therapy.

Using cell lines and primary patient tumors, we have investigated the molecular and functional characterization of HK1 isoforms and demonstrated that HK1b isoform is a critical enzyme in glycolysis metabolism and cell survival. We showed the first time that only HK1b isoform is predominantly expressed in A549 cells and NSCLC patient tumors; this distinguishes NSCLC cells from the normal surrounding lung cells and HK1b is associated with poor NSCLC patient survival. We observed that HK1b expression was changed based on p53 status using p53 null and p53 WT NSCLC cells. In order to identify the mechanisms of HK1b regulation by p53, we deleted the p53 or HK1b isoform in

human NSCLC A549 cells using CRISPR/Cas9 system. Surprisingly, we showed that p53 positively regulates HK1b. Interestingly; HK1b ablation inhibits proliferation and *in vivo* tumor growth of NSCLC cells. Finally, HK1b knockout in NSCLC cells show sensitization to cisplatin, which in turn leads to p53-mediated apoptotic and autophagic cell death. Collectively, these findings suggest that therapeutic strategies to modulate the Warburg effect, such as targeting HK1b isoform, may interfere with growth and therapeutic sensitivity of NSCLC.



3 MATERIALS AND METHODS

3.1 Chemicals, solutions antibodies and reagents

Cisplatin (50 mg/100mL) was purchased from Koçak Farma.

4x Laemmli loading buffer: Tris-HCl (pH 7.5, 62.5mM), SDS 2%, Glycerol 10%, Bromophenol Blue 0.01% and β -Mercaptoethanol 5% is added freshly.

5x Electrophoresis Running Buffer, pH 8.3: Tris base 36g, Glycine 172.8g, SDS 12g 2400ml with deionized water added.

1x Transfer Buffer: TRIS base 12.11g (25mM), Glycine 57.65g (192mM), Methanol 800ml, H₂O 3200ml. Final volume 4000ml. Store at 4°C.

Tris-buffered Saline 10x (TBS) Stock: TRIS base 12.11g (100mM), NaCl 87.75g 1.5mM, HCl to pH 7.5. Final volume 1000ml. Store at RT.

Tris-buffered Saline 10x (TBS) Stock: TRIS base 12.11g (100mM), NaCl 87.75g 1.5mM, Tween 20 10ml (1%), HCl to pH 7.5. Final volume 1000ml. Store at RT. Tris base, Glycine, NaCl, Tris-HCl (pH 7.5), Tween 20, SDS, β -Mercaptoethanol, Methanol, HCl, Bromophenol Blue are from Fisher Scientific, USA.

Agarose Gel: For 100 ml 1% w/v gel, 1 g of agarose powder was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

Tris-Borate-EDTA (TBE) Buffer: For 1 L 10X stock solution, 104 g Tris-base, 55 g boric acid, and 40 ml 0.5M EDTA (pH 8.0) were dissolved in 1 L of ddH₂O. The solution is kept at room temperature.

FACS Buffer: For 500ml 1X solution, 2.5 g bovine serum albumin (BSA) and 0.5 g sodium azide were mixed in 500 ml 1X PBS and the solution was kept at +4°C.

Plasmids: pSpCas9(BB)-2A-GFP (PX458) CRISPR Cloning plasmid, developed from Zhang Lab, purchased from Addgene.

Antibodies: Rabbit monoclonal primary antibodies against HK1 (C35C4), HK2 (C64G5), Phospho-Akt (Ser 473), total Akt, Phospho-STAT3(Tyr705), total STAT3, Cleaved PARP (Asp214) (D64E10), PARP (46D11), Cleaved Caspase-3 (Asp175) (5A1E), Caspase-3 (D3R6Y), Phospho-GSK-3 β (Ser9) (D85E12), total GSK-3 β (D5C5Z), Phospho-p53 (Ser15), Phospho-mTOR (Ser2448) (D9C2), Phospho-AMPK α (Thr172) (D4D6D), total AMPK α (D63G4), Atg13 (E1Y9V), β -Actin (13E5), GAPDH (D16H11) and horseradish

peroxidase (HRP)-conjugated rabbit and mouse secondary antibodies purchased from Cell Signaling Technologies (USA). Mouse monoclonal primary antibodies against p53 (DO-1), Bcl-2 (C-2), MAP LC3 β (G-2), and BECN1 Antibody (G-11) purchased from Santa Cruz Biotechnologies (USA). Cell culture medium (RPMI-1640), FBS, glutamine, penicillin, and streptomycin were acquired from Gibco (Grand Island, NY).

3.2 Cell culture and treatment

Human non-small cell lung cancer (NSCLC) cell lines, including A549, H1299, were obtained from Dr. Engin Ulukaya's laboratory (University of Istinye) and propagated in monolayer culture in RPMI 1640 supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine. All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. All cell lines were tested for Mycoplasma routinely every 3 months using MycoProbe Mycoplasma Detection Kit (R&D Systems).

3.2.1 Cell Proliferation by FACS Analysis

A549 and A549 HK1b^{-/-} cells were trypsinized and harvested at 50%–70% confluency. Cells were fixed with ice-cold 70% ethanol dropwise while vortexing and incubated at minus 20 degree for a minimum of 24 hr. Fixed cells were pelleted and washed twice with PBS. Anti-Human Ki-67, FITC kit is used to stain cells according to the manufacturers' protocol (BD Biosciences). Unstained and isotype-control stained cells are shown as controls. Cells were analyzed with a FACS AriaII flow cytometer and processed using FlowJo software

3.2.2 Cell viability assay

Twenty-four hours following seeding (15,000 cells/well) in a 96-well plate, A549 and A549 HK1b^{-/-} cells were treated with phosphate buffered saline, or cisplatin (0.9% NaCl w/w) with concentrations ranging from 0–100 μ M/ml and the cells were incubated for a further 48 h. The cell viability was determined using the CellTiter-Blue assay kit (Promega) according to the manufacturer's instructions. IC₅₀ was defined as the concentration causing a 50% reduction in absorbance relative to the negative control. IC₅₀ was determined by non-linear regression analysis using Graphpad Prism v8

(Graphpad Software, CA, USA). IC50 concentration of cisplatin are used in A549 (75 μ M) and A549 HK1b^{-/-} (47 μ M) cells and after 48hrs treatment cells were harvested by trypsinization, collected by centrifugation, washed once with 1 ml of PBS and stained with propidium iodide (BD Biosciences) according to the manufacturers' protocol and number of death cells was measured using fluorescence-activated cell sorting.

3.2.3 Measurement of oxygen consumption rate and extracellular acidification rates

For Seahorse XFe96 assays, cells were seeded at pre-optimized final concentration 1.5 x 10⁴ cells/well onto Seahorse XF-96-well plates and glycolysis stress and mito Stress tests were performed following the manufacturer's specifications (Seahorse Bioscience, North Billerica, MA). Each datum was determined minimally in triplicate. OCR and ECAR were reported as absolute rates (pmoles/min and OCR or mpH/min for ECAR) or normalized against cell counts, or expressed as a percentage of the baseline oxygen consumption

3.3 Gene knockout in lung cancer cells

A549-HK1b isoform-knockout cells were generated by using the CRISPR/ Cas9 genome editing system. Briefly, the sgRNAs specific for *HK1b isoform only* was designed to target the 8th exon of human *HK1* gene. pX458 vector with GFP selection marker from Addgene, as described previously [19], was used to deliver Cas9 and a sgRNA targeting *HK1b* (namely sgHK1b) into the target cells. The expression of single-cell clones expressing sgHK1b were screened for HK1b isoform elimination and specific indel mutations at target loci was confirmed with PCR, DNA fragmentation analyses. The CRISPR guide sequences targeting *HK1b* were sense: GAGATTTAGAGAGAGCGCAG, antisense: CTGCGCTCTCTCTAAATCTC

3.4 Patient Tissue collection and immunohistochemical staining

This study was approved by the Ethics Committee of Istanbul Medipol University. 18 cases of clinically and immunohistologically verified frozen NSCLC primary tumor and normal lung tissue samples were collected from affiliated hospital of Istanbul Medipol University (Istanbul, Turkey), and were agreed by all patients. All studies were conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice.

The sections were stained with indicated antibodies, and staining of HK1 and Ki-67 was scored by pathologists based on the number of positive cells per area and staining intensity. Patients' clinical characteristics are listed in Supplementary Table 1.

3.5 *In vivo intra-tracheal xenograft tumorigenesis assay.*

NSG mice were purchased from The Jackson Laboratory and experiments were performed at the Istanbul Medipol University. All mice were maintained in a specific pathogen-free facility in a temperature-regulated room ($23\pm1^{\circ}\text{C}$) with a 12 h light/dark cycle. The protocols used were approved by the Institutional Animal Care and Use Committee of Istanbul Medipol University (Istanbul, Turkey), and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory 6-8 weeks old animals. 5×10^6 x A549 WT and A549 HK1b^{-/-} cells were *intra-tracheally* injected into the right pleural cavity of NSG male mice. Approximately 3rd-4th intercostal space in the midclavicular line is used for injection. *Relative tumor volume* was measured 8 weeks after implantation and, is calculated as follows: $(\text{length} \times \text{width} \times \text{height}) \times \pi/6$. End points were reached, and mice were killed once the tumor size measured 2000 mm³.

3.6 RNA isolation and quantitative PCR

RNA was isolated from human NSCLC cells and human clinical NSCLC tumor and normal samples by homogenization in Trizol reagent (Invitrogen) according to the manufacturer's instructions, followed by purification on an RNeasy column (Qiagen). RNA purity was assessed by Thermo NanoDrop 2000 (Thermo Fisher Scientific, Inc.) by standard absorbance ratios as $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} \geq 1.5$. Complementary DNAs were synthesized from 2 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) Real time Quantitative PCR (RT-*qPCR*) results obtained with *Bio-Rad's* iTaq Universal SYBR Green Supermix..The threshold cycle (Ct) for individual reactions were identified using iCycler IQ sequence analysis software (Bio-Rad). β -actin was used for normalization of the data. All experiments were performed in triplicate. The following primers were used in this study with the following sequences:

HK1a isoform Fwd., 5'- TGGCAAGCGCTGTTCTTTGC-3'
 Rev., 5'- CTGGCTCTTCAACGCAGTG-3'

HK1b isoform Fwd., 5'- CCCTGAACACGGAGATTGACA -3'
 Rev, 5'- GCACCGGCACATTTCGATAG -3'

HK1c isoform Fwd, 5'- GCTCAGTGCCAGGGTGATTT-3'
 Rev., 5'- TGCCAAGATAGTCCTCTTCTATTT-3'

HK2 Fwd, 5'- CATCTTCCGTGTGCTATGGG-3'
 Rev., 5'- TGACACCAGGAACTGTCGT-3'

p53 Fwd, 5'- GGACCACTAAGCGAGCACTG
 Rev., 5'- TCTCGGAACATCTCGTAGCG-3',

H₂O-actin Fwd, 5'- AGAGCTACGAGCTGCCTGAC -3'
 Rev., 5'- AGCACTGTGTTGGCGTACAG -3'

3.7 Western blot analysis

3.7.1 Protein extraction

Cells were harvested and subjected to lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, 25 mM NaF), containing proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche) and then subjected to sonication for total lysates recovery. The lysates were subjected to centrifugation at 10,000 g at 4°C for 20 minutes and the supernatants removed. For immunoblotting of tissue samples, proteins were extracted in the same buffer with a tissue homogenizer after thawing frozen tissues collected by liquid nitrogen snap freezing.

3.7.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Using SDS-PAGE, proteins were separated under denaturing conditions according to their molecular weight. 50 µg of protein lysates were re-suspended in 4x Laemmli loading buffer, incubated at 100°C for 5 minutes, and then allowed to cool to RT for 2 minutes. The total protein (50 µg) was fractionated by 10% SDS-PAGE. Gels were run at 80V for approximately 2.5 hrs at room temperature by BioRad Mini Protean II System (BioRad, USA).

3.7.3 Protein Transfer and Immunoblotting

0.45µM pore size (Millipore, USA) Immobilon-P transfer membranes were first dipped into methanol for 2 minutes, then washed in ddH₂O for 5 minutes, and then incubated in transfer buffer for 10 minutes. After the gelelectrophoresis, gels were incubated for 5min in a cold transfer buffer. Gel and membrane were assembled in a Mini Trans-Blot Cell (BioRAD, USA) according to the manufacturer's instructions. Proteins were transferred at 90V (240mA to 400mA) for 90 minutes.

After transferred, the membrane was blocked with blocking buffer (Li-Cor) at room temperature for 1 hour, and then incubated with appropriate primary antibody at 4C overnight. After washing with TBS containing 0.05% Tween 2 (TBST), the membrane was incubated with 1:2500 dilutions of appropriate secondary antibody for 2 hours at room temperature. The membrane was washed with TBST again, and Pierce ECL Western Blotting Substrate (Thermo Scientific) was used for development of immunoreactive bands.

3.8 The Cancer Genome Atlas NSCLC cohort analyses and GSEA analysis

The publicly available Cancer Genome Atlas (TCGA) RNA-seq data on adenocarcinoma (n = 511) and SCC (n = 501) was used as an external cohort to verify DEGs between adenocarcinoma and SCC based on the RG cohort and to further analyze the expression of the HK1b isoform (transcript variant-8) between normal and TCGA Lung adenocarcinoma samples (RefSeq id NM_001322366). Lung Adenocarcinoma project contains 426 RNA sequencing of lung tumor tissues and matched with 602 normal lung tissues from the GTEx Project. For each database the supplied expected transcript read counts that are generated using the RSEM software package is used for the comparison. The read counts are normalized using the DESeq2 variance stabilized normalization method.

Gene expression data of lung cancers was extracted from the GEO database (accession number: [GSE31210](#)). Before GSEA, all lung cancer samples were divided into two groups according to their HK1 expression levels: HK1 high and HK1 low. GSEA was then performed based on normalized data using GSEA v2.0 tool

(<http://www.broad.mit.edu/gsea/>) for identification of enriched gene sets between the HK1 high and HK1 low groups.

3.9 Statistical analysis.

Unless otherwise stated, data are expressed as mean $\pm$ SEM. GraphPad Prism 8 and SPSS 18.0 software were used for graphing and statistical analysis. After calculating normality by Shapiro-Wilk test, Student *t* or Mann-Whitney test were used to compare two samples. For multiple group comparisons, One-way ANOVA analysis and followed by Tukey's multiple comparison tests were used. Statistical parameters including exact value of *n*, statistical test and significance are reported in the figures and figure legends. Values of $p < 0.05$ were considered statistically significant.



4 RESULTS

4.1 HK1 expression is upregulated in human NSCLCs and is associated with poor clinical outcome in patients

To validate specific upregulation of HK1 in human NSCLCs, we analyzed human NSCLC tumor tissues and adjacent non-tumor lung *tissues* for the expressions of HK1, HK2 and p53. Clinical details of patients included in this study are provided in Table 4.1. We found that in a small cohort of patients (12 tumor and 10 adjacent non-tumor samples), HK1 and HK2 were upregulated in NSCLC tumor tissues as compared to normal lung tissues as indicated with two immunoblotting assays (Fig. 4.1.1A and B). Quantification of band intensities of HK1 and HK2 revealed that human NSCLC samples have significantly higher HK1 (n=12) ($p<0001$) expression than HK2 (n=12) ($p=0037$) (Fig. 4.1.1C), suggesting that HK1 is more ubiquitously overexpressed. We further confirmed *in situ* expression of HK1 in NSCLC cells by using the immunohistochemistry assay. All the noncancerous epithelium showed weak staining of HK1, while tumor cells showed moderate to strong staining of HK1 (Fig. 4.1.2A). To investigate whether gene sets that have prognostic value are enriched in *HK1*-expressing NSCLC samples, we carried out Gene Set Enrichment Analysis (GSEA) by using the GSE31210 dataset [114, 115]. Indeed, survival analysis showed that higher expression of HK1 was associated with decreased relapse-free survival ($p=0.01$; Fig. 4.1.2B), suggesting that elevated HK1 expressions associated with disease progression.

Table 4-1 List of total 18 NSCLC patients' clinical details is included in this study.

Patient #	Patient ID	Gender	Age (Years)	Histological type	Grade	Stage	p53	Used in experiment
1	20141-16	Male	64	ADC	2	pT2aN1	pozitif (%95)	WB
2	1103-17	Male	67	ADC	3	pT3N0	negatif (%1)	WB
3	2201-17	Female	75	ADC	2	pT1bN0	negatif (%1)	WB
4	4333-17	Male	68	ADC	3	pT2bN1	pozitif (%90)	WB,q-RT
5	14011-17	Female	53	ADC	1	pT2aN0	negatif (%1)	IHC,WB,q-RT
6	16814-17	Male	54	ADC	3	pT2aN2	negatif (%0)	IHC,WB,q-RT
7	18729-17	Male	54	ADC	3	pT3N0	negatif (%1)	WB,q-RT
8	24555-17	Male	62	Solid ADC	3	ypT2bN0	pozitif (%95)	IHC,WB
9	27384-17	Male	66	Enteric type ADC	2	pT1aN1	negatif (%0)	IHC
10	27796-17	Male	65	Invasive mucinous ADC	1	pT4N1	negatif (%0)	IHC,WB
11	12066-18	Male	66	Enteric type ADC	2	pT2bN0	pozitif (%95)	IHC,WB
12	12867-18	Male	72	Acinar dominant type ADC	2	pT2N1	pozitif (%90)	WB
13	15612-18	Male	68	Solid ADC	3	pT2N1	pozitif (%90)	IHC,WB,q-RT
14	18053-18	Female	76	Acinar dominant type ADC	2	pT2aN0	negatif (%0)	WB,q-RT
15	21673-18	Male	48	Enteric type ADC	2	pT2aN1	negatif (%0)	IHC,WB,q-RT
16	14196-16	Male	62	Basaloid SCC	2	pT3N0	pozitif (%100)	WB,q-RT
17	17682-17	Male	40	SCC	3	pT2aN2	negatif (%1)	WB,q-RT
18	6369-18	Male	69	Solid ADC	3	pT2aN1	negatif (%0)	IHC

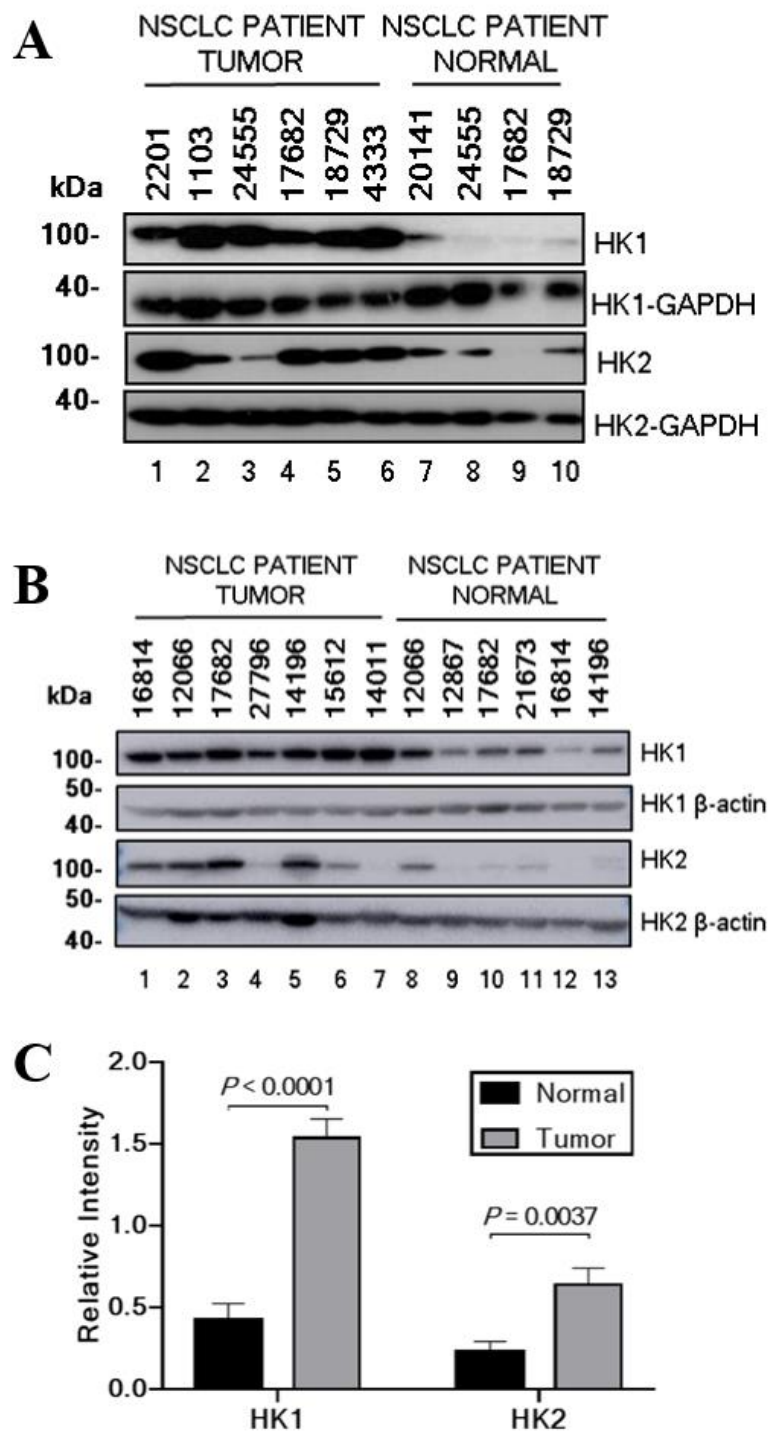


Figure 4.1.1 HK1 expression is upregulated in human NSCLC from western blot analyses. Protein was isolated from NSCLC tumor and adjacent-non tumor samples and HK1 level was determined by WB analyses **A)** protein lysates of human NSCLC tumor (n=6), normal lung (n=4); **B)** protein lysates of human NSCLC tumor (n=7), normal lung (n=6). **C)** Quantification of HK1 and HK2 levels in NSCLC tumor (n = 13) and normal lung samples (n=10).

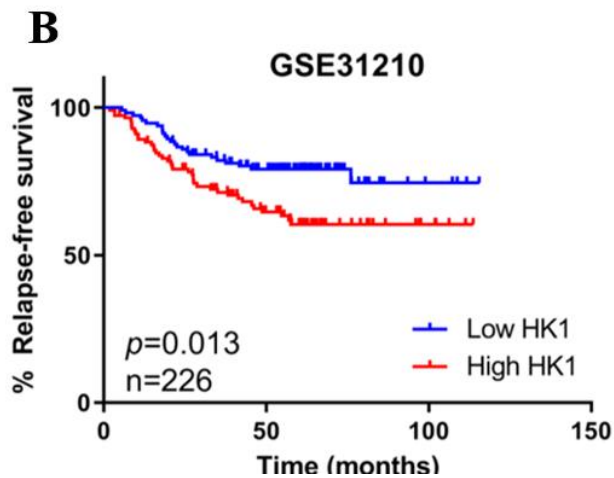
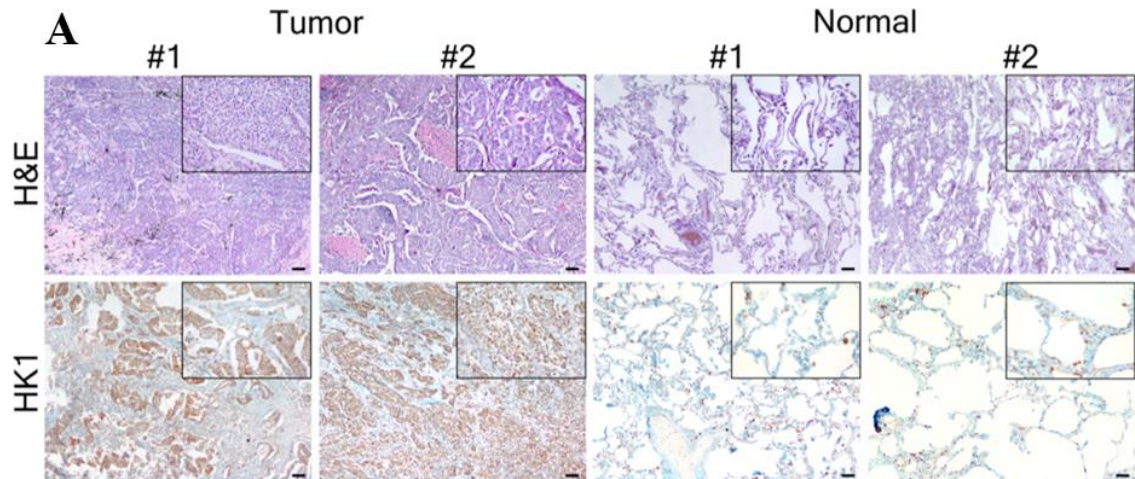


Figure 4.1.2 HK1 expression is upregulated in human NSCLC from IHC analyses and is associated with a poor overall survival. **A)** Immunohistochemical staining of HK1 expression and H&E staining in representative human NSCLC tumor (left) and the surrounding normal tissue (right). Images are 40X magnification; insets are 200X magnification. Scale bars, 100 μm. **B)** the prognostic values of HK1 in NSCLC patients (n=226). Tumors were separated into two groups according to HK1 expression levels (high or low). The high expression of HK1 is correlated to worse survival (p=0.013).

4.2 Human NSCLC cells express one of the HK1 isoforms- hexokinase1b (HK1b) as a poor prognostic factor

The HK1 gene spans approximately 131 kb and consists of 25 exons. According to RefSeq gene annotations, alternative splicing of *HK1* gene at its 5' end produces different transcripts in different cell types. The testis-specific exons are located at approximately 15 kb upstream of the erythroid-specific exon R. The first 5 exons of the *HK1* gene encode the

testis-specific sequences. The 6th exon is the erythroid-specific exon R [112]. When these tissue specific transcripts are excluded, 3 remaining transcripts are; *HK1a*, *HK1b* and *HK1c*. On the other hand the *HK2* gene has one transcript in the RefSeq gene annotations (Fig. 4.2.1A). Although, there are a few studies on the testis specific transcripts [113], the differential expressions of these three *HK1* isoforms in different tissues (including the cancer tissues) and their functional role have not been reported. When we analyze the *HK1* transcripts, it is possible to differentiate them using the differentially used exons. *HK1a* transcript contains different exons at the 5' end and therefore it is possible to distinguish this transcript. Both *HK1b* and *HK1a* isoforms possess one extra exon, which is missing in the *HK1c* isoform. Using these exon-exon junctions around this extra exon, we were able to design specific genomic probes to differentiate *HK1b* and *HK1c* isoforms. Therefore, designing specific primers for these exons will allow us to quantify the expressions of these isoforms in different tissues. Moreover, the extra exon in *HK1b* codes for 32-amino acid long alpha-helix that is part of the small sub-domain (Fig. 4.2.1B).

To identify new, HK1 isoform specific metabolic target for the treatment of NSCLC, we first analyzed HK1 isoforms (*HK1a*, *HK1b*, and *HK1c*) along with *HK2* and *p53* in NSCLC adenocarcinoma cell lines (*p53* WT-A549 and *p53* null-H1299). Semi-q-PCR data of both A549 and H1299 cells showed that *HK1a* isoform is not specific to NSCLC because of its absent expression. *HK1b* isoform predominantly expressed in *p53* WT A549 cells, but not in *p53*^{-/-} H1299 cells. However, we observed no difference in the levels of *HK1c* isoform and *HK2* expressions in A549 and H1299 cells (Fig. 4.2.2A). Since there is no available isoform specific antibody to detect HK1 isoforms, we used *HK1* antibody to detect the level of its expression in both A549 and H1299 cells. Therefore, we further validated the PCR results by immunoblot analysis that *HK1* protein expression was higher in A549 cells than H1299 cells and *HK2* expression levels were not changed in both cells (Fig. 4.2.2B), indicating that *HK1b* expression was specific to A549 cells. Importantly, to evaluate the clinical relevance of *HK1b* upregulation in human NSCL cancer, we performed semi-q-PCR and qRT-PCR analyses on tumors and adjacent non-tumor samples from NSCLC patients. In particular, *HK1b* was significantly ($p=0.005$) upregulated than *HK2* transcript ($p=0.03$) in the NSCLC tumor when compared to normal adjacent tissue ($p=0.005$) (Fig. 4.2.2C and D). *HK1c* isoform expression was not significant, supporting

the idea that Hk1b was uniquely upregulated in comparison to HK1c isoform and HK2. Interestingly, p53 expression also increased ($p=0.005$) similar to HK1b. In addition, NSCLC patient tumor cells showed no HK1a isoform expression (Fig. 4.2.2C and D). In order to further validate the increased expression of the HK1b isoform between the NSCL adenocarcinoma and the normal lung tissues, we merged the most extensive public databases TCGA (The Cancer Genome Atlas) Project and the GTEx (Genotype-Tissue Expression) Project. TCGA Lung Adenocarcinoma project contains 426 RNA sequencing of lung tumor tissues. These tumor samples, adenocarcinoma ($n = 511$) and SCC ($n = 501$), are matched with 602 normal lung tissues from the GTEx Project. Consistent with RT-PCR analyses, the comparison of the expression of the HK1b isoform (transcript variant-8) between normal and TCGA Lung adenocarcinoma samples (RefSeq id NM_001322366) showed a significant upregulation of *HK1b* in NSCLC tumor tissues compared with those in normal lung tissues ($p= 1.85e-07$) (Fig. 4.2.3A). Altogether, these data reveal that elevated HK1b expression is specific to NSCLC across a wide selection of lung cancer patients when compared to normal lung tissues. Moreover, higher expression of the HK1b isoform is associated with decreased survival probability for both adenocarcinoma (Fig. 4.2.3B) and squamous cell carcinoma (Fig. 4.2.3C) types of NSCLC, albeit the latter is not significant ($p= 2.69e-02$, $p=9.5e-02$, respectively). Collectively, our data provide compelling evidence for the specificity of HK1b isoform in NSCLC which may be utilized an attractive metabolic target.

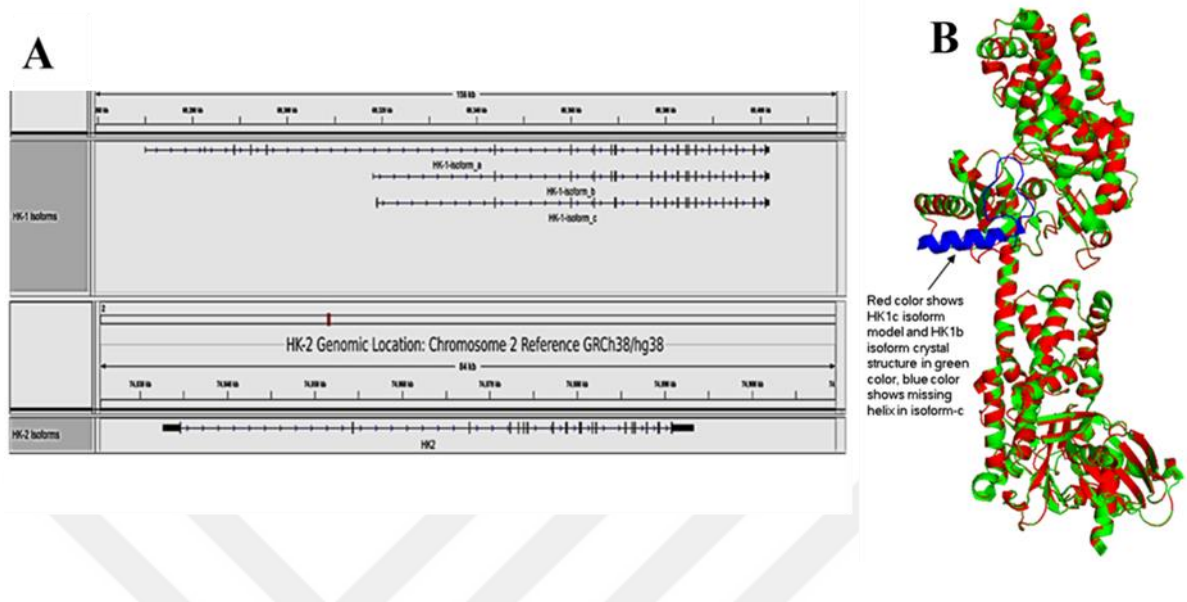


Figure 4.2.1 **A)** RefSeq gene annotations of HK1 isoforms (HK1a, HK1b and HK1c) and HK2. HK1a and HK1b isoforms possess one extra exon, which is missing in HK1c. **B)** Structural alignment between crystal structures of HK1b and HK1c model. The extra exon in HK1b, which is shown in blue, codes for 32-amino acid long alpha-helix that is part of the small sub-domain of N-terminus. For the sake of simplicity only one monomer is shown. HK1b isoform levels were elevated in NSCLC tumors compared with normal lung tissue.

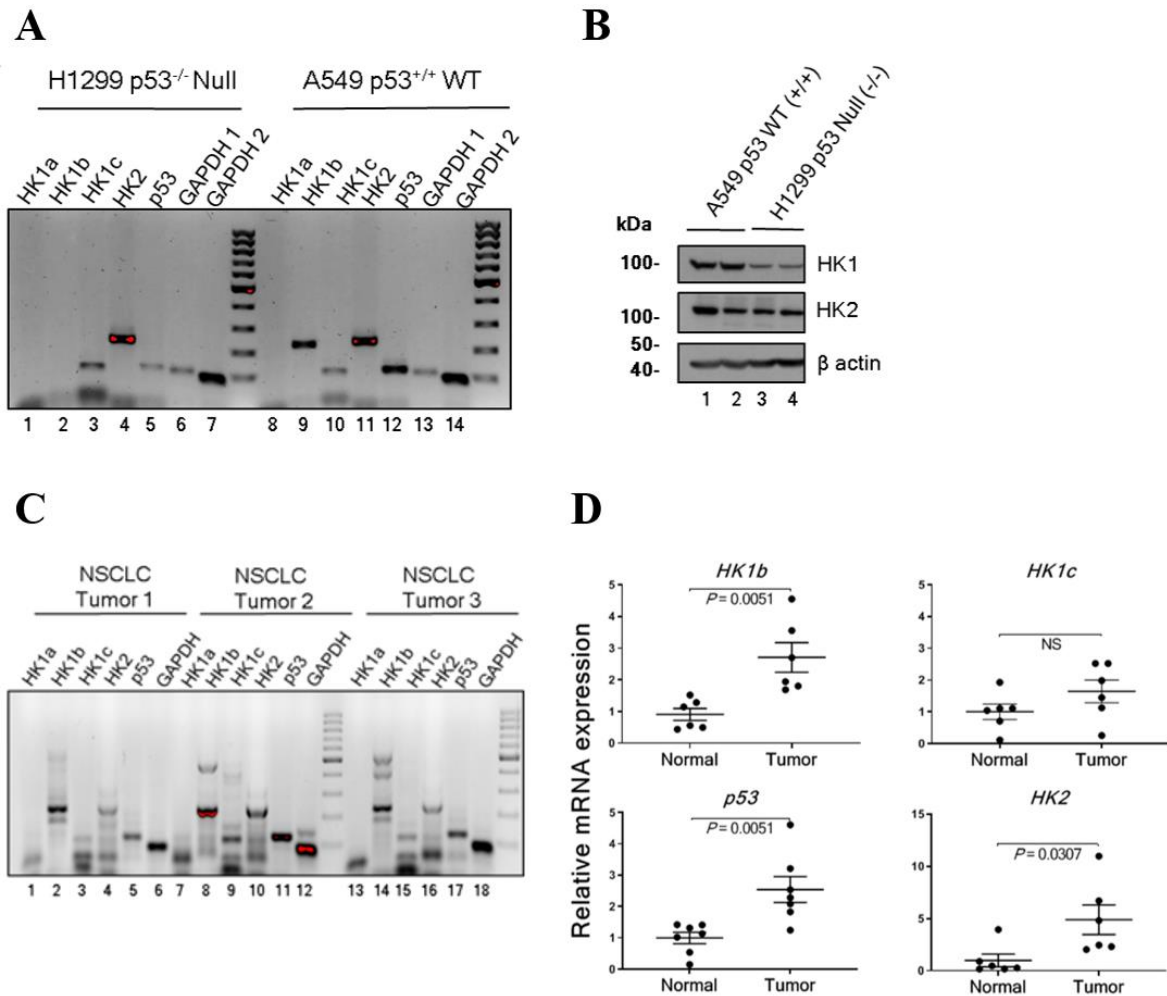


Figure 4.2.2 HK1b isoform upregulation in NSCLC patients **A)** Representative semi q-PCR amplification results for HK1 isoforms, HK2 and P53 in H1299 and A549 cells. **B)** Immunoblot analysis of HK1 and HK2 in A549 and H1299 cells. **C)** Representative semi q-PCR amplification results for HK1 isoforms, HK2 and P53 in NSCLC patient tumors (n=3). **D)** RNA was extracted from NSCLC patient normal lung and tumor samples, HK1b, HK1c, HK2 (normal n=6, tumor n=6) and p53 (normal n=7, tumor n=7) levels were determined by qRT-PCR. mRNA levels were normalized to ACTB mRNA. Data are from two or three independent experiment as triplicate, presented as mean $\pm$ SEM, Student's t test

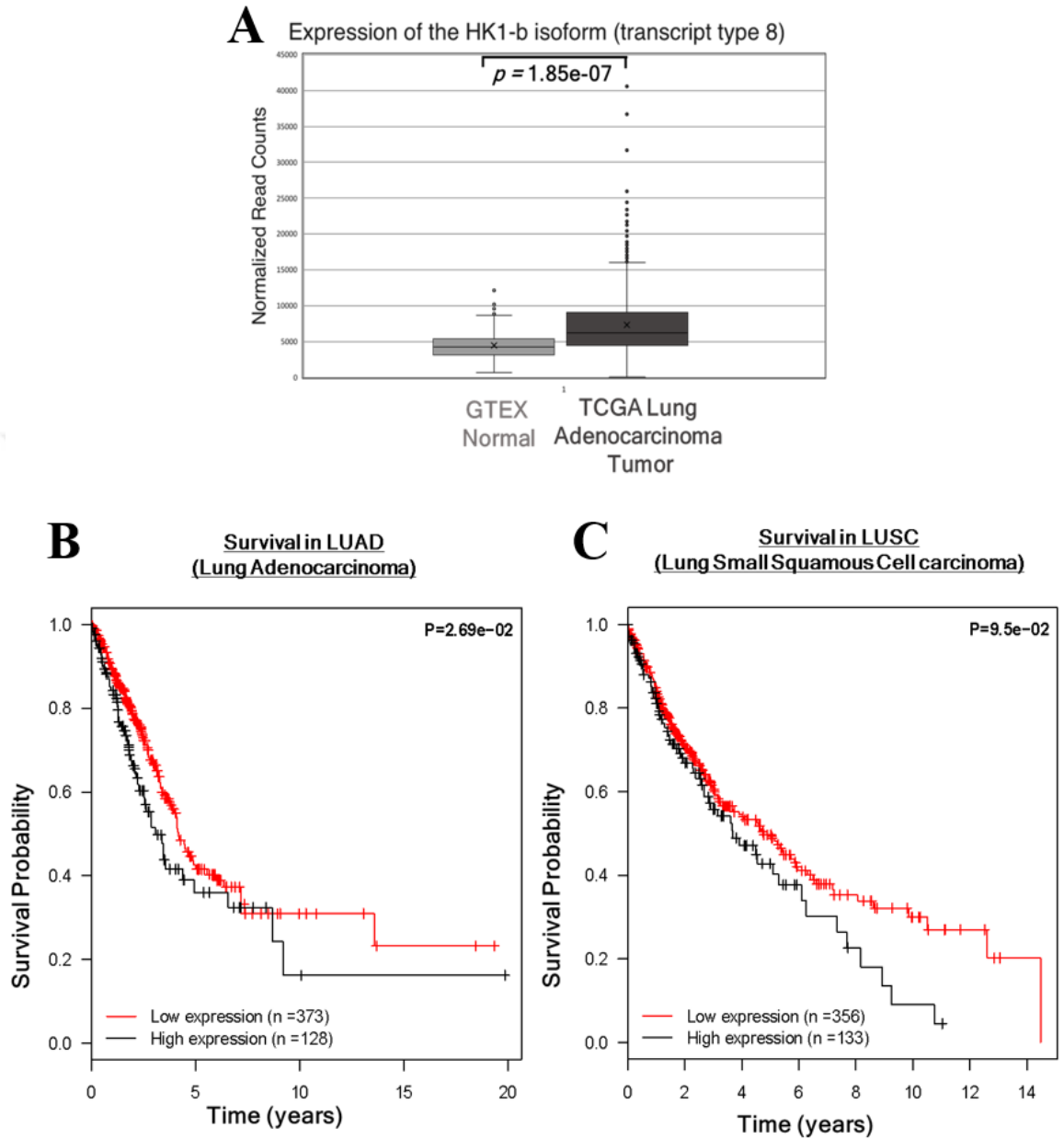


Figure 4.2.3 Increased expression of the HK1b isoform is associated with worse prognosis from TCGA NSCLC patient data **A**) Kaplan–Meier plots showing the association of HK1b isoform overexpression between normal and TCGA Lung adenocarcinoma samples in patients from the TCGA cohort. Kaplan–Meier survival curves **B**) adenocarcinoma (n = 501) and **C**) squamous cell carcinoma (n = 489), patients stratified according to low or high HK1b expression levels. P values indicate significance levels from the comparison of survival curves using the Log-rank (Mantel-Cox) test.

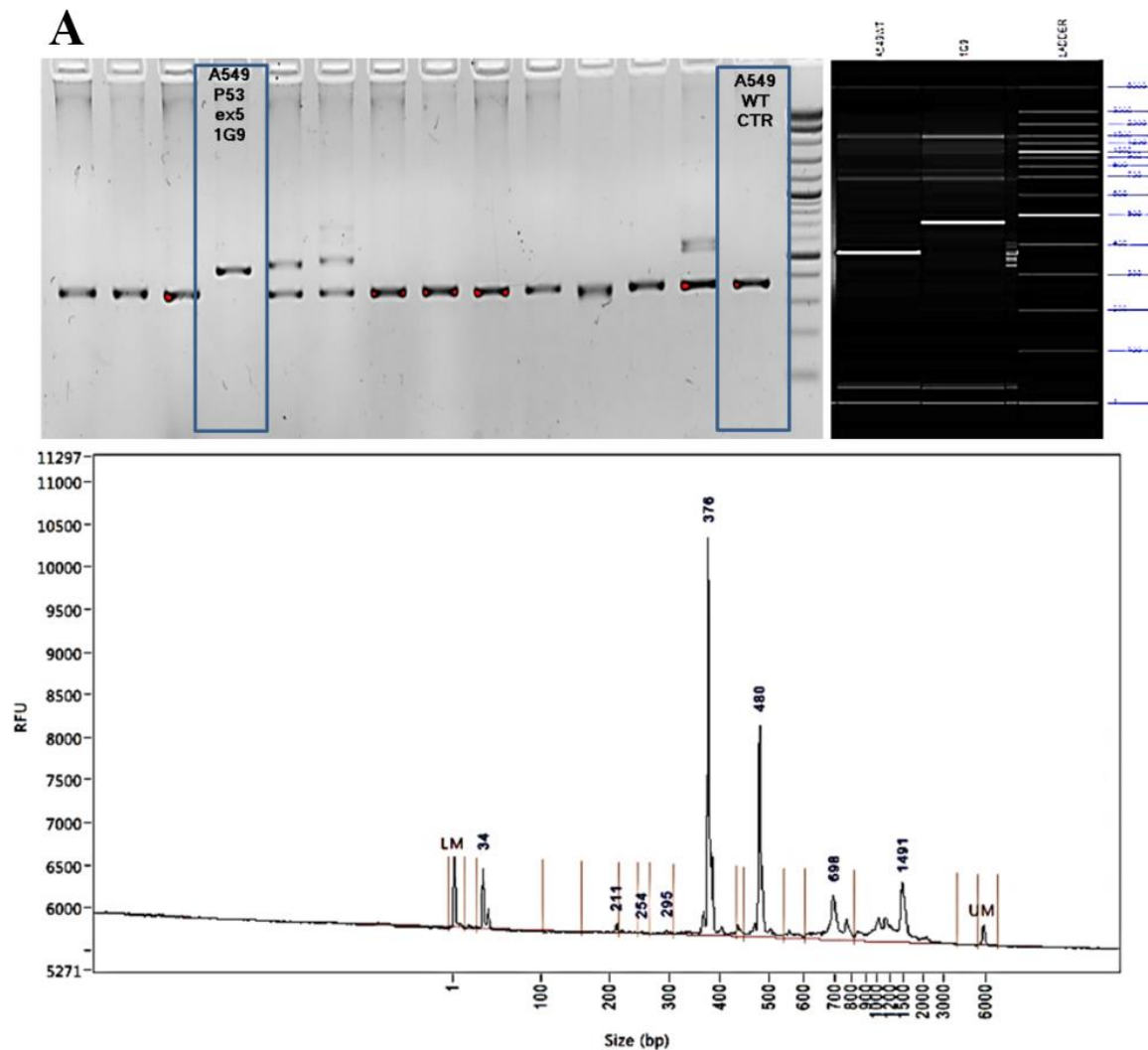
4.3 p53 positively regulates HK1b expression

p53 has several functions in regulating cell metabolic pathway by regulating the levels of series of gene products that balance the use of glycolysis and oxidative phosphorylation. This includes activation of hexokinase and phosphoglycerate mutase (PGM), which could increase glycolysis under some circumstances [25, 88]. We observed the change in expression of the HK1b based on p53 status of NSCLC cells. A549 NSCLC cells expressing WT *p53* show high levels of HK1b expression, whereas H1299 NSCLC cells that are *p53* null show absence of HK1b expression.

To understand if the association of HK1b expression with p53 is cell specific or p53 related event, we used CRISPR/Cas9 -based method to genetically disrupt p53 or HK1b isoform in human NSCLC A549 cells as an isogenic system using CRISPR/Cas9 system. While the sgRNAs specific for *p53* was designed to target the fifth exon of human *p53* gene, the sgRNAs specific for *HK1b* isoform only was designed to target the eighth exon of human *HK1* gene. pX458 vector with GFP selection marker, as described previously [116], was used to deliver Cas9 and a sgRNA targeting *p53* or *HK1b* (namely *sgp53* or *sgHK1b*) into the target cells. Single-cell clones expressing *sgp53* or *sgHK1b* were screened for p53 or HK1b isoform elimination at the expense of specific indel mutation generation at target loci. PCR and DNA fragmentation analyses showed insertion of 104bp nucleotides in *p53* (Fig. 4.3.1A) and deletion of 14 bp nucleotides in *HK1b*, resulting in a frame-shift mutation (Fig. 4.3.1B).

To elucidate the regulation of HK1b expression with p53, we first validated the knock-outs of p53 and particularly HK1b isoform transcriptionally. Both semi-qPCR and qRT-PCR of A549 WT and A549 *p53*^{-/-} cells transcription analysis revealed that A549 WT cells showed absence of endogenous p53. Interestingly, as a result of p53 deletion we observed total absence of HK1b isoform expression, reduced expression of HK2, and HK1c, which was not significant (Fig. 4.3.2A). Further, both Immunoblot and immunofluorescence analyses of A549 WT and A549 *p53*^{-/-} cells, using anti-p53 and HK1 antibodies showed the total absence of endogenous p53 and significantly decreased level of endogenous HK1 protein (Fig. 4.3.2B and E).

We further analyzed isogenic clones expressing sgHK1b with qRT-PCR. Of note, we were able to delete HK1b isoform specifically, without altering the transcriptional expressions of HK1c isoform, HK2 and p53 (Fig. 4.3.2C). Moreover, we confirmed by Immunoblot and immunofluorescence analyses that level of HK1 protein was absent due to permanent loss of HK1b isoform (Fig. 4.3.2D and E).



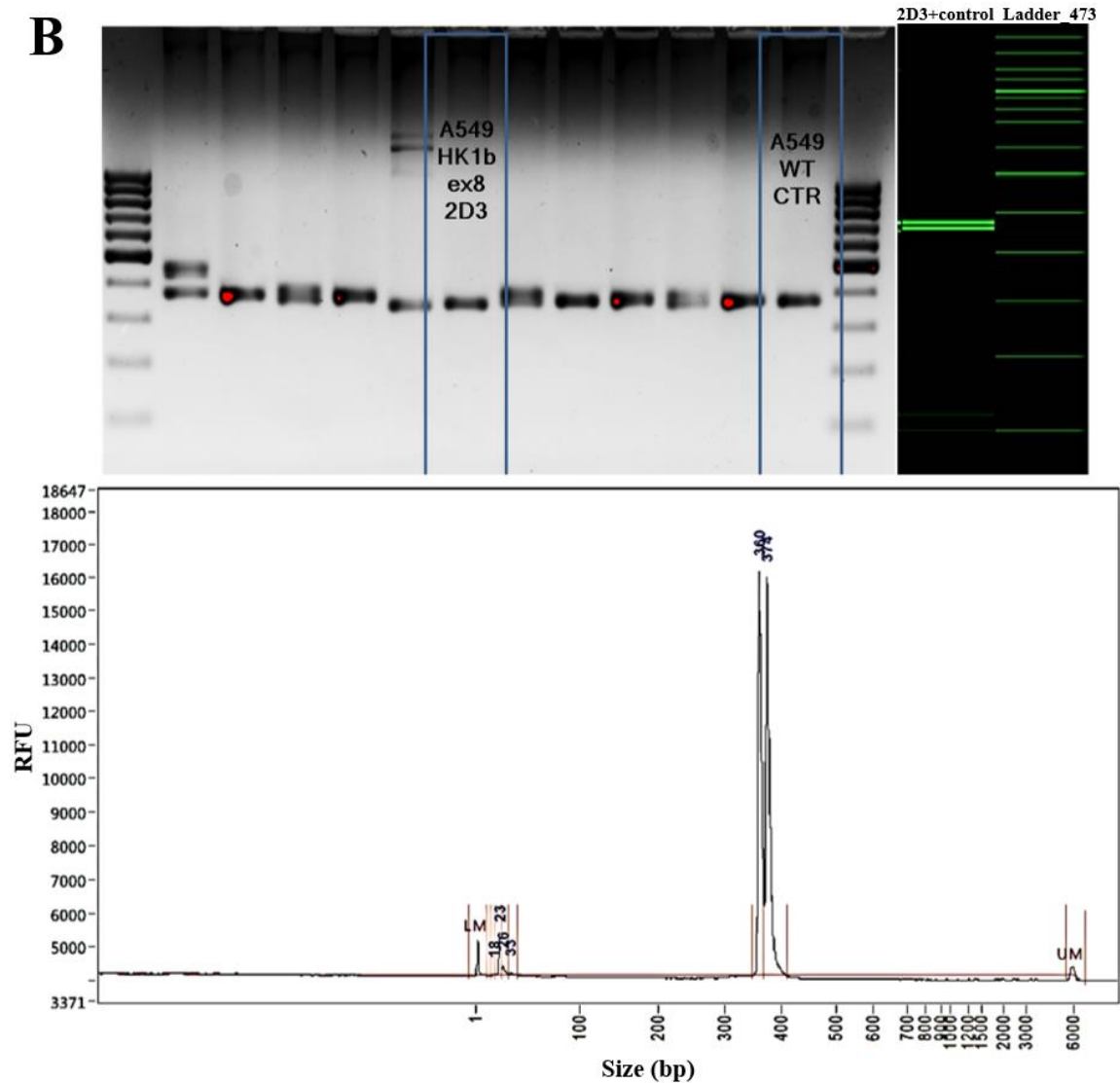
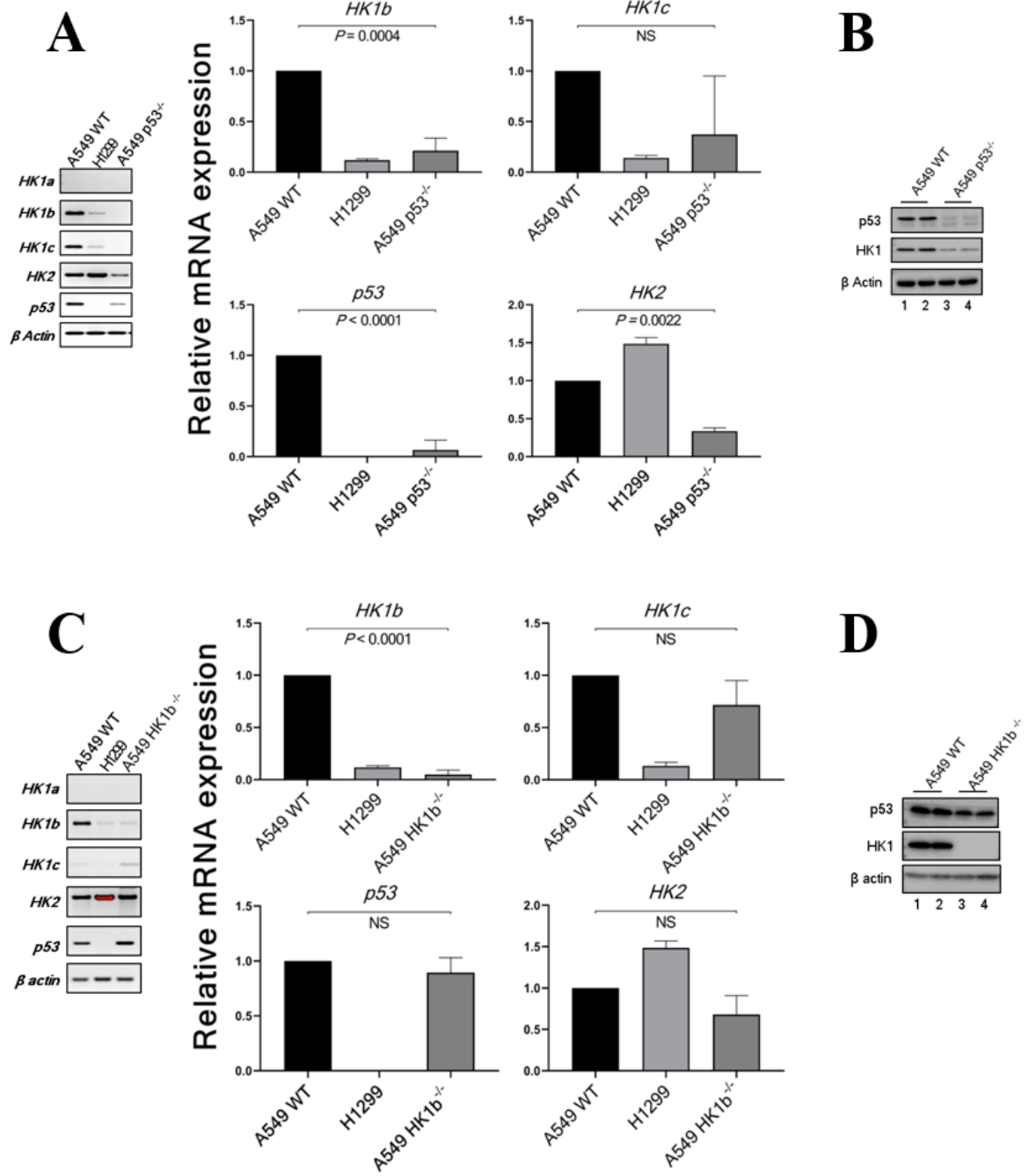


Figure 4.3.1 PCR and DNA fragmentation analysis of **A)** p53 elimination at the target loci, indicating insertion of 104 bp nucleotides resulting in a frame-shift mutation. **B)** HK1b isoform elimination at the target loci, indicating deletion of 14 bp nucleotides resulting in a frame-shift mutation.



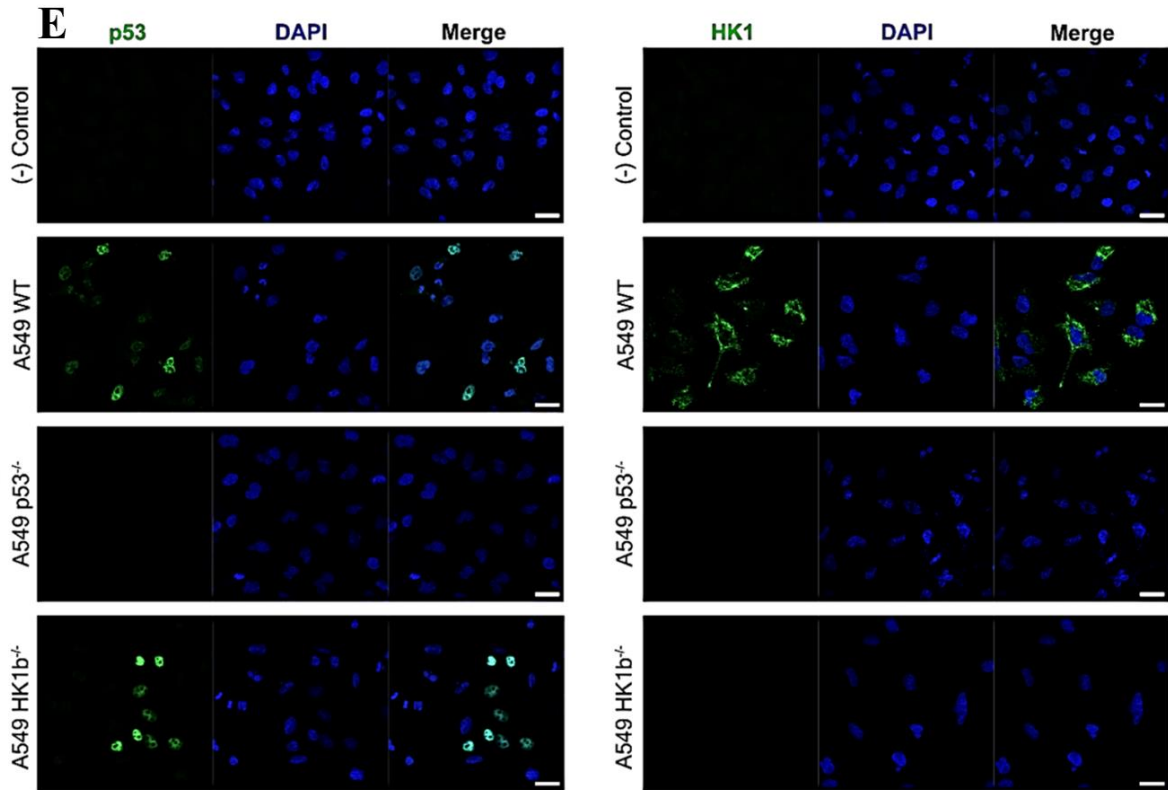


Figure 4.3.2 Elimination of p53 and HK1 b isoform. A549 p53^{-/-} and A549 WT cells were analyzed by **A)** qRT-PCR for level of HK1 b, c, HK2, and p53 in A549 p53^{-/-} cells. Error bars indicate mean $\pm$ SEM, (n=3) or **B)** Immunoblot analysis of p53 KO in A549 cells. A549 HK1b^{-/-} and A549 WT cells were analyzed by **C)** qRT-PCR for level of HK1 b, c, HK2, and p53 in A549. Error bars indicate mean $\pm$ SEM, (n=3) or **D)** Immunoblot analysis of HK1b KO in A549 cells. E) Immunofluorescence analysis of p53 KO and HK1b KO in A549 cells.

4.4 Deletion of HK1b suppresses cell proliferation and tumorigenesis in NSCLC cells via regulating glycolytic activity

N-terminal domains stabilize HK1 and HK2 enzymes and are involved in their binding to the outer mitochondrial membrane [117, 118]. Although HK1a, b, c and HK2 have structural similarities, major differences in N-terminal sequence distinguishes from one another and thus provide distinct biochemical and functional properties in glycolytic pathway and tumor development [118-120]. We predict that an extra alpha-helix that is in the sub-domain of N-terminus of HK1b isoform might provide additional stability to the protein as shown by root-mean square fluctuation analysis (Fig. 4.4.1A). Consequently,

this may impact the strength of the interaction between mitochondria and HK1b as this helix was shown to be at interface of mitochondria and the HK1 [118].

To understand the mechanism and the functional role of HK1b isoform in proliferation, survival, and tumorigenesis, we analyzed the metabolic consequences of HK1b isoform deficiency. Changes in glycolysis and respiration were measured using the Seahorse metabolic analyzer. Analysis of the mitochondrial respiration rate following HK1b knockout (KO) resulted in nonsignificant changes in oxygen consumption rate (OCR) and basal respiration (Fig. 4.4.2A), suggesting that HK1b ablation did not alter the mitochondria-driven oxidative phosphorylation (OXPHOS). However, measuring extracellular acidification rate (ECAR) showed significant reductions in glycolytic activity in HK1b isoform-deficient A549 cells (A549 HK1b^{-/-}) when compared to parental control cells (A549 WT) ($p=0.0001$) (Fig. 4.4.2B).

In order to evaluate the effect of reduced glycolysis on proliferation *in vitro*, flow cytometry analyses of Ki67 was performed and A549 HK1b^{-/-} cells showed an approximately 35% decrease in Ki-67 positivity (Fig. 4.4.2C). In order to test the potential role of HK1b *in vivo*, A549 WT and A549 HK1b^{-/-} cells were implanted into immunodeficient NSG male mice via intra-tracheal injection. Mice were necropsied at 8 weeks post-implantation and *relative* tumor volumes were measured. Tumor size was significantly reduced in mice bearing A549 HK1b^{-/-} cells ($p=0.01$) (Fig. 4.4.3A). Histopathological evaluation of A549 WT or A549 HK1b^{-/-} tumors confirmed that they are both NSCLC lung adenocarcinomas. HK1 staining confirmed complete depletion of HK1 expression in A549 HK1b^{-/-} tumors, while A549 WT tumors expressed high levels (Fig. 4.4.3B). In situ analyses of tumor tissues showed significantly lower Ki-67 staining in A549 HK1b^{-/-} tumors compared to parental control tumors ($p=0.001$) (Fig. 4.4.3C), indicating that HK1b KO cells have less proliferation ability. In conclusion, all these data show that inhibition of HK1b reduces cell proliferation and tumor cell growth in NSCLC and might be because of reduced glycolysis.

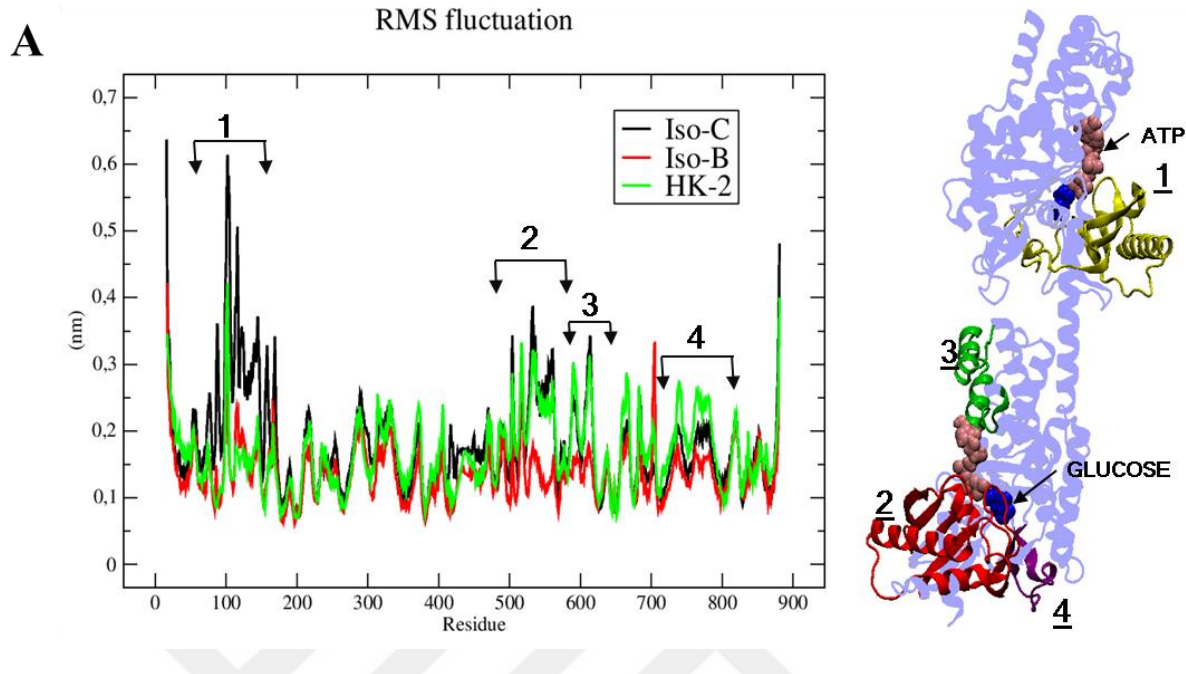


Figure 4.4.1 RMSF Plot shows four major regions fluctuating in HK1c and HK-2 while HK1b is more stable. Structure of HK1c is shown in the figure, first region (1) responsible for ligand binding in N-terminal and a part of small sub-domain (which is responsible for opening and closing of ligands binding pocket), second region (2) ligand binding site in C-terminal part of small sub-domain, third region (3) C-terminal part of large domain and involved in ATP binding, fourth region (4) near to Glucose binding site of C-terminal large domain.

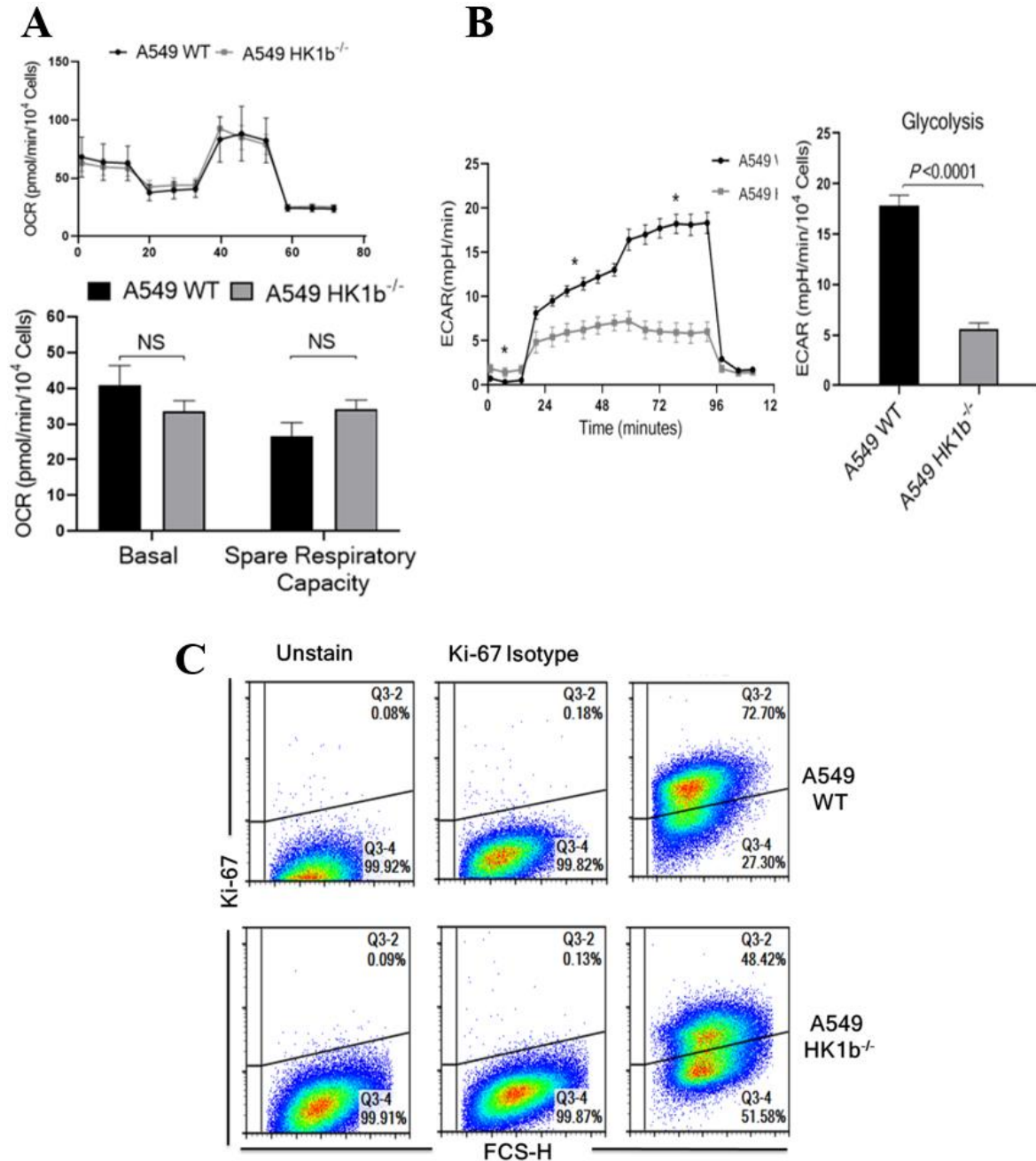
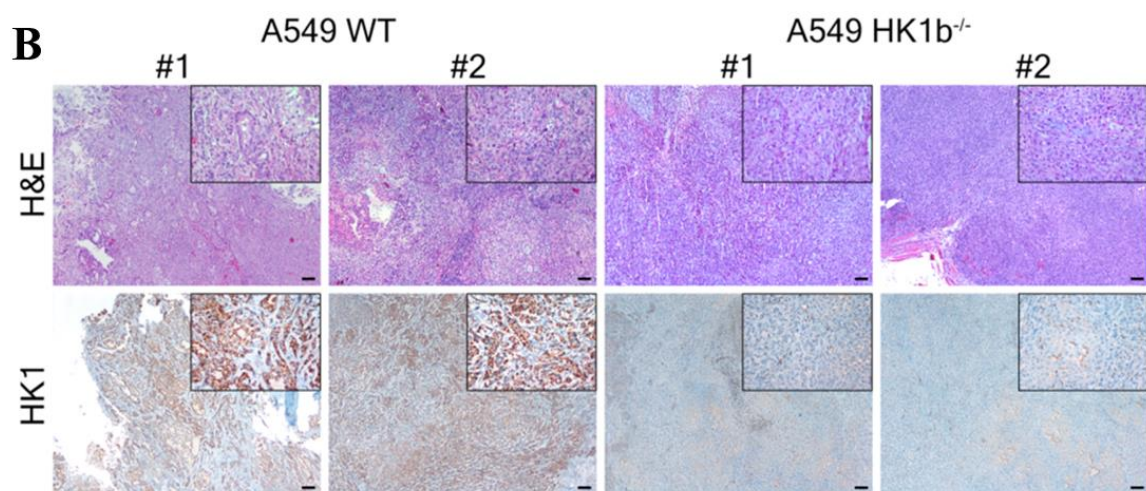
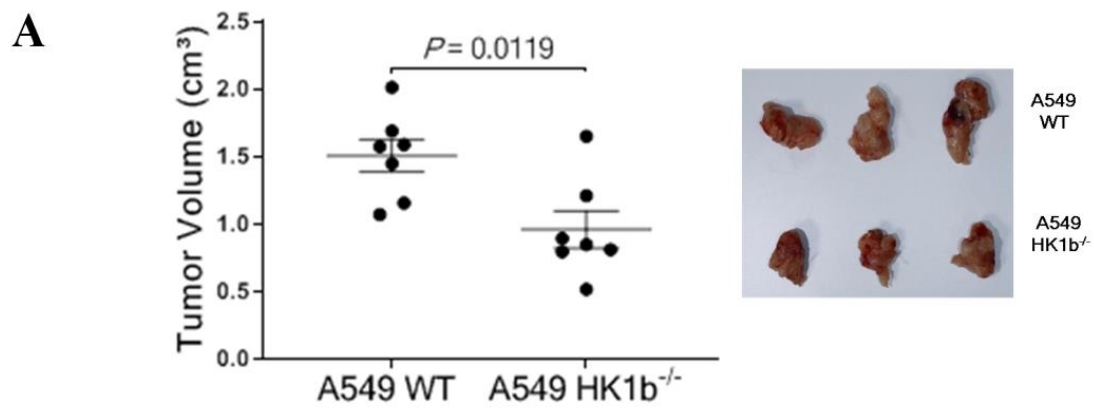


Figure 4.4.2 Effect of HK1b knockout on metabolism and proliferation of A549 cells. Seahorse metabolic analysis **A)** of OCR values from mitochondrial stress test, and **B)** of glycolysis stress test in A549 HK1b^{-/-} and A549 WT cells. Experiments were performed at least twice in quadruplicates for OCR and once for quintuple for ECAR. The results are presented as the mean $\pm$ SEM and means compared with Student's t test. **C)** Ki-67 proliferation flow cytometry analysis of A549 WT and A549 HK1b^{-/-}.



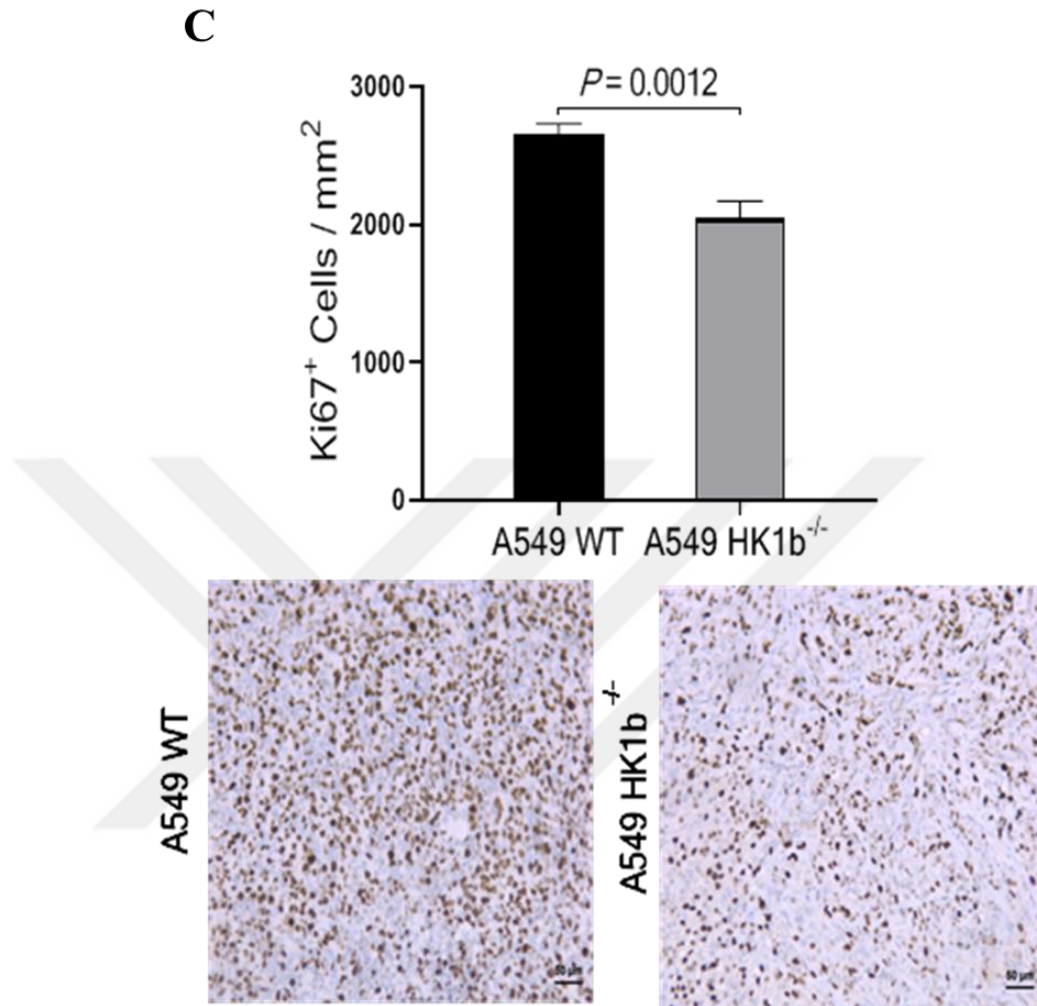


Figure 4.4.3 Effect of HK1b knockout on tumorigenicity of A549 cells. **A)** A549 WT or A549 HK1b^{-/-} cells were intra-tracheally injected into immunodeficient NSG male mice. Graphs on the left show tumor volume in (n=7, each) A549 WT (control) and A549 HK1b^{-/-} (n=7) mouse and representative images of resected tumors on the left. Tumor volumes presented as mean $\pm$ SEM, two-tailed unpaired t-test. **B)** Immunohistochemical staining of HK1 expression and H&E staining in representative mouse xenograft A549WT tumor (left) and A549 HK1b^{-/-} tumor (right). Images are 40X magnification; insets are 200X magnification. Scale bars, 100 μ m. **C)** Representative photographs of Ki-67 expression in xenografts of A549 WT (left) and A549 HK1b^{-/-} (right) stained by IHC in upper panel. Number of Ki-67 positive cells is counted per area (mm²) from 5 different fields in 8 different slides. The results are presented as the mean $\pm$ SEM and means compared with Student's t test.

4.5 HK1b plays critical role in cisplatin response, metabolism, and survival in NSCLC cells

The treatment options for NSCLC are mainly based on several factors but in general, chemotherapy is often one of the first line therapies in NSCLC patients. *Cisplatin* is slightly more effective platinum-based treatment for NSCLC patients. However, cisplatin has been associated with wide range of side effects [121, 122]. Moreover, inhibitors targeting metabolic changes have been developed as a potential therapeutics and there are now several early-phase clinical trials testing metabolic inhibitors [123]. However, the efforts that have been made so far not successful because of adverse side effect of these drugs [11]. Therefore, there is a clinically unmet need for identification of specific and effective metabolic targets in cancer cells to improve survival and reduce disease-related adverse events in NSCLC patients. We first hypothesized that HK1b elimination in combination with cisplatin could synergize to decrease glycolysis further that could potentially have therapeutic utility for NSCLC patients. Indeed, we observed further reduction in basal glycolysis and glycolytic capacity though not significant, and total absence of glycolytic reserve on cisplatin treated A549 HK1b^{-/-} cells (Fig. 4.5.1B). No significant change of basal respiration rate was observed between both cisplatin treated A549 WT and A549 HK1b^{-/-} cells, suggesting that HK1b ablation maintains the OXPHOS. However, only spare respiratory capacity was diminished in cisplatin treated A549 WT cells ($p=0.02$) (Fig. 4.5.1A).

We next investigated whether the HK1b deletion could sensitize NSCLC cells to cisplatin treatment. Cell viability assay was performed in A549 HK1b^{-/-} cells showed significantly lower IC₅₀ value for cisplatin (47 μ M) than the A549 WT control group (75 μ M) (Fig. 4.5.2.A). Based on these results, A549 WT and A549 HK1b^{-/-} cells were treated with cisplatin at 75 μ M and 47 μ M, respectively for 48 hours. Flow cytometric quantification of cell death measured by propidium iodide (PI) staining. HK1b deletion when combined with cisplatin treatment significantly augmented cell death in A549 HK1b^{-/-} cells (Fig. 4.5.2B). Furthermore, we found that cisplatin treatment induces cleavage of PARP and caspase 3 but induction of cleavage was significantly higher in A549 HK1b^{-/-} cells (Fig. 4.5.3A). Additionally, we used rapamycin as a control and found that cisplatin but not rapamycin treatment induces apoptosis. However, cisplatin promoted greater cell death in

HK1b deleted cells (Figure 4.5.3A). Interestingly, HK1b deletion slightly induces down-regulation of Bcl-2 expression in untreated A549 HK1b^{-/-} cells and further down-regulation of Bcl-2 expression was significant with the cisplatin treatment (Fig. 4.5.3B). Indeed, HK1 plays an important role in protecting against mitochondrial regulated apoptosis through direct interaction with mitochondria [124]. Surprisingly, HK1 expression was significantly upregulated in cisplatin treated A549 WT cells (Figure 4.5.3B). Altogether, these results suggest that loss of HK1b sensitizes NSCLC cells to cisplatin induced apoptosis.

In order to identify the underlying molecular mechanisms of synergistic apoptosis induction, we examined several signaling pathways. Upon silencing of HK1b, we detected that AKT, ERK (extracellular signal-regulated kinase), and GSK3 β (Ser-9) phosphorylation were increased but mTOR phosphorylation was maintained in A549 HK1b^{-/-} cells potentially due to glycolysis depletion in comparison to A549 WT cells (Fig. 4.5.3C and 4.6.2A). Moreover, cisplatin treatment of A549 HK1b^{-/-} cells resulted in significant reduction of AKT phosphorylation (Fig. 4.5.3C) as well as mTOR (Fig. 4.6.2A), and STAT3 phosphorylation (Figure 4.5.3C). However, ERK and GSK3 β phosphorylations were further increased by cisplatin treatment in A549 WT cells and A549 HK1b^{-/-} cells (Fig. 4.5.3C). It was previously reported that AKT plays critical role in enhancing glycolysis through increasing HK activity while ERK signaling has role in promoting the aerobic glycolytic phenotype via induction of transcriptional regulators of glycolysis [73, 125, 126]. On the other hand, ERK phosphorylation might also be involved in cell death particularly when there is DNA damage [127]. Our results confirmed that cisplatin was shown to induce DNA damage as well as ERK phosphorylation. Furthermore, HK1b ablation in combination with cisplatin treatment induces greater apoptosis through down regulation of Bcl-2 by blocking its anti-apoptotic function. Taken together these results indicate that, HK1b depletion sensitizes NSCLC cells to cell death and synergizes to cisplatin.

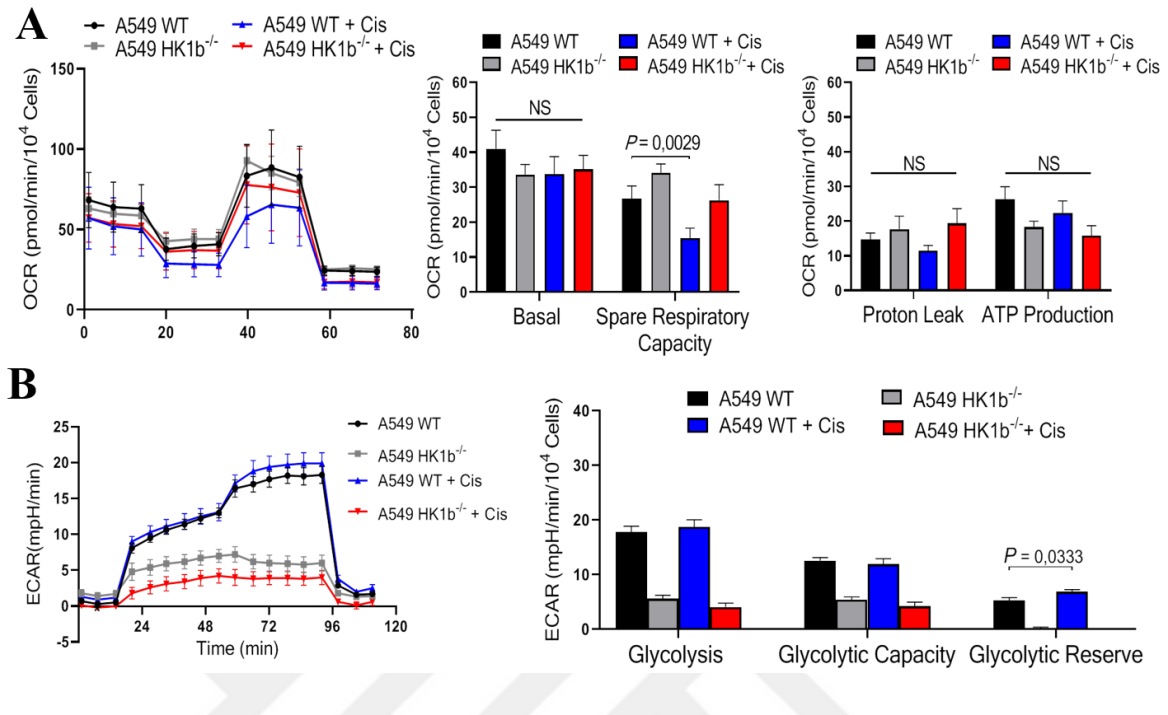


Figure 4.5.1HK1b KO role in cisplatin response on metabolism, **A)** OCR analysis of untreated or 6hrs cisplatin treated A549 WT and A549 HK1b^{-/-} cells. Experiments were performed twice in quadruplicates for OCR. The results are presented as the mean $\pm$ SEM and means compared with one-way ANOVA and Tukey's post hoc test. **B)** ECAR analysis of untreated or 6hrs cisplatin treated A549 WT and A549 HK1b^{-/-} cells. Experiments were performed once for quintuple for ECAR. The results are presented as the mean $\pm$ SEM and means compared with one-way ANOVA and Tukey's post hoc test

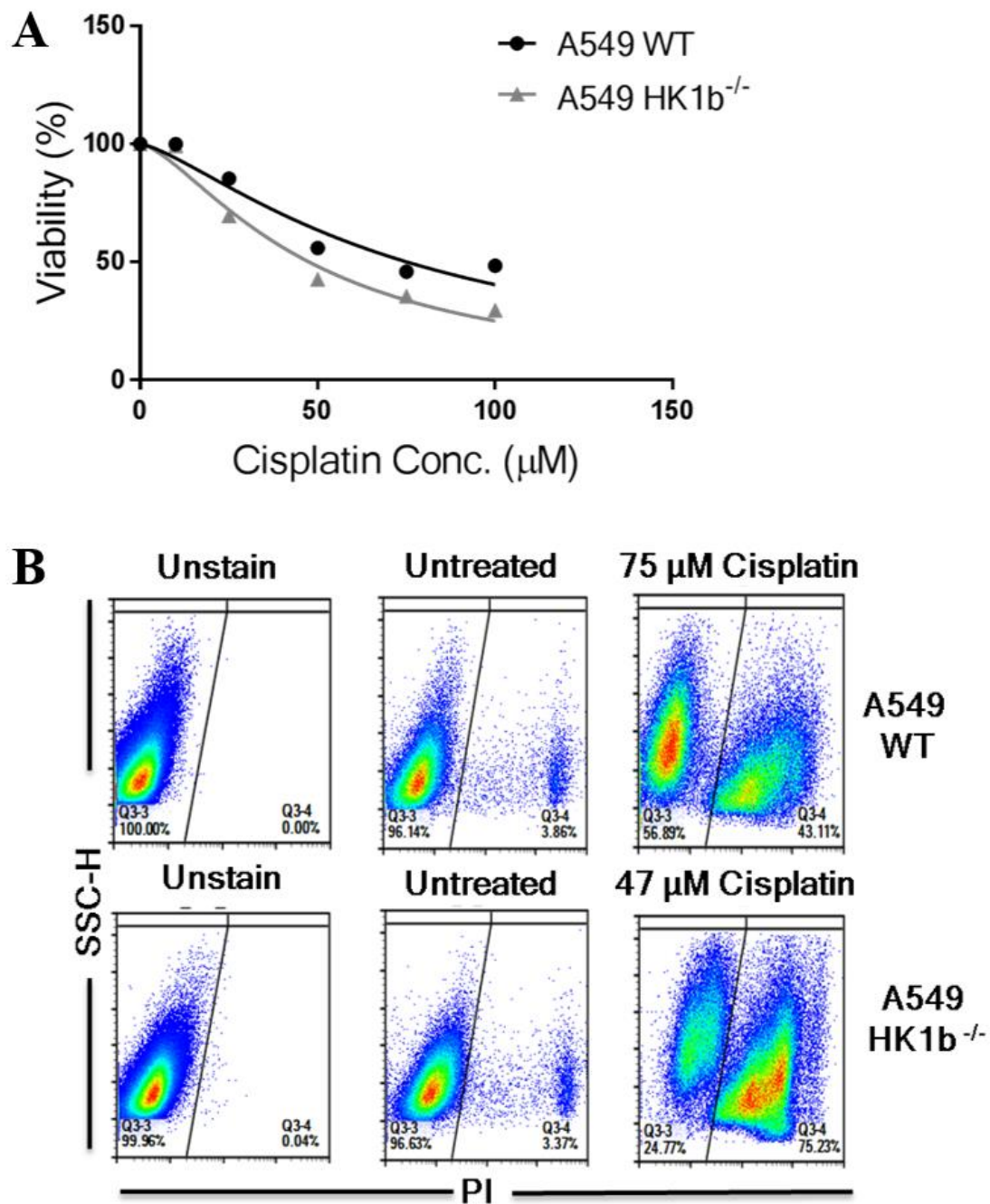


Figure 4.5.2 HK1b KO role in cisplatin response **A**) A549 WT and A549 HK1b^{-/-} cells were treated with increasing concentrations of cisplatin (0.1 μM–100 μM) for 48 h. Dose-response curves were generated from which IC₅₀ values were deduced (n=2). **B**) Flow cytometric quantification analysis of propidium iodide (PI) staining in A549 WT and A549 HK1b^{-/-} cells as unstained, untreated, and treated with cisplatin (75μM and 47 μM, respectively), (n=2).

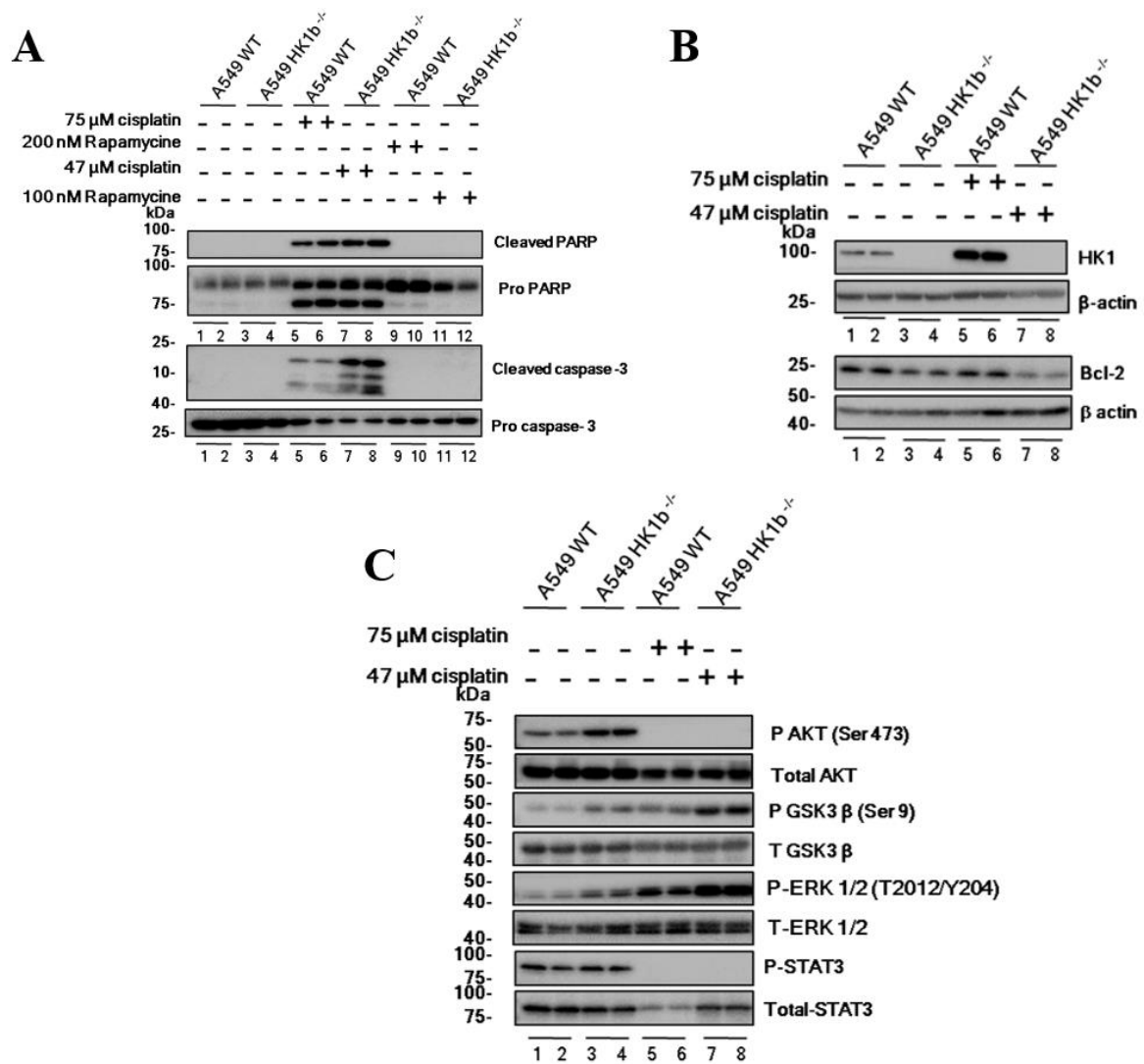


Figure 4.5.3 Immunoblot analysis of cells treated with cisplatin (75 μ M and 47 μ M, respectively) or rapamycin (100 μ M and 47 μ M, respectively). **A)** Immunoblot analysis of apoptosis markers. **B)** Immunoblot analysis of HK1 and Bcl-2. Anti-beta-actin is used as a loading control. **C)** Immunoblot analysis of AKT, GSK3 β , ERK, and STAT3.

4.6 Deletion of HK1b sensitizes cells to cisplatin induced cytotoxicity via p53 mediated apoptotic and autophagic cell death.

Because of the fact that HK1b deletion results in glycolysis reduction and synergize cells to cytotoxic cell death, we investigated the underlying mechanism of apoptotic cell death in HK1b deleted cells upon treatment with cisplatin. We found that H2AX phosphorylation is inhibited by both cisplatin and rapamycin treatments (Fig. 4.6.1A). Indeed, kinetics of H2AX phosphorylation is time dependent since cells must enter S phase in order for

H2AX stimulation [128]. We showed that cisplatin increases apoptosis through both activation through phosphorylation of Ser15 and high level of expression of p53 in HK1b deleted cells. In contrast, p53 expression was not induced by rapamycin treatment (Fig. 4.6.1A), indicating that only HK1b depletion and cisplatin synergize the induction of p53 mediated apoptosis. Furthermore, characteristics of apoptosis have been detected by electron microscopy (Fig. 4.6.1B). Recent reports suggested a complex and multifaceted relationship between autophagy and apoptosis. Moreover, glucose deprivation typically results in autophagy induction to maintain energy homeostasis [129]. However, autophagy might be growth inhibitory after a certain threshold [130]. In order to determine whether the combination of HK1b deletion and cisplatin led to the activation of autophagy in NSCLC cells, we examined the markers of autophagic signaling pathway upon treatment with cisplatin and rapamycin as a control. Expectedly, HK1b elimination induces autophagy in response to glucose deprivation since formation of LC3II significantly elevated in A549 HK1b^{-/-} cells. Moreover, expression of the autophagy marker LC3-II was markedly induced in HK1b deleted cells when treated with cisplatin or rapamycin treatment in A549 HK1b^{-/-} cells (Fig. 4.6.2A). Furthermore, cisplatin significantly inhibits phosphorylation of mTOR in A549 HK1b^{-/-} cells (Fig. 4.6.2A). Furthermore, upon cisplatin or rapamycin treatment, AMPK phosphorylation was diminished in A549 WT cells but the phosphorylation is totally inhibited in A549 HK1b^{-/-} cells (Fig. 4.6.2A). Although we observed higher LC3II expression level in cisplatin or rapamycin treated A549 HK1b^{-/-} cells, expression levels of other autophagic indicators of beclin-1 and ATG13 expression levels were downregulated in both A549 WT, A549 HK1b^{-/-} cells. However, concomitantly beclin-1 and ATG13 levels were not changed with rapamycin treatment (Fig. 4.6.2A). We further demonstrate that cisplatin treatment induces significant numbers of autophagic vesicles and myelinoid bodies in HK1b deleted cells as demonstrated by electron microscopy (Fig. 4.6.2B). Our findings suggest that while autophagy was induced mainly by reduction of glycolysis, apoptotic cell death was induced mainly by cisplatin. These data imply that combination of HK1b deletion with cisplatin induces greater cell death through increased activity of apoptotic and autophagic pathways.

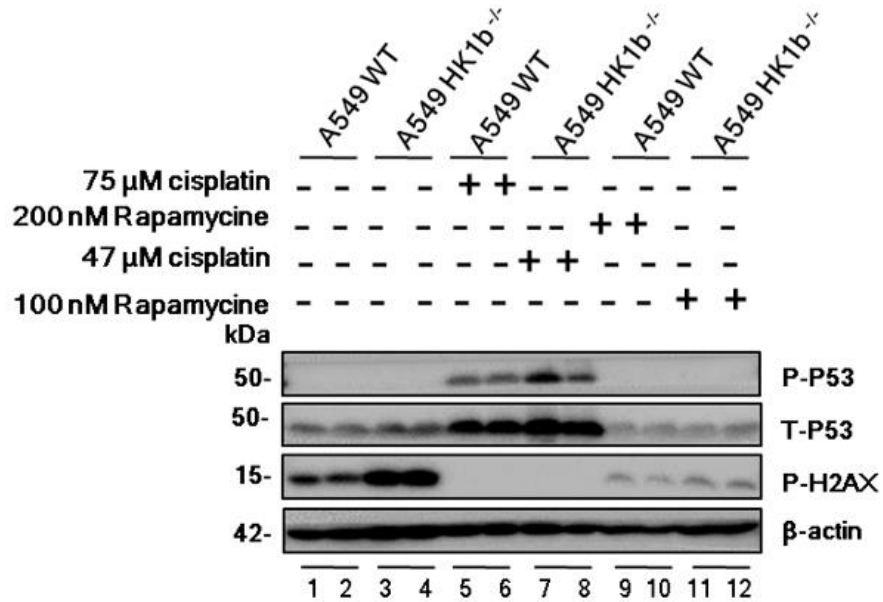
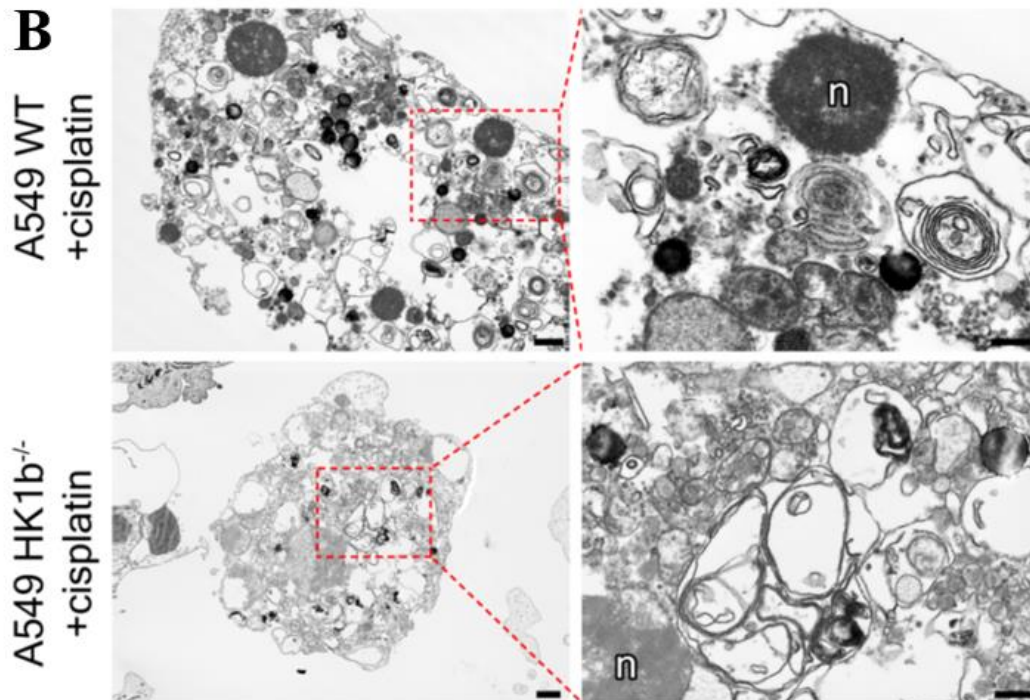
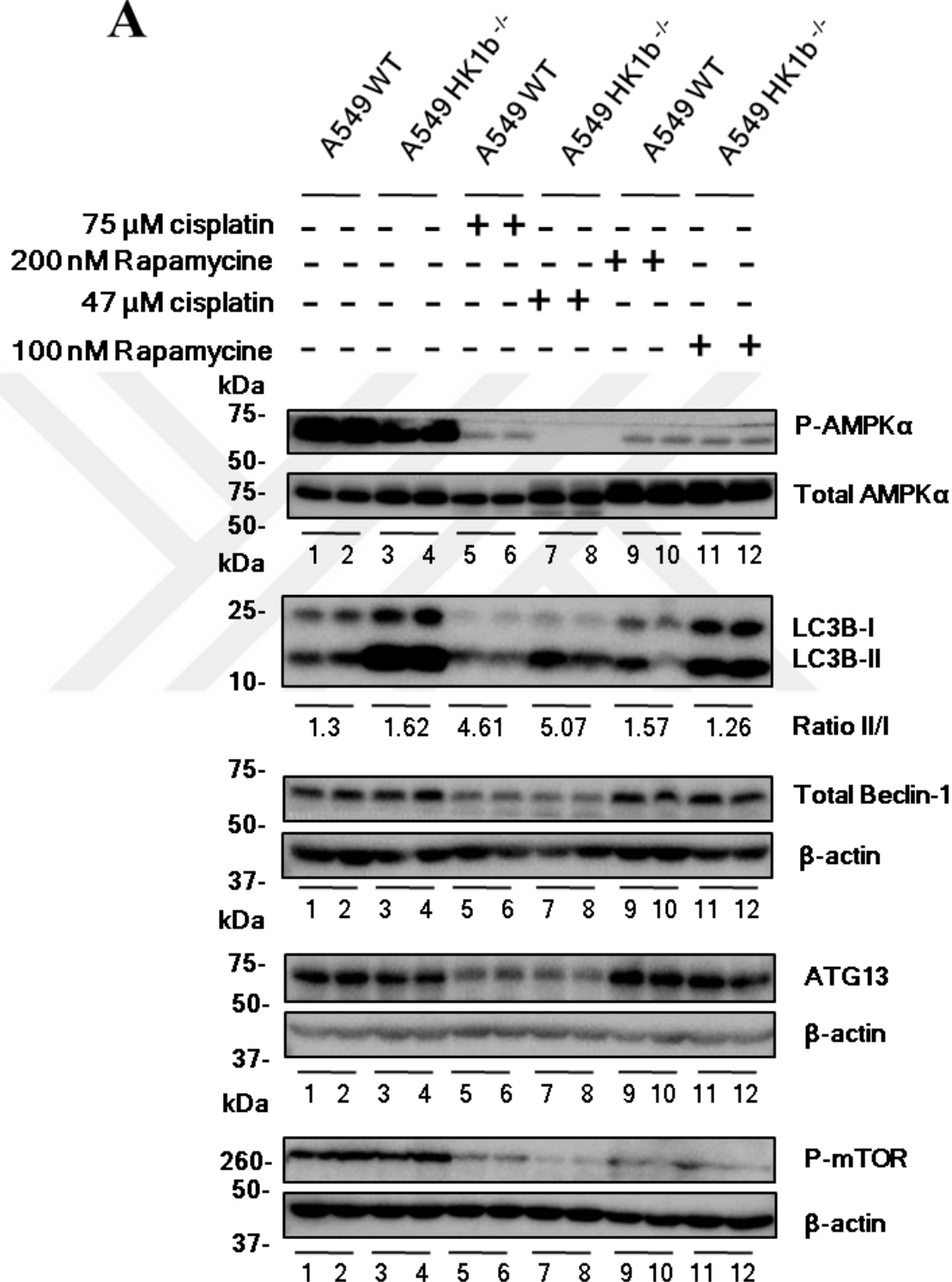
A**B**

Figure 4.6.1 Synergetic effect HK1b ablation and cisplatin increases p53-mediated apoptotic cell death. Immunoblot analysis of cells treated with cisplatin (75 μ M and 47 μ M, respectively) or rapamycin (100 μ M and 47 μ M, respectively) **A**) Immunoblot analysis of p53 and H2AX **B**) Representative transmission electron microscopy (TEM) micrographs displaying characteristics of apoptosis; images are representative of two independent experiments; scale bar, 1 μ m (left) and 400 nm (right).

A



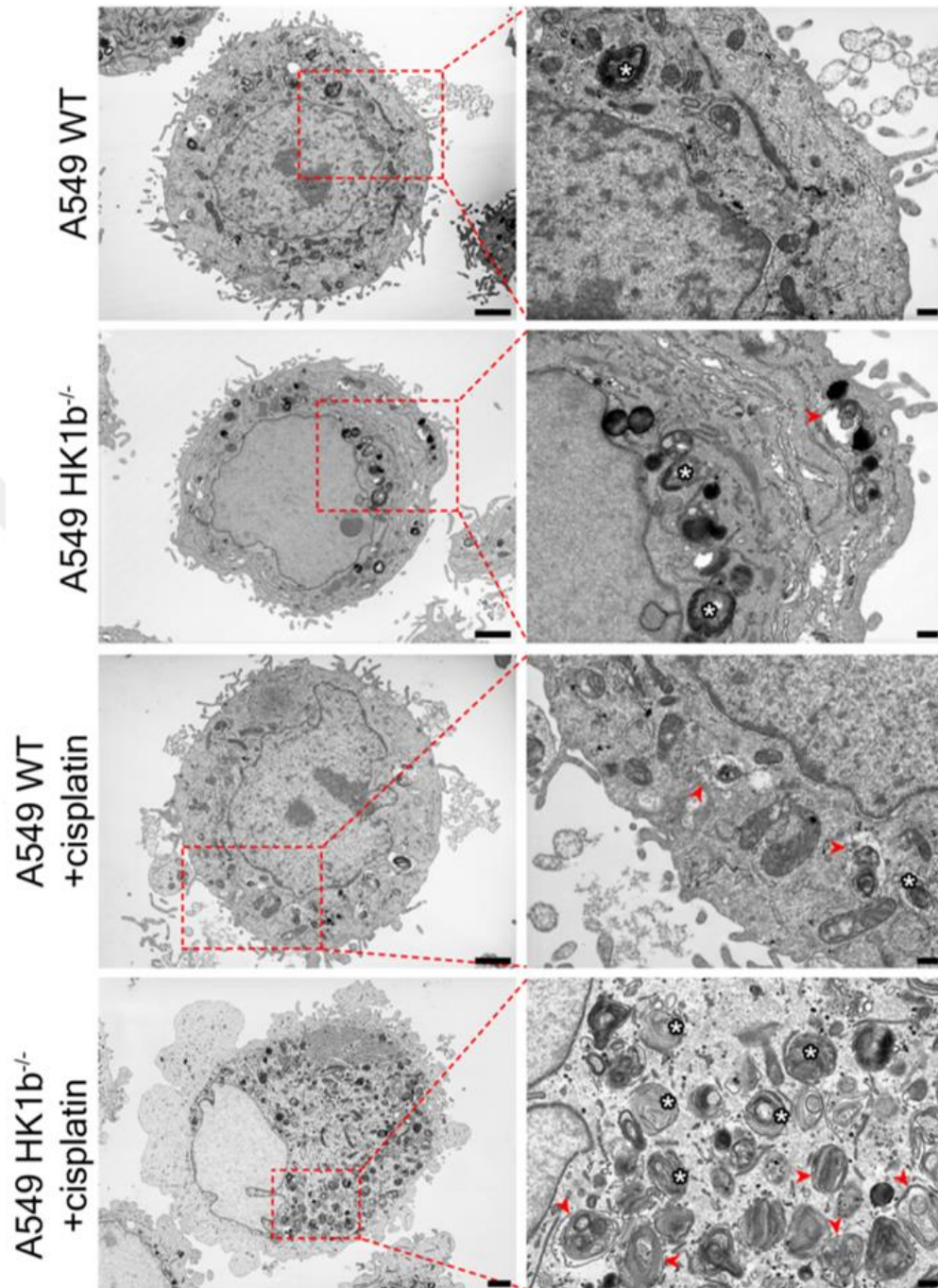


Figure 4.6.2 Synergetic effect HK1b ablation and cisplatin increases autophagic cell death. Immunoblot analysis of cells treated with cisplatin (75 μ M and 47 μ M, respectively) or rapamycin (100 μ M and 47 μ M, respectively) **A)** Immunoblot analysis of AMPK α , LC3B I/II, Beclin-1, ATG13, mTOR. Anti-beta-actin is used as a loading control. **B)** Representative transmission electron microscopy (TEM) micrographs displaying characteristics of autophagy; images are representative of two independent experiments; scale bar, 2 μ m (left) and 400 nm (right). Red arrows represent autophagic vesicles, black starts represent myelinoid bodies and n refers to nuclear fragment.

5 DISCUSSION

Like most cancers, NSCLC display comprehensive multiple metabolic alterations such as reprogramming of glucose metabolism and increased dependency on glycolysis [131, 132]. This is manifested by turning on the expression of certain enzymes such as Hexokinases (HKs) that are required for the accelerated glucose metabolism in NSCLC cells. Selective HK isoforms that regulate glucose metabolism in NSCLC cells but dispensable or absent in normal lung cells would be an attractive molecular target. In this line of work, we demonstrated that HK1 expression was significantly elevated in comparison to HK2 expression in NSCLC patient tumor samples.

It is known that alternative splicing of HK1 gene results in several transcripts encoding different isoforms, while HK2 gene has only one transcript according to RefSeq data. To the best of our knowledge, although specific HK1 isoforms has been reported in testis, there have been no reports on the role of these isoforms in other tissues (including cancer) [112]. In order to understand whether alternative isoforms tend to display distinct expression patterns, we analyzed the expression of 3 different isoforms of HK1 (HK1a, b, and c) in NSCLC cells and primary tumor tissues along with HK2, and p53. We found that only HK1b isoform was predominantly expressed in NSCLC cells and it was higher in the p53 WT A549 cells. Significantly elevated HK1b isoform in patient tumors as compared to normal tissue has also been detected, indicating a higher dependency on glycolysis and resistance to cell death in these tumor cells. Our data suggests that HK1b isoform may have therapeutic utility targeting metabolic pathway in NSCLC.

Despite several activities of p53 in cellular response, the role of p53 in the regulation of metabolism is complex. Several studies have shown that p53 regulates glycolysis by upregulating genes or increasing activity of enzymes such as hexokinases (HK1 and HK2) [25, 88]. Although, a few studies suggest that p53 might have direct connection with HK, how p53 regulates hexokinase (HK), whether is a direct regulation or not is still elusive. In reference to p53, an early study suggested that p53 increases HK2 mRNA transcription through p53-responsive elements, suggesting that p53 can promote at least some steps in glycolysis by regulating HKs [133]. Moreover, recent study found that depletion of WT p53 resulted in decreasing glycolysis and proliferation in hepatoma carcinoma cells

(HCC), suggesting that WT p53 may direct role in regulating genes that are responsible for glycolysis. Additionally, study showed that WTp53 is required to maintain growth and glycolysis by examining several different human cancer cells other than HCC cells. Altogether study revealed the oncogenic role of WTp53 in promoting the cancer metabolic switch by dominantly suppressing OXPHOS [134]. We first observed that HK1b isoform expressed in only in p53WT-A549 cells but not in p53null-H1299 cells. To find out if this finding was due to whether cell specific or p53 related event, we deleted p53 in P53WT-A549 cells using CRISPR/Cas9 system. In our study we found that p53 regulates HK1b isoform as a result of genetic disruption of *p53*.

To extent the clinical relevance of HK1b data, we examined the functional role of HK1b by deleting HK1b isoform in A549 cells using CRISPR/Cas9 system. We found that HK1b deletion inhibits proliferation and tumorigenesis *in vivo* and synergizes with cisplatin to sensitize NSCLC cells to cell death because of significant reduction of glycolysis and downregulation of the anti-apoptotic proteins. The treatment options for NSCLC patients are based mainly on several factors, including the stage of the cancer and availability of molecular markers for targeted therapy. Currently, cisplatin is widely used as a one of the main platinum-based drug, but low efficacy and considerably side effects as a single agent would not benefit NSCLC patients [122]. Our results provide ample evidence that the combination of cisplatin and HK1b silencing markedly increased NSCLC cell death. Cisplatin induced cytotoxicity in HK1b deleted cells is regulated by induction of p53-mediated apoptosis and increased autophagy by depletion of glycolysis. Together our data suggest that HK1b deletion could significantly synergize cells to cisplatin. The mechanism by which HK1b deletion synergizes cells to cisplatin cytotoxicity may be through blocking its binding ability to mitochondria. Our work is consistent with the previous report, suggesting that the increased expression of HK1 attenuates apoptosis in a glucose-dependent manner because of its anti-apoptotic effect and also induction of increased HK activity [124]. As previously reported, mitochondrial binding of HKs near voltage-dependent ion channel (VDAC) may be required for access to ATP to efficiently phosphorylate glucose [135].

Furthermore, we showed that N-domain of HK1b isoform is more stable around the glucose and ATP binding site compared to HK1c isoform and HK2 using molecular dynamic simulations. Our result might provide a better explanation for higher stability of HK1b over HK2 by showing that HK1b deletion induces significant depletion of glycolysis. Moreover, HK1b ablation alone or with cisplatin increases AKT, ERK, and GSK3 β signaling. AKT and ERK both have role in promoting glycolysis [136]. AKT activity, particularly through the phosphorylation of T473 residue promotes glucose metabolism by stimulating the activity of hexokinase [137]. ERK increases glycolysis by upregulating transcriptional expressions of several metabolic genes [84, 138]. The inhibition of GSK3 β (Ser9) is required for the stimulation of glycogen and protein synthesis and, was also suggested as a mechanism for increased resistance to apoptosis of cancer cells [139]. Recent studies strongly indicate that mitochondrial-bound HK1 and HK2 play a major role in preventing tumor apoptosis by blocking mitochondrial outer membrane permeabilization (MOMP) and the association is regulated by GSK3 β [120, 140]. It has been reported that serine phosphorylation of GSK-3 is mediated by protein kinases such as AKT and ERK1/2/p90RSK [141]. Here, we found that synergetic effect of HK1b ablation and cisplatin induces higher activation of ERK inhibition of GSK3 β despite the inhibition of mTOR and AKT activation because of significant reduction in glycolysis. It was suggested that, cisplatin induces DNA damage thereby activates ERK, and extensive DNA damage-induced by ERK activation causes apoptosis [142, 143]. Consistent with these studies, we also found that upregulation of HK1 in parental A549 cells upon cisplatin treatment. Furthermore, we showed downregulation of anti-apoptotic Bcl-2 upon HK1b deletion which was further reduced by cisplatin treatment. These results suggest that HK1b has an important role in not only glycolysis but also apoptotic resistance mechanism by induction of p53-mediated apoptosis.

To determine whether HK1b plays a regulatory role in autophagy under glucose deprivation, we evaluated NSCLC cells after ablation of HK1b and determined the possible change with cisplatin treatment. We found that HK1b deletion induces autophagy in A549 HK1b^{-/-} cells with increased *LC3B-II* formation regardless of change in expression of AMPK, Beclin1, and ATG13. We also observed that autophagy was significantly

elevated in A549 HK1b^{-/-} cells in combination with cisplatin at multiple levels. Furthermore, electron microscopy images revealed significantly increased numbers of autophagic vesicles and myelinoid bodies in HK1b deleted cells upon cisplatin treatment. Interestingly, we observed inhibition of the AMPK phosphorylation and downregulation of autophagic markers such as Beclin-1 and ATG13 in NSCLC cells upon cisplatin treatment. In line with our results, it was previously reported that AMPK inhibition sensitizes several cancer cells to cisplatin via hyper-induction of p53 [144]. Taken together, our studies provide first evidence that HK1b is a compelling metabolic therapeutic target that may also potentiate the efficacy of cisplatin in NSCLCs.

6 CONCLUSION AND FUTURE WORK

The identification of isoform-specific contributors to cancer cell glucose metabolism that could be selectively targeted to eliminate cancer cells without compromising systemic homeostasis or corresponding metabolic functions in normal cells could be a very attractive approach for cancer therapy

To identify novel potential mediators and drivers of NSCLC, we analyzed HK1 isoforms (HK1a, HK1b, and HK1c) along with HK2, and p53 expression in NSCLC cell lines (A549 and H1299) and patient samples. Since there is no isoform specific antibody to detect HK1 isoforms at protein level, we first analyze the HK1 transcripts and its association to survival in clinical NSCLC tissue samples. We found that HK1 is abundantly expressed in tumors of lung cancer patients compared to their adjacent non-tumor tissues and showed that higher expression of HK1 associates with poor survival. We showed the first time that only HK1b isoform is predominantly expressed in A549 cells and NSCLC patient tumors; this distinguishes NSCLC cells from the normal surrounding lung cells and HK1b is associated with poor NSCLC patient survival.

We observed that HK1b expression was changed based on p53 status using p53 null and p53 WT NSCLC cells. In order to identify the mechanisms of HK1b regulation by p53, we deleted the p53 or HK1b isoform in human NSCLC A549 cells using CRISPR/Cas9 system. Surprisingly, we showed that p53 positively regulates HK1b. To understand the role of HK1b in NSCLC, we used HK1b knock out A549 NSCLC cells. We found that HK1b ablation inhibits glycolysis significantly, hence proliferation, and *in vivo* tumor growth of NSCLC cells. HK1b deletion caused increased in survival signaling through AKT and GSK3 β to compensate significant reduction of glycolysis.

Finally, HK1b ablation and cisplatin were synergistic in decreasing glycolysis, increasing apoptosis through upregulation of p53, and inhibiting tumor growth *in vivo*. Cisplatin also synergized with HK1b deficiency to inhibit AKT, STAT3, SAPK/JNK, and mTOR in an AMPK-independent manner. Finally, HK1b deficiency with cisplatin treatment markedly increased the susceptibility to cell death by not only induction of apoptosis, but also

inducing significantly increased number of autophagic vesicles and myelinoid bodies with increased LC3-II lipidation regardless of downregulation of autophagic markers such as Beclin 1 and ATG13. Collectively, these findings suggest that therapeutic strategies to modulate the Warburg effect, such as targeting HK1b isoform, may interfere with growth and therapeutic sensitivity of NSCLC.



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8 APPENDIX A SUPPLEMENTARY MATERIAL and METHOD

8.1 Sample Preparation for TEM

Cells were fixed with fixative mixture (0.08 M cacodylate pH 7.4, 2.5% paraformaldehyde, 1.25% glutaraldehyde and 2 mM calcium chloride) for 2 h at 4 °C washed and, were post-fixed in 2% OsO₄ aqueous solution for 20 min. Cells were incubated in 2.5% ferrocyanide for 20 min. Sequentially, the cells were incubated in 1% thiocarbohydrazide at 40 °C for 15 min, 2% OsO₄ for 20 min and 1% uranyl acetate at 4 °C for 30 min. Cells were warmed up to 50 °C for 30 min in uranyl acetate, washed twice in nanopure filtered water, were incubated in lead aspartate at 50 °C for 20 min. The samples were then dehydrated with graded alcohol series and then embedded in epoxy resin. After sectioning using an ultramicrotome (Leica EM UC7), samples were analyzed using an electron microscope (Carl Zeiss, Gemini 500).

8.2 DNA Fragmentation analysis

Standard Sensitivity NGS Fragment Analysis Kit (DNF-473-0500) is used for DNA fragmentation experiment and for dsDNA 905 Reagent Kit ((DNF-905-K0500) is used for visualization of 1-500bp PCR (Advanced Analytical Technologies, Inc., USA), according to manuscript protocol.

8.3 Structure Preparation

We used crystal structures of HK1b (PDB ID: 1QHA) and HK2 (PDB ID: 2NZT), whereas HK1c was modeled using SWISS-Homology-Modeling-Software. Unresolved parts of the crystal structures were modeled with Swiss-Modeller, and loop regions were refined with ModLoop. The protonation states of residues were determined using PROPKA. Glucose was used in beta conformation. ATP and Mg²⁺ were translated to each system from crystal structure of Glucokinase (PDB ID: 3FGU).

8.4 Molecular Dynamics Simulations

MD simulations were performed with GROMACS 5.1.4 employing CHARMM 36 force-field. Systems were subjected minimized using a conjugate gradient algorithm. All systems

were energy relaxed with 1000 steps of steepest-descent. The systems were equilibrated using NVT ensemble with Berendsen- algorithm. The final structures were run in NPT ensemble at 1 atm and 310 K. MD simulations were run for 300-ns. All coordinates were saved at 2-ps intervals for analysis. Long-range electrostatic forces were handled using PME, and Van der Waals forces treated with a 0.9-nm-cut-off. Water molecules were modelled with TIP3P model.

