Universidade do Minho
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Role of RKIP protein in the modulation of lung cancer cell metabolism

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Trabalho efetuado sob a orientação da
Doutora Olga Catarina Lopes Martinho e da
Doutora Sara Costa Granja

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Direitos de autor e condições de utilização do trabalho por terceiros

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Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Resumo

Os tumores sólidos são caracterizados por uma dependência exacerbada de glucose, sendo a reprogramação metabólica das células tumorais considerada um *hallmark* do cancro. Hoje é sabido que esta reprogramação em tumores é frequentemente controlada e induzida pela ativação oncogénica de vias de sinalização celular. O cancro do pulmão, é um exemplo clássico dos tipos tumorais em que já foi identificada uma alta frequência de mutações oncogénicas, cujo papel na ativação aumentada de vias de sinalização celular, está mais que estabelecido. A complexa maquinaria de ativação concomitante de várias vias de sinalização celular tem sido associada à aquisição de um fenótipo metabólico altamente heterogéneo. Desta forma, o uso da reversão metabólica como abordagem terapêutica torna-se muito mais complexa, sendo necessário a contínua investigação na procura de novas moléculas que possam estar envolvidas nesta reprogramação metabólica heterogénica. A proteína supressora tumoral RKIP, alterada em vários tipos tumorais como o cancro de pulmão, é funcionalmente uma proteína sinalizadora intracelular impactante na agressividade tumoral, através da regulação de várias vias de sinalização celular, que entre outras, podem controlar o metabolismo tumoral. Assim, juntamente com o conhecimento de que a RKIP poderá estar diretamente associada a alterações metabólicas, surge-nos a hipótese de que a RKIP pode ser uma das proteínas subjacentes à heterogeneidade metabólica em cancro do pulmão.

Para explorar e validar a nossa hipótese, começamos por fazer uma análise *in silico*, utilizando a base de dados TCGA, para determinar se existe uma assinatura molecular de vias metabólicas associadas à expressão de RKIP, em pacientes com adenocarcinoma pulmonar. Ademais, foi primeiramente realizada a caracterização metabólica dos modelos *in vitro* escolhidos para o estudo, a fim posteriormente manipular geneticamente a expressão da RKIP e determinar quais as alterações metabólicas especificamente associadas à RKIP.

De forma geral, observamos *in silico* que a RKIP está associada à alteração de expressão de genes, proteínas e vias de sinalização mais relacionadas com fosforilação oxidativa do que glicólise. Funcionalmente, a manipulação genética da RKIP alterou principalmente os padrões de expressão de proteínas metabólicas, e discretamente os níveis de glucose e lactato *in vitro*, indicando de uma forma geral que a RKIP inibe o metabolismo glicolítico.

Em conclusão, os nossos resultados forneceram as primeiras provas funcionais de que a RKIP poderá de facto modular o metabolismo celular em cancro do pulmão, abrindo uma enorme janela para trabalhos futuros.

Palavras-chave: Cancro do pulmão, Reprogramação metabólica, RKIP

Abstract

Solid tumours are characterized by an exorbitant glucose metabolism, being tumour cell metabolic reprogramming now considered a hallmark of cancer. Oncogenic signalling has been linked to metabolic rewiring of cancer cells. Lung cancer in specific, is often driven by mutations that lead to oncogenic activation of tyrosine kinases, known to produce metabolic alterations in tumour cells. Growing evidence suggest that tumour cells acquire heterogeneous metabolic phenotypes to withstand the hindrances associated to tumour growth and progression, which makes the search for novel players in tumour metabolic reprogramming of most importance. As a stablish tumour suppressor, RKIP downregulation has been associated with tumoral aggressiveness and patient's poor prognosis, in several tumour types, including lung cancer. Being a master regulator of several intracellular signalling pathways, together with recent findings suggesting that RKIP could potentially be associated with alterations in tumour cell metabolism, RKIP arises to us as one of the potential proteins behind cancer metabolic heterogeneity. which give support to our hypothesis. Therefore, it was herein aimed to explore RKIP role as a modulator of lung cancer metabolism.

First, using the TCGA database, an *in silico* analysis was performed to determine whether there is a RKIP-associated metabolic signature in lung cancer patients. Further, it was performed a metabolic characterization of the *in vitro* models chosen for the work, in order to genetically manipulate their RKIP expression and determine the specific RKIP-associated metabolic changes.

In general, we observed that RKIP is significantly associated with alterations in metabolism-related genes and signalling pathways involved in the regulation of metabolic processes, both at mRNA and protein level. Specifically, RKIP expression is positively correlated with oxidative phosphorylation-related genes, and inversely with glycolysis-related genes, in lung adenocarcinoma patients. Functionally, we found that, although not significant, RKIP genetic manipulation *in vitro* led to some metabolic alterations, mainly in the expression patterns of metabolic proteins, but also in glucose and lactate levels, which in general indicate that RKIP leads to inhibition of glycolytic metabolism.

In conclusion, our results provided the first functional evidence that RKIP could in fact modulate lung cancer cell metabolism and opened a huge window for future work.

Keywords: Lung cancer, Metabolic reprogramming, RKIP

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List of abbreviations

A

AC- Adenocarcinoma

ACO2 - Aconitase

AKT - Protein Kinase B

AMPK - AMP-Activated Protein Kinase

ATP - Adenosine Triphosphate

B

BACH1 - BTB Domain and CNC Homolog 1

C

ccRCC - Clear Cell Renal Cell Carcinoma

CD147 - Cluster of Differentiation 147

cDNA – Complementary DNA

CoA - Coenzyme A

COX - Cytochrome c Oxidase

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CS - Citrate Synthase

CSC - Cancer Stem Cell

CTR - Control

D

DAPI - 4',6-Diamino-2-Phenylindone

DG - Deoxyglucose

DLST - Dihydrolipoamide S-succinyl Transferase

DMEM- Dulbecco's Modified Eagle's Medium

DNA - Deoxyribonucleic Acid

E

EGFR - Epidermal Growth Factor Receptor

EMT- Epithelial-Mesenchymal Transition

ERK - Extracellular Signal-Regulated Kinase

ETC - Electron Transport Chain

F

F1,6BP - Fructose 1,6-Biphosphate

F6P - Fructose 6-Phosphate

FBS - Fetal Bovine Serum

FDG-PET - Fluorodeoxyglucose - Positron Emission Tomography

FH - Fumarate Hydratase

G

G6P - Glucose 6-Phosphate

GLUT - Glucose Transporter

GPCR - G Protein–Coupled Receptors

GRK2 - G Protein-Coupled Receptor Kinase 2

GSK3β - Glycogen Synthase Kinase 3 Beta

H

HDR - Homology Direct Repair

HIF-1α - Hypoxia Inducible Factor-1 alpha

HK - Hexokinases

HO-1 - Heme Oxygenase 1

I

ICI - Immunological Checkpoint Inhibitors

IDH - Isocitrate Dehydrogenase

IKB - Inhibitory Kinase B

IκB - Inhibitory kappa B

IKK - Inhibitory kappa B Kinase

K

KO - Knockout

KRAS - Kirsten rat sarcoma viral oncogene homolog

L

LC- Lung Cancer

LCC - Large Cell Carcinoma

LDHA - Lactate Dehydrogenase A

M

MAPK - Mitogen-Activated Protein Kinase

MCT - Monocarboxylate Transporter

MEK - Extracellular Signal-regulated Kinase Kinase

miRNA - MicroRNA

MMP - Matrix Metalloproteinase

mRNA – Messenger RNA

N

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NCID - Notch Intracellular Domain

NFKβ - Nuclear Fator Kappa Beta

NIK - Nuclear Fator Kappa Beta Inducing Kinase

NO - Nitric Oxide

NSCLC- Non-Small Cell Lung Cancer

O

OE - Overexpression

OXPHOS - Oxidative Phosphorylation

P

PD-1 - Programmed Cell Death - 1

PDH - Pyruvate Dehydrogenase

PDK - Pyruvate Dehydrogenase Kinase

PD-L1 - Programmed Cell Death Ligand - 1

PEBP1 - Phosphatidylethanolamine-binding protein 1

PEP - Phosphoenolpyruvate

PFK - Phosphofructokinase

PI3K - Phosphoinositide 3-Kinases

PK - Pyruvate Kinase

PKC- Protein Kinase C

PPP – Pentose Phosphate Pathway

R

RKIP- Raf Kinase Inhibitor Protein

RT- Room Temperature

RTK- Receptor Tyrosine Kinase

S

SCC - Squamous Cell Carcinoma

SCLC - Small Cell Lung Cancer

SDH - Succinate Dehydrogenase

Shh - Sonic hedgehog

SMO - Signal Transducer Smoothened

SRB - Sulforhodamine B

STAT - Signal Transducer and Activator of Transcription

T

TCA - Tricarboxylic Acid

TCGA - The Cancer Genome Atlas

TKI - Tyrosine Kinase Inhibitor

TME - Tumour Microenvironment

TP53 – Tumour Protein p53

W

WB - Western Blot

WT - Wildtype

Y

YY1- Yin Yang 1

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CHAPTER 1:

General Introduction

1.1 Cancer

Cancer is a heterogeneous and complex disease that remains one of the main causes of death worldwide, with rapid growth both in incidence and mortality. About 18.1 million new cases of cancer and 9.6 million cancer deaths were estimated to have occurred in 2018[1]. Thus, understanding the biology and complexity underlying the evolution of this disease is important for reducing its global burden.

In short, cancer is a disease of the genome, caused by the acquisition of somatic mutations in several genes that collectively dictate malignant growth[2,3]. In the early 2000s, Hanahan and Weinberg proposed the hallmarks of cancer as an organizing principle for rationalizing the complexities of neoplastic disease[4]. The hallmarks of cancer comprise several biological capabilities acquired during the multistep development of human tumours. Among them are, sustaining proliferative signalling, evading growth suppressors, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, genome instability and mutation, tumour-promoting inflammation, avoidance immune destruction and deregulation of cellular energetics[5] (Figure 1).

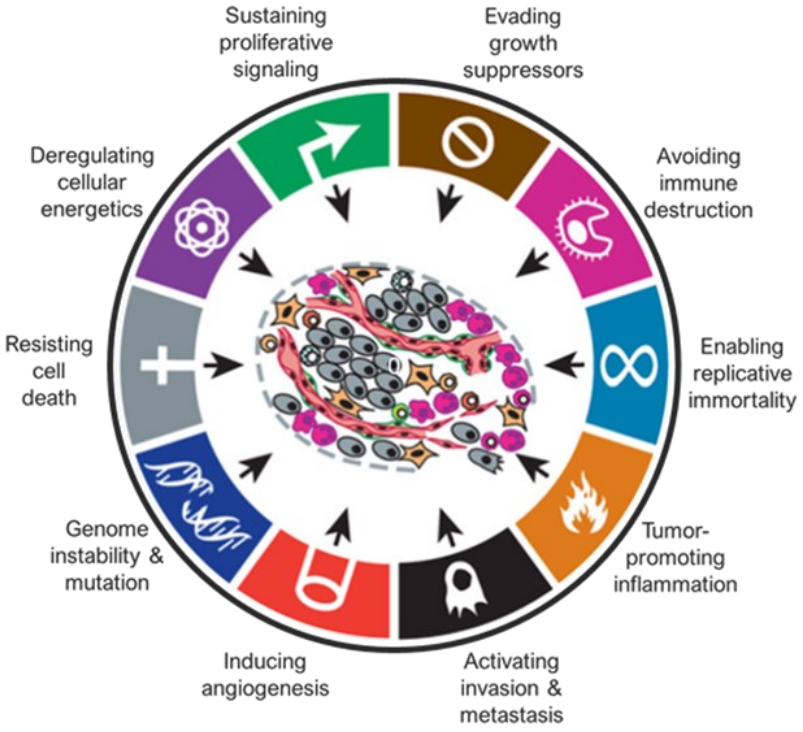

Figure 1: Hallmarks of cancer. Schematic illustration of the set of features acquired by tumours cells that enables tumour growth and metastatic development. Adapted from[5].

1.1.1 Lung cancer

Lung cancer remains the most diagnosed cancer and the leading cause of cancer deaths worldwide in both sexes combined, with 2.1 million (11.6% of the total cases) new cases and 1.8 million (18.4% of the total cancer deaths) deaths predicted in 2018[1]. The main risk factor of lung cancer is tobacco use, accounting for more than 80% of the cases in Western populations[6]. Although the numbers are high, major shifts in the global distribution of this disease have occurred, reflecting the temporal changes in the tobacco consumption patterns[7]. Declines in smoking, due to tobacco control measures applied in the last decades, as well as improvements in early detection and treatment, have resulted in a continuous decline in the lung cancer incidence and mortality rates. This incidence decrease has shown to be much faster in men than in women, which may be due to the historical and gender differences in tobacco uptake and cessation[1,6]. Besides that, was recently reported that women have a higher risk to develop lung cancer upon smoking than men, which can also explain this tendency[8].

Lung cancer can be categorized in two main categories based on their histological features: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter, which accounts for approximately 85% of all lung cancer cases, is further divided into three subtypes, adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC)[9,10]. Of all the types, lung adenocarcinomas are the most common and have the poor survival rates, with an average five-year relative survival rate of 5%, mainly because of late-stage detection and lack of treatments in advanced stages[6,11].

Although smoking is unquestionably the leading cause of lung cancer, approximately 25% of lung cancer cases worldwide are not attributable to tobacco consumption[10]. Striking differences in the epidemiological, clinical and molecular characteristics of lung cancer that arises in never smokers versus smokers have been identified in the last decade[10]. Globally, lung cancer in never smokers is more common in women and in East Asia, and has been associated with environmental exposures, pollution, other occupational carcinogens, and inherited genetic susceptibility[1,12]. Despite all the major histological types are associated with tobacco use, the association is stronger for SCLC and SCC than for adenocarcinoma[10]. Molecular epidemiology studies demonstrate that the genomic landscape of lung tumorigenesis between never smokers and smokers, providing more evidence that these cancers arise through different molecular mechanisms (Figure 2). As example, adenocarcinomas in never smoker patients frequently present mutations in the epidermal growth factor receptor (*EGFR*) gene. By contrast, smokers' patients often harbour mutations in *KRAS* and *TP53* genes[10,12,13]. This can be explained by the exposure of smokers to highly carcinogenic agents like tobacco smoke, that contain agents that are known to bind to DNA

creating DNA adducts which, eventually, can lead to mutations in *KRAS* or *TP53* genes, which are key events in lung cancer pathogenesis[10,14,15].

Although *EGFR* and *KRAS* mutations in lung tumours are most entirely mutually exclusive, both lead to constitutive activation of receptor tyrosine kinase (RTK) signalling[9,10] (Figure 2). RTK signalling pathways, namely RAS/MAPK, PI3K/AKT and STAT pathways, govern fundamental physiological processes, such as cell proliferation, differentiation, metabolism and cell death and survival[16,17]. Notably, dysregulation and constitutive activation of these signalling pathways have been implicated in the initiation, progression and metastatic spread of lung cancer, either by mutations in RTKs (e.g., *EGFR*) or in its downstream effectors (e.g., *KRAS*), which makes them an attractive target for lung cancer therapy[16].

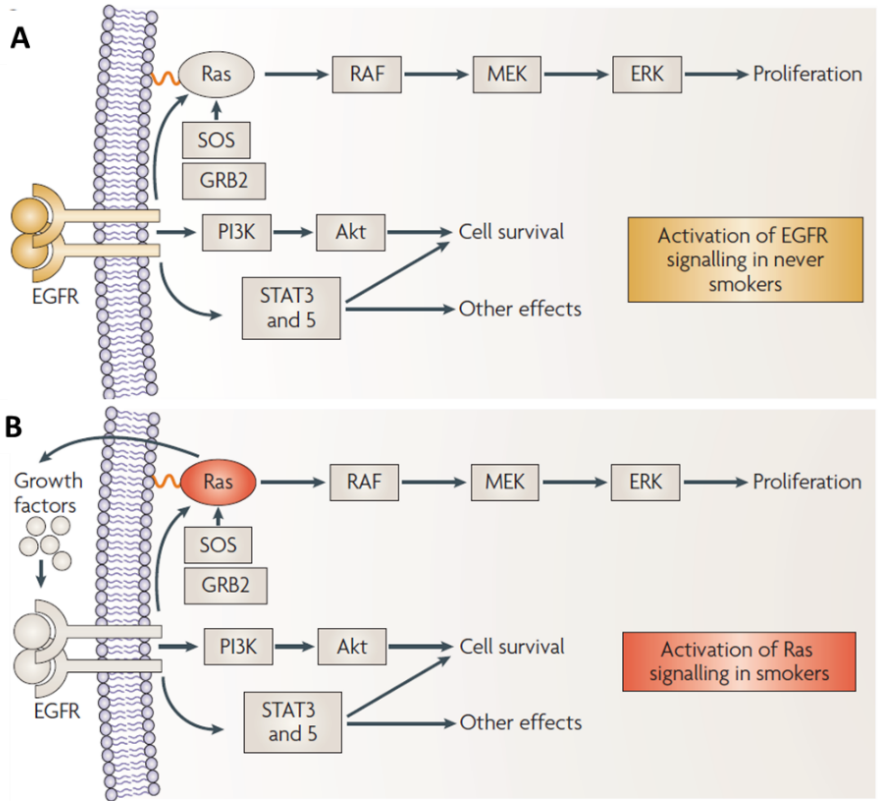

Figure 2: Two pathways to adenocarcinoma. A) Ligand binding to EGFR induces homo- and hetero-dimerization of the receptor, resulting in activation of downstream effectors including the RAS/MAPK (mitogen activated protein kinase), PI3K (phosphatidylinositol 3-kinase)/AKT, and STAT (signal transducer and activator of transcription) pathways that lead to cell proliferation, survival and many other effects associated with carcinogenesis. The EGFR pathway is frequently activated in never smokers by mutations in the *EGFR* gene. **B)** In smokers, mutations of the *KRAS* gene often occur, resulting in the release of growth factors that bind to EGFR, activating its pathway. In addition, Ras directly activates the PI3K/AKT pathway. The result of *KRAS* or *EGFR* mutations are virtually identical, and mutations of both genes in adenocarcinomas of the lung are rarely seen. Other methods of activation of these pathways include gene amplification and mutations in *BRAF*, *PIK3CA* (a subunit of PI3K), and mutations in other receptors tyrosine kinase (RTKs), such as *ERBB2* (also known as *HER2*). Adapted from[10].

Concerning lung cancer treatment, the first approach is usually surgical resection, followed by chemotherapy or radiotherapy, depending on the staging of the tumour[18]. Despite the high relevance and use of the traditional cytotoxic treatments, they present several limitations such as the lack of selectivity to tumour cells, the gain of resistance and systemic toxicity[19]. Fortunately, over the past two decades, important advancements in lung cancer treatment have been achieved. An expansion on the understanding of these malignancies, coupled with advances in the molecular characterization of tumours, enable the development of targeted therapies that introduce the era of personalized medicine[20-22]. In fact, it is now the standard approach, in the treatment of lung cancers, to genotype tumours at diagnosis, that allow individualized therapy, leading to higher efficiency of the treatment, less secondary effects, and ultimately an improvement of patient life quality and survival[13,23]. For instance, the use of small molecule tyrosine kinase inhibitors (TKI), such as erlotinib and gefitinib, for lung adenocarcinoma patients harbouring *EGFR* mutations, show longer progression-free survival and more favourable tolerability[24,25]. Although promising, NSCLC tumours treated with these first-generation TKIs inevitably develop resistance. Several resistance mechanisms were already described and other target therapy drugs, such as second and third generation TKIs (afatinib and Osimertinib, respectively), were clinical developed and are now used in lung cancer patients treatment[26]. Besides that, the emergence of immunological checkpoint inhibitors (ICIs) has signalled a new direction for lung cancer treatment. Current approved ICIs for NSCLC treatment include anti-PD-1 antibodies, nivolumab and pembrolizumab, as well as anti-PD-L1 antibody atezolizumab[18,27-29]. Unfortunately, the clinical benefits of ICIs have been proven limited and unsatisfactory, with the overall response rate of monotherapy about 10-20%, which may be due to the complexity of the tumour immune microenvironment[26].

Despite the major progress in understanding the biology and management of lung cancer in the past decades, the overall cure and survival rates remain low, particularly in metastatic disease. Therefore, understanding the mechanisms behind treatment resistance and a continued search for key molecules involved in the regulation of lung tumorigenesis is necessary.

1.2 Raf Kinase Inhibitor Protein (RKIP)

Raf kinase inhibitor protein (RKIP), first discovered as phosphatidylethanolamine-binding protein 1 (PEBP1), is a small (23kDa) cytosolic protein originally purified from bovine brain, that belongs to a highly conserved family of proteins[30,31]. This protein is widely expressed in normal human tissues and has been recognised for its important role in multiple physiological processes, such as membrane biosynthesis, spermatogenesis, cardiac output, neural development, inflammation and others[31-33]. Therefore,

dysregulated RKIP expression has been related with some human diseases, such as Alzheimer's disease, metabolic and inflammatory disorders and cancer[30-33]. This multifunctional capacity of RKIP is associated with its involvement in the modulation of several signalling pathways (Figure 3) that control important cellular processes[34-36].

1.2.1 RKIP as a signalling modulator

Regarding RKIP role as a signal transduction modulator, this protein was first described as an endogenous inhibitor of the Raf-MEK-ERK (MAPK) pathway, accounting for its current name. Yeung *et al.* first reported that RKIP was able to block Raf-1/MEK interaction by acting as a competitive inhibitor of MEK phosphorylation and by reducing the affinity between Raf-1/MEK[37] (Figure 3). Moreover, it was described that RKIP binds directly to the N-region of the Raf-1 kinase domain, preventing Ser338 and Tyr340/341 phosphorylation by PAK and Src kinases, needed for Raf-1 activation[38,39].

RKIP has been also reported to indirectly interfere with upstream activators of Raf-1, such as G-protein coupled receptors (GPCR), being the nature of this interference dictated by the phosphorylation status of RKIP. Protein Kinase C (PKC)-mediated phosphorylation of RKIP at serine 153 decreases RKIP affinity for Raf-1 and increase its affinity for G protein-coupled receptor kinase 2 (GRK2), which is an endogenous inhibitor of GPCR activation[40,41]. Thus, the binding of phosphorylated RKIP to GRK2 leads to a dissociation of GRK2 from GPCR, allowing GPCR activation and phosphorylation of downstream targets, including Raf-1, which implies a role of RKIP as an endogenous modulator of cell response to growth factor stimuli[42,43] (Figure 3).

Furthermore, RKIP was identified as a negative modulator of nuclear factor kappa B (NF-κB) signalling, by antagonizing its upstream signal transducers[44] (Figure 3). The inhibitory effect of RKIP is exerted by its association with the upstream kinases TAK, NIK, IKKα and IKKβ, abolishing their kinase activity, which results in the elimination of inhibitory kappa B (IκB) phosphorylation and degradation, blocking NF-κB translocation to the nucleus and consequently NF-κB-mediated transcription of several genes with anti-apoptotic features[44,45].

Likewise, it has recently described that RKIP acts as a negative modulator of the signal transducer and activator of transcription 3 (STAT3), Sonic hedgehog (Shh) signalling and Notch1 (Figure 3). Concerning STAT3, RKIP blocks its activation by preventing its phosphorylation by upstream kinases, controlling this way the transcription of genes related to cell growth, apoptosis, survival and differentiation[46,47]. Additionally, it was demonstrated that RKIP can bind to signal transducer Smoothened (SMO), keeping it inactive and consequently blocking the activation of the zinc-finger transcription factor

Gli1, the final target of Shh signalling, which is involved in the regulation of proliferation, differentiation and cancer stem cells (CSCs) activation[48]. Moreover, RKIP directly interacts with Notch1 preventing its proteolytic cleavage and the release of Notch intracellular domain (NCID), which stimulates the epithelial to mesenchymal transition (EMT) and metastasis[49].

In contrast, RKIP can also act as a positive modulator intracellular signalling, as it able to activate glycogen synthase kinase-3β (GSK3β) (Figure 3), that is involved in the regulation of many cellular functions besides its initially described role in glycogen metabolism[50,51]. Al Mulla and colleagues described that RKIP stabilizes GSK3β expression by preventing its phosphorylation at the inhibitory T390 residue by p38 MAPK, which is activated under oxidative stress augmented upon RKIP depletion or downregulation. The loss of RKIP de-represses GSK3β inhibition of oncogenic substrates causing stabilization of cyclin D, which induces cell-cycle progression, Snail, and Slug, which promote EMT[50].

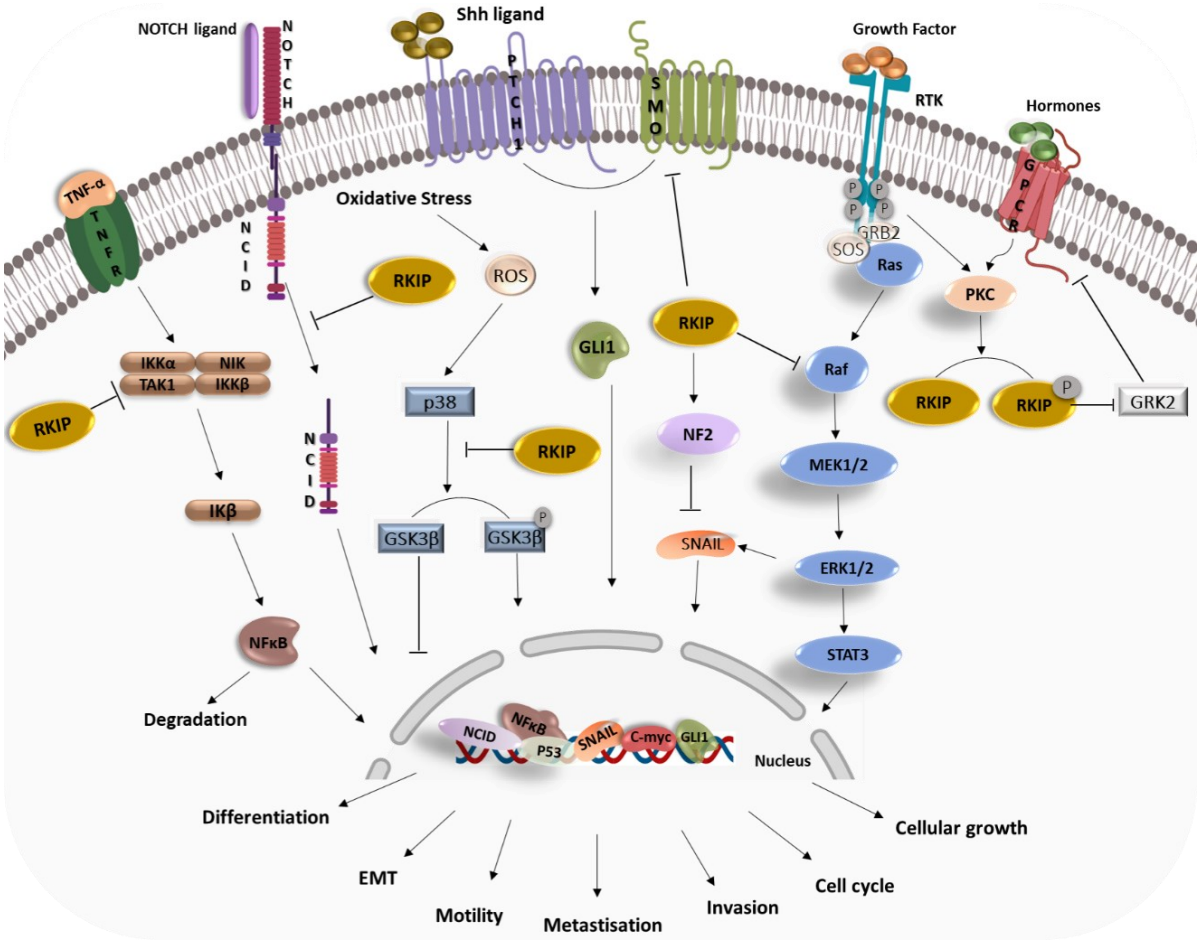

Figure 3: RKIP as a modulator of intracellular signalling pathways. On the left, RKIP binds to IKK complex preventing Ikβ phosphorylation and degradation which ultimately blocks the translocation of NF-kβ to the nucleus. Next, RKIP binds to the Notch Intracellular Domain (NICD) preventing its proteolytic cleavage, inhibiting the translocation of NICD to the nucleus. In the middle, RKIP act as an inhibitor of the Shh signalling pathway by binding to the SMO receptor, keeping it inactive and preventing Gli1 transcription. Also in the middle, RKIP depletion enhances oxidative stress–mediated activation of the p38

MAPK, which, in turn, inactivates GSK3β by phosphorylating it at the inhibitory T390 residue. On the right, RKIP is bound to Raf preventing the phosphorylation of MEK by Raf leading to the inhibition of the Raf/MEK/ERK/STAT3 signalling. Further, RKIP blocks Snail through MAPK inhibition and NF2 stabilization. In the nucleus, Snail acts as a p53 suppressor GPCRs are desensitized and internalized in response to phosphorylation by GRK2. After cells stimulation (e.g. growth factors), PKC-mediated phosphorylation of RKIP, at S153, inactivates RKIP as an inhibitor of Raf-1, and converts it to a GRK2 inhibitor. GPCR signalling through ERK/MAPK can therefore persist. Adapted from[52].

1.2.2 RKIP role in cancer

Given the major role of RKIP as a modulator of important intracellular signalling pathways that are often deregulated in cancer, RKIP has proved to play a crucial role in controlling both tumour aggressiveness and therapeutic response, being nowadays considered a well-establish tumour suppressor[43]. The first association between RKIP and cancer was established in prostate metastatic cell lines, which displayed lower RKIP expression levels when compared to primary tumour cell lines[53]. Besides that, Fu *et al.* also demonstrated that reestablishment of RKIP expression in the metastatic cell lines lead to an inhibition of their invasion capability, a decreased development of lung metastases, but did not affect the growth of the primary tumour[53]. This suggested that RKIP may not have an essential role in the primary tumour, but instead, has great importance as a metastasis suppressor, as evidenced in further studies on different tumour types[43,54,55]. In accordance, loss or reduction of RKIP expression has been associated with malignancy and poor prognosis in several solid tumours, as described by our group[56-62] and others[36,43,63]. In fact, due to its negative association with metastasis, RKIP has been implicated as a strong predictive biomarker for metastatic risk in patients as well as an independent prognostic marker for overall survival and disease-free survival in many solid tumours (reviewed by us in[52]).

Despite the growing importance of RKIP as a metastasis and prognostic marker in human tumours, the mechanisms behind RKIP downregulation remains elusive, however, it was already demonstrated that RKIP expression can be regulated at multiple levels[36]. For instance, at the epigenetic level, promotor methylation was pointed as a mechanism behind RKIP loss of expression in some tumour types, yet this mechanism seems to be tumour-type specific[43,64-66]. Regarding the regulation at the transcriptional level, some transcription factors, such as Snail and BACH1, can bind directly to RKIP promoter suppressing its transcription and expression[36,67,68]. Moreover, it was found that RKIP mRNA can be targeted and consequently suppressed by microRNAs, such as miR-27a[69], miR-534[70], miR-23a[71] and miR-224[72]. Ultimately, as mention before, RKIP can be modulated at the post-transcriptional level by PKC-mediated

phosphorylation at Serine 153, which also accounts for the loss of RKIP activity in several cancer types[40,41,73].

As a master metastasis suppressor, RKIP exerts its inhibitory functions at different steps of the metastatic process, including those involved in its initiation, such as angiogenesis, EMT, cell migration and invasion[43]. It is now known that RKIP targets signalling networks that directly or indirectly regulate metastatic functions and tumour progression, which are mostly under the control of the central signalling pathways described above to be regulated by RKIP (Figure 3). For instance, Rosner and colleagues identified a pro-metastatic signalling cascade involving MYC/LIN28/let-7 as an RKIP target in breast cancer models. They demonstrated that, through its inhibitory action in the Raf/MEK/MAPK pathway, RKIP leads to inhibition of LIN28 transcription by MYC, which in turn, enhances let-7 expression[74]. The potentiation of let-7 expression by RKIP leads to a negative regulation of its targets, such as BACH1, a transcription factor that induces matrix metalloproteinase1 (MMP1) expression and promotes metastasis[75,76]. Furthermore, it has also been describe that RKIP can negatively regulate the early metastatic events, such as EMT, through the NF-κB/Snail/YY1/RKIP circuitry[77,78]. Snail, a well-described EMT inducer, is transcriptionally regulated by NF-κB signalling, that is known to repress both RKIP and E-cadherin transcription, two key proteins in cancer metastasis[67]. In turn, RKIP was demonstrated to inhibit NF-κB pathway, leading to negative regulation of EMT inducers, such as Snail and YY1 and to an upregulation of E-cadherin, supressing this way the EMT process[79,80].

Besides that, recent findings strongly suggest that RKIP's anti-metastatic properties can also be mediated through modulation of the tumour microenvironment (TME), as recently reviewed by us[33]. It was demonstrated that RKIP modulates the breast cancer TME by controlling the infiltration of specific immune cells and secretion of pro-metastatic factors, for example by blocking the recruitment of pro-metastatic macrophages, through vital regulation of chemokines expression[81,82]. Moreover, Bainer *et al.* also demonstrated that gene expression in metastatic breast tumours was significantly correlated with gene expression in local stroma, and that alterations in stromal gene expression elicited by tumours positive or negative for RKIP expression is a better predictor of breast cancer subtype and patient survival that tumour gene expression alone[83]. Together, this emphasizes the importance of exploiting RKIP's microenvironmental functions for a better understanding of its role in carcinogenesis.

It is acknowledged that tumour cells often develop therapeutic resistance along with increased metastatic potential, resulting in a poorer overall survival. Notably, downregulation of RKIP expression has been associated to the development of resistance to conventional cytotoxic drugs in tumour cells[61,84,85]. In that sense, RKIP expression has also been implicated in the regulation of tumour cells resistance to

conventional chemotherapy, radiotherapy, and more recently to immune-mediated cytotoxicity[43,86,87]. As an apoptotic inducer, RKIP has shown to cause the re-sensitization of resistant tumours to therapy and also sensitivity to host immune-surveillance, via multiple interactions with signalling modules[33,43,88]. Examples of these RKIP-modulated cascades are the NF-κB, STAT3, Shh, MAPK and PI3K/AKT signalling pathways, which are involved in the regulation of both metastasis and tumour cell sensitivity to apoptotic stimuli[43,48]. Since RKIP is commonly downregulated in many human cancers, its induction could be an attractive approach to sensitize tumour cells to therapy response. In fact, some agents have already been reported as able to induct RKIP expression contributing this way to cells sensitization to apoptosis, such as rituximab[89], nitric oxide (NO) donors[79], didymin[90] and the proteosome inhibitor NPI-0052[80].

Concerning lung cancer in specific, the role of RKIP as a prognostic marker in these malignancies is not yet clear, as the studies regarding its expression and clinical significance are still scarce and not concordant among them, however a clinical association between low expression of RKIP and higher TNM stage or presence of lymph node metastasis is found across the studies (as reviewed in[62]). Nonetheless, the implications of RKIP in these tumours related signalling, progression and therapy resistance is quite evident[62]. For instance, it was demonstrated, using NSCLC cell lines, that RKIP is able to inhibit ERK and STAT3 phosphorylation and, consequently, supress cell migration and tumour metastasis[46]. Moreover, RKIP has shown to modulate EMT, invasion and metastasis in NSCLC through the modulation of EMT-controlling pathways such as Nocth1 and the NF-κB/Snail/YY1/RKIP loop[49,91]. Further, RKIP was also identified as a p53 modulator in lung cancer by blocking Snail, that acts as a p53 suppressor, through MAPK signalling inhibition and NF2 stabilization[92]. Beyond this, it was demonstrated that RKIP reduction enhances radioresistance by activating the Shh signalling pathway in NSCLC[48]. Importantly, RKIP show to be a key molecular player in the modulation of NSCLC cells response to conventional therapy, such as the chemotherapeutic agents adriamycin and cisplatin, as well as radiotherapy[48,69,93]. Finally, Giovannetti *et al.* demonstrated that RKIP is also implicated in lung cancer cells response to targeted therapies. By studying the synergistic interaction between sorafenib, a multikinase inhibitor, and erlotinib, an EGFR inhibitor, they found that sorafenib-related reduction of AKT/ERK phosphorylation in erlotinib-resistant cells was associated with significant RKIP upregulation[94]. Taking these together, RKIP has already shown to be an important modulator of relevant signalling pathways, including MAPK, as well as to be both a driver and predictor of therapy response in lung cancer, which emphasizes the importance of a continued study of RKIP biological role in these malignancies.

1.3 Cancer metabolism reprogramming

Over the past decades, it has become clear that tumorigenesis is highly dependent on the reprogramming of cellular metabolism as both direct and indirect consequence of oncogenic mutations[95]. In fact, metabolic reprogramming is now considered a hallmark of cancer and has been an area of accelerated research on cancer biology[5,96].

The first evidence of altered cancer metabolism was reported by Otto Warburg in the 1920s, who described that tumour cells, rely mainly on glycolysis to generate energy, even under sufficient oxygen conditions, in a phenomenon known as "Warburg effect"[97]. Thus, unlike normal cells, tumours cells obtain most of their energy from aerobic glycolysis, converting most of the incoming glucose into lactate, rather than metabolizing it in mitochondria through oxidative phosphorylation (OXPHOS)[98] (Figure 4).

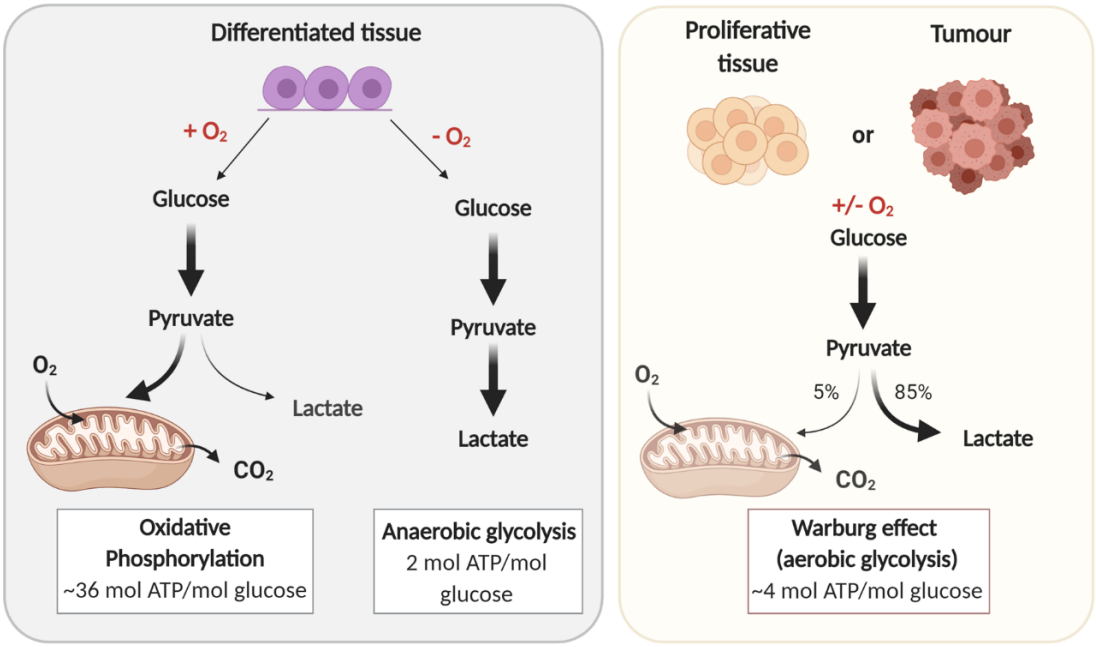

Figure 4: Schematic representation of the metabolic differences between normal cells and proliferative or tumour cells. In the presence of oxygen, non-proliferating (differentiated) tissues metabolize glucose to pyruvate and oxidize it in mitochondria through oxidative phosphorylation (OXPHOS). On the other hand, when oxygen levels are limited, pyruvate is converted to lactate (anaerobic glycolysis). In proliferative tissues, such as tumour cells, glucose is mainly metabolized to lactate, even in the presence of oxygen, in a process denominated aerobic glycolysis or Warburg effect. Adapted from[98].

Although aerobic glycolysis seems inefficient in terms of ATP generation per glucose when compared to mitochondrial respiration (Figure 4), the rate of glycolysis is must higher in this process, in such a way that the production of lactate from glucose occurs 10 to 100 times faster than the complete oxidation of glucose in the mitochondria[99]. This leads to an increased glucose consumption providing metabolites for anabolic processes needed to support the biosynthetic requirements linked to cancer cells deregulated

growth and proliferation[100]. Besides that, elevated glucose metabolism promotes the acidification of the tumour microenvironment due to excessive lactate secretion, which confers a higher aggressiveness to cancer cells by increasing several malignant features such as, migration and invasion[101,102]. Moreover, aerobic glycolysis has an important role in the protection of cancer cells from the oxidative stress, by promoting antioxidant glutathione and NADPH production to maintain redox homeostasis[99,103].

Warburg initially proposed that cancer cells adopted this glycolytic phenotype as a result of impairments on mitochondrial function however, nowadays we know that most tumours maintain mitochondrial activity and respiration[104]. In fact, it has become clear that metabolic reprogramming is an oncogene-driven cell adaptation to support cancer cell proliferation and survival, rather than a compensatory mechanism for mitochondrial dysfunction[105-107].

Henceforward, several works demonstrated that mutations in oncogenes and tumour suppressor genes can mediate the reprogrammed metabolism, through constitutive activation of signalling pathways involved in cell growth, such as RTK pathways. This oncogene pathway regulation allows cancer cells to maintain a growth factor–independent glycolysis and survival through the expression of oncogenic kinases[103,107]. Within the pathways altered in cancer, glucose metabolism is consistently reprogrammed by mutations in *MYC*, *TP53*, *KRAS*, and the PI3K/AKT and MAPK signalling pathways (Figure 5), among others[107-110]. Besides the intrinsic regulation by oncogenic mutations, the metabolic reprogramming of cancer cells is dependent on several extrinsic factors, such as microenvironment oxygen and nutrient conditions[103]. Hypoxia-inducible factor-1 (HIF-1) is considered the master regulator of the metabolic reprogramming in cancer cells response to low oxygen conditions (hypoxia), a common feature of solid tumours[111,112].

1.3.1 Molecular basis of cancer metabolism reprogramming

The metabolic reprogramming of cancer cells towards the glycolytic phenotype is carried by changes in expression and activity of several metabolic enzymes and other glycolytic markers. Thus, the upregulation of several metabolite transporters and metabolic enzymes are frequently observed in the tumorigenic context, being this regulated through oncogenic signalling activation[107,113,114].

The uptake of extracellular glucose is facilitated by the glucose transporters (GLUTs)[115]. There are multiple mechanisms described for the upregulation of glucose transporters in cancer cells, specially GLUT1, whose expression is often associated with tumour progression and poor prognosis[116,117]. Besides the positive regulation by the transcription factors HIF-1α and MYC, oncogenic KRAS and activated PI3K/AKT pathway also showed to increase both GLUT1 expression and its translocation to the plasma

membrane[113,118-120]. In contrast, it was demonstrated that tumour suppressor p53 downregulates the glycolytic rates by directly suppressing the transcription of GLUT1 and others[121] (Figure 5).

Hexokinases (HKs) catalyse the first committed step in glucose metabolism, which consists in the phosphorylation of glucose to glucose 6-phosphate (G6P), trapping glucose inside the cell to fuel the glycolysis and other anabolic pathways, such as pentose phosphate pathway (PPP)[122]. Hexokinase 2 (HK2) is overexpressed in several cancers, and it has been described that its expression is markedly induced by numerous mechanisms and oncogenic drivers, being transcriptionally upregulated by MYC and HIF-1α[113]. Moreover, it was also described that AKT promotes HK2 association with mitochondria, increasing its intracellular activity[113,123] (Figure 5). The next committed step of glycolysis, generation of fructose 1,6-biphosphate (F1,6BP) from fructose 6-phosphate (F6P), is catalysed by one of the most regulated enzymes of glycolysis, phosphofructokinase 1 (PFK1). Given its important role in controlling glycolysis rates, PFK1 is often upregulated in cancer cells being its activity induced by HIF-1α and AKT signalling[113,123] (Figure 5).

Moreover, the last irreversible step of the glycolytic pathway is dictated by the pyruvate kinase (PK), that catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate, which can then enter into TCA cycle or be converted into lactate, as it happens on aerobic glycolysis. PKM2 is overexpressed in many cancers and has been demonstrated to play a crucial role in controlling the glycolytic flux and promoting aerobic glycolysis[124,125]. Notably, it has been described that constitutive activation of MAPK pathway, by *EGFR* mutation, promotes the Warburg effect through regulation of PKM2 activity[110,126] (Figure 5). Yang *et al.* reported that ERK phosphorylation-dependent nuclear translocation of PKM2 is required for autoregulation of PKM2 expression and PKM2-depedent expression of glycolytic genes, which are crucial for the EGFR-promoted Warburg effect and tumorigenesis[126].

Finally, lactate dehydrogenase A (LDHA) plays a critical role in regulating glycolysis by catalysing its final step, conversion of pyruvate to lactate, allowing NAD+ regeneration that helps to maintain the glycolytic flux of cancer cells[127]. Both HIF-1α and MYC transcriptionally increase LDHA expression in cancer cells[108,113]. Furthermore, HIF-1α and MYC regulate the export of lactate from cancer cells by inducing the expression of monocarboxylate transporters (MCTs), MCT4 and MCT1, respectively, that play an important role in glycolysis maintenance[113,128-130](Figure 5).

Notably, most human tumours present an increased uptake and use of glucose, therefore, attempts to target the glucose metabolism for cancer diagnosis and therapy have emerged in the past decades[131-133]. The most impressively clinical utility of the Warburg effect until now is the widespread use of FDG-PET (18F-fluorodeoxyglucose positron emission tomography) scanning in cancer evaluation. Importantly, this

non-invasive technique allows the detection of accelerated and aberrant glycolysis providing information about the pathological differentiation, tumour staging and dissemination, and enabling the monitorization of therapy response in cancer patients[134,135].

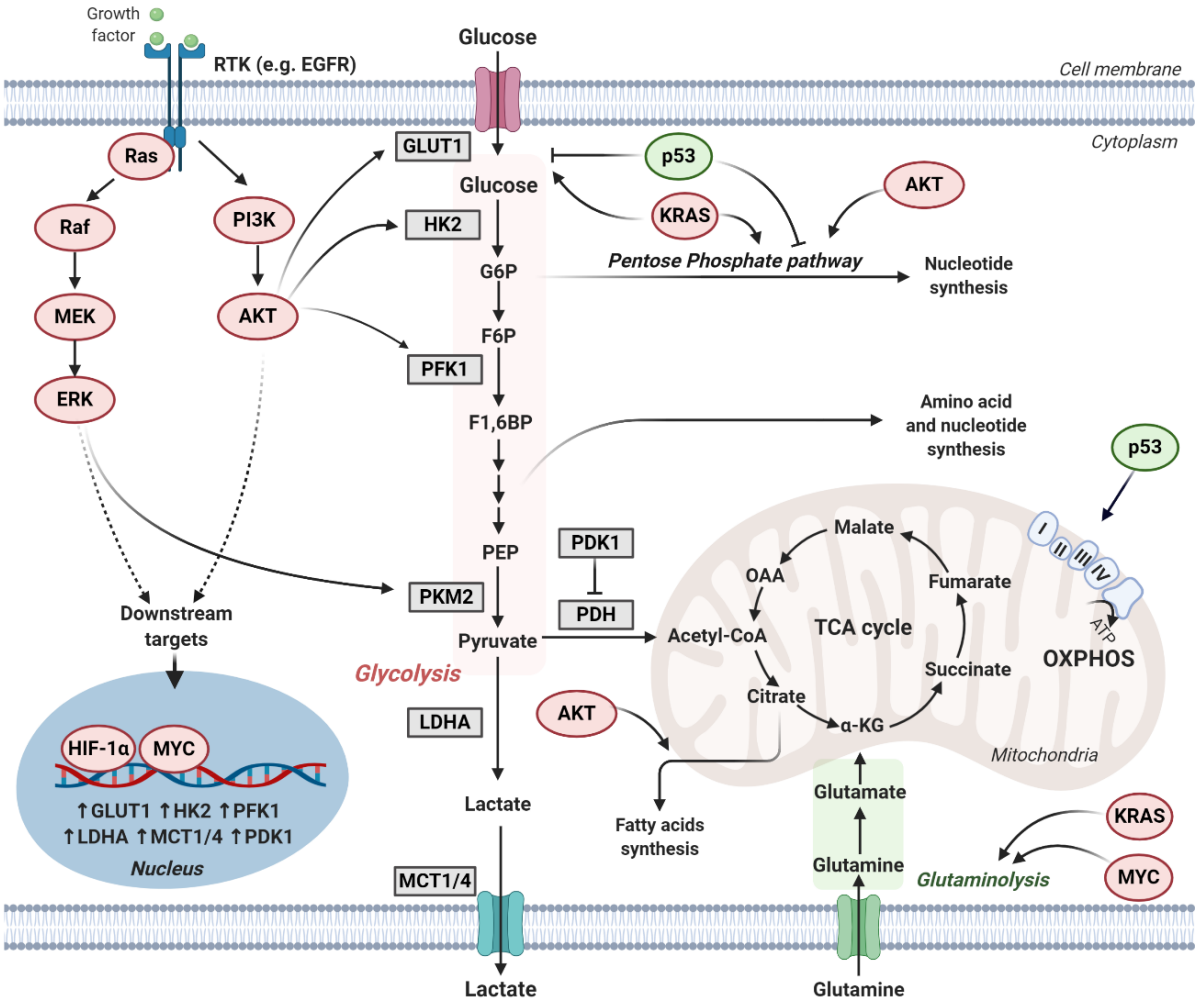

Figure 5: Molecular basis of cancer metabolism reprogramming. Mutations in oncoproteins, such as KRAS, and activation of oncogenic pathways, such the ones downstream of RTKs, MAPK and PI3K/AKT pathway, lead to increased glucose uptake and enhanced lactate production through the Warburg effect, either directly, by regulation of glycolytic proteins function or indirectly by regulation of downstream targets (e.g., MYC and HIF-1α). In the nucleus, MYC and HIF-1α, that are also often mutated in cancer, upregulate the transcription of glycolytic proteins, contributing to increased glycolysis. In addition, KRAS and MYC promote glutamine metabolism to fuel the TCA cycle. Intermediates from glycolysis and the TCA cycle supply biosynthetic pathways to produce macromolecules necessary for cell proliferation. Tumour suppressors, such as p53, inhibit the glucose and biosynthetic metabolism and induce OXPHOS. Adapted from[107,108,110,114].

Even though it has been described that some tumours acquire the ability to undergo enhanced glycolysis while maintaining OXPHOS[104,136,137], mitochondrial alterations can be also found at different levels. Actually, increased glycolytic metabolism can be a result of mitochondrial metabolism impairment, due

to abnormalities in components of the tricarboxylic acid (TCA) cycle and electron transport chain (ETC), that can lead to OXPHOS malfunction[107,138,139].

Pyruvate dehydrogenase (PDH) is a mitochondrial enzyme that controls the entry-point of the TCA cycle by promoting pyruvate oxidative decarboxylation into acetyl-CoA[140]. PDH is inhibited by the pyruvate dehydrogenase kinases (PDK) through phosphorylation, which results in a reduced pyruvate flux across PDH and downstream metabolic pathways, namely the TCA cycle[141]. In these sense, high PDK expression leads to a shift in glucose metabolism towards glycolysis instead of OXPHOS. Notably, overexpression of PDK1 is found in many cancers as a result of increased activation of HIF-1α[142,143] (Figure 5).

In addition, germline or somatic mutations in the TCA cycle enzymes isocitrate dehydrogenase (*IDH*), succinate dehydrogenase (*SDH*) and fumarate hydratase (*FH*), which are found in some human tumours, have been described as leading to accumulation oncometabolites, meaning that their accumulation is sufficient to initiate and/or sustain tumour growth and metastasis[96,139,144]. Moreover, several studies have demonstrated that other cycle enzymes, such as citrate synthase (CS), aconitase (ACO2) and dihydrolipoamide S-succinyltransferase (DLST) are also found deregulated in cancer[144]. Emphasizing the importance of TCA dysfunction not only for glycolysis and OXPHOS uncoupling but also for the promoting tumour growth and progression.

Despite the earlier belief that cancer cells bypass the TCA cycle, emerging evidence suggests that many cancer cells also rely on the TCA cycle to meet their high biosynthetic needs[144]. Therefore, the replenishment of TCA intermediates, a process named anaplerosis, is crucial for tumour cells proliferation. Reductive glutamine metabolism has been reported as a major source of citrate, a substrate for fatty acids synthesis, in the presence of mitochondrial defects and hypoxia[107]. Notably, both *MYC* and *KRAS* oncogenes have been described to promote glutamine-addiction in cancer cells[108,145] (Figure 5).

Moreover, abnormalities in ETC components can also promote the Warburg effect. For instance, loss of tumour suppressor *TP53*, can lead to OXPHOS malfunction, by interfering with the activity of cytochrome c oxidase (COX) synthesis, a crucial component of ETC[121]. In turn, mitochondrial respiration dysfunction can lead to increased production of reactive-oxygen species (ROS) triggering the accumulation of potentially oncogenic DNA defects (e.g. *KRAS* mutations) and the activation of oncogenic signalling pathways, such as MAPK, thus promoting tumorigenesis[139].

One of the striking characteristics of cancer cells is the great metabolic plasticity that allows them to adapt to the stressful and dynamic microenvironment of solid tumours[146]. Tumour vasculature is structurally and functionally abnormal, and combined with the intrinsically altered cancer cell metabolism, gives rise to spatial and temporal tumour heterogeneity in oxygenation, pH, and the concentrations of

crucial nutrients such as glucose[103,147,148]. Together these factors dictate cellular stress responses that contribute to cancer cell metabolic plasticity which promotes tumour growth and metastasis[149].

Hypoxia is a common feature of solid tumours that has been strongly associated to metastasis, poor prognosis and therapy resistance in several malignancies[103,111,112,150]. HIF-1α is a master regulator of cellular oxygen sensing and a ubiquitous transcriptional activator that mediates the cells adaptation to hypoxia conditions. Therefore, HIF-1α plays a critical role in the metabolic reprogramming of tumour cell metabolism by enhancing the glycolytic metabolism and decreasing the mitochondrial respiration[111,112,147].

Thus, it is becoming more evident that heterogeneous metabolic phenotypes support tumour growth and progression, and that this metabolic heterogeneity is a result of a combination of cell-intrinsic factors (e.g., genetic lesions) and cell-extrinsic factors (e.g., nutrient and oxygen availability, pH, metabolic competition)[136,151], which seems to be context dependent and tumour specific[152-154].

1.3.2 Targeting cancer cell metabolism

Considering the vital role of metabolic reprogramming in tumour development and progression, many studies have demonstrated that target cancer bioenergetics can be a promising approach for the development of anti-cancer therapies[128,133,155,156]. Indeed, several drugs been already developed to selectively target metabolic enzymes that are found deregulated in cancer, such as the ones mention above[128,156,157] (Figure 6).

The glucose analogue 2-deoxyglucose (2-DG) is one of the most widely used glycolysis inhibitors (Figure 6). It competes with the glycolytic enzyme HK, inhibiting this way its activity, leading to the inhibition of both glycolytic rate and ATP production, ultimately inducing cell death[156,158]. As a competitive inhibitor, the single use of 2-DG is limited since it requires high concentrations to compete with glycolytic pathway products. Besides that, targeting glycolysis alone has shown to be limited due to the high metabolic plasticity associated to tumour cells which allows them to induce compensatory routes[157]. Several reports have demonstrated that metabolic rewiring of cancer cells can be a mechanism behind resistant to either chemo/radiotherapy or oncogene-targeted therapies[128,157,159]. Therefore, the clinical potential of some of these therapies can be enhanced by combining them with glycolysis inhibitors, such as 2-DG[160,161].

Aside from this, recent findings suggest that mitochondrial metabolism is active and necessary for tumour growth, as a matter of fact, reliance on enhanced OXPHOS has been reported as a mechanism of drug resistance in oncogene-addicted cancers[156,162]. Therefore, mitochondrial bioenergetics has also emerged as a key target for cancer therapy (Figure 6). Metformin is a well-tolerated biguanide derivate

and one of the most prescribed drugs in the world for the treatment of type 2 diabetes mellitus. Notably, retrospective clinical studies suggested that the use of metformin improve the prognosis of diabetic patients with multiple cancers and prevent tumour initiation and reoccurrence[163,164]. Moreover, metformin has shown to inhibit proliferation, cell survival and induce apoptosis in several cancer models[165,166]. It is now recognized that metformin acts as an anticancer agent by inhibiting mitochondrial ETC complex I (NADH: ubiquinone oxidoreductase), blocking this way mitochondrial ATP production also its biosynthetic capacity, which can lead to an increase of cellular energy stress, and ultimately cell death[107,165]. Furthermore, several studies demonstrated synergistic effects of metformin in cancer cells when combined with other treatments, such as glycolysis inhibitors, chemotherapeutic drugs, and targeted therapies[155,167-169].

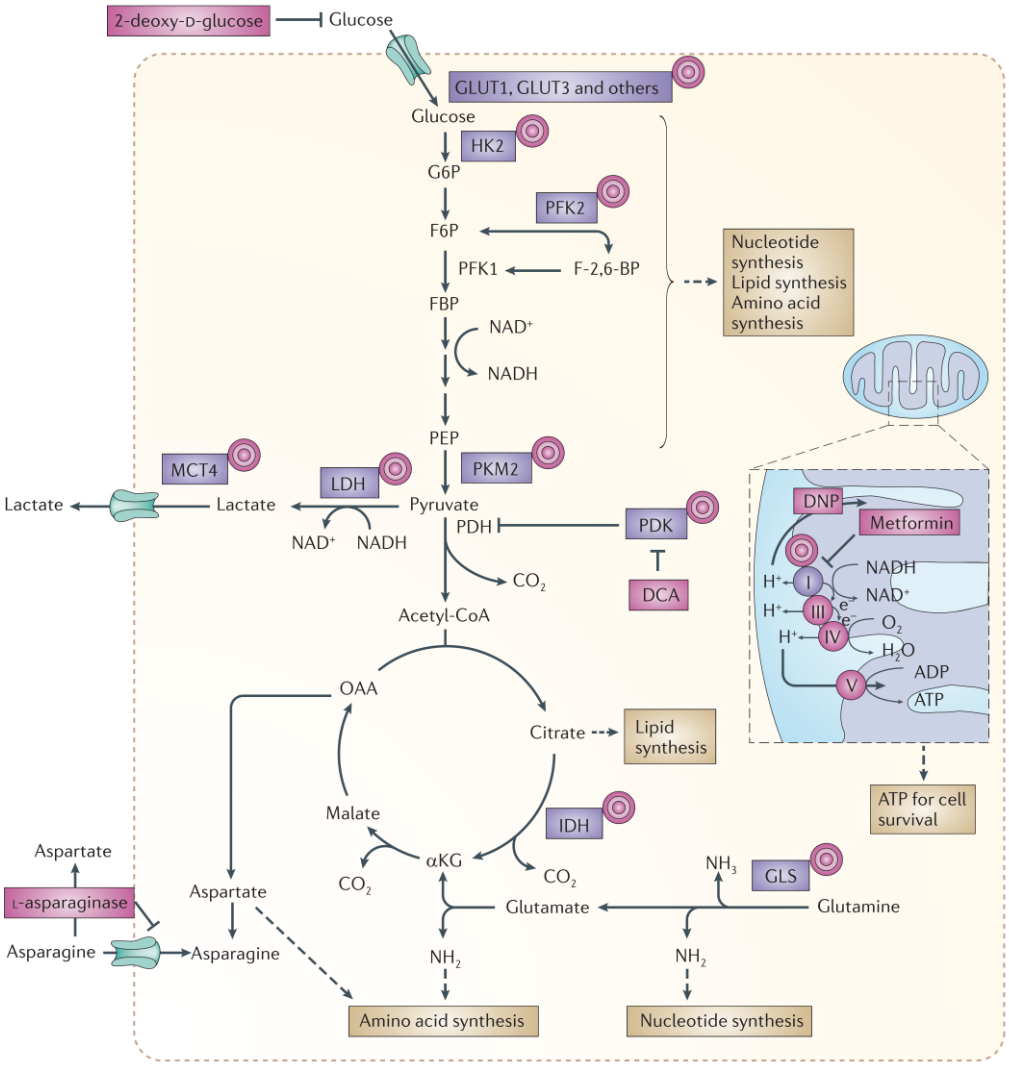

Figure 6: Potential molecular targets in cancer cell metabolism. The pathways of central carbon metabolism are presented. Some of the metabolic enzymes deregulated in cancer that are currently being considered as molecular targets for therapy are marked with a target (shown as a pink circle in the figure). Five drugs that influence metabolism and have been tested in humans are shown in pink boxes. Adapted from[156].

1.3.3 Metabolic reprogramming in lung cancer

Lung cancer is a molecularly heterogeneous disease, therefore, understanding its biology is crucial for the development of effective therapies[13]. Like many other solid tumours, lung cancer requires a high intake of glucose, and FDG-PET scan already became a standard diagnostic tool to assess tumour extension[9]. Moreover, data from patients indicate that lung tumours are dependent on glucose metabolism, being the increased expression of glycolytic markers correlated with poor prognosis in lung cancer patients[170-172]. Despite the clear association between high glucose metabolism and poor prognosis, the pathways through which glucose is metabolized in this type of cancer remain unclear.

Studies using surgical resections from NSCLC patients after infusion with radioactive glucose demonstrated that these tumours displayed enhancement of both glycolysis and OXPHOS, which was translated in the high levels of lactate and TCA cycle intermediates in tumour samples compared with normal tissue[137,173]. These observations suggested that glycolysis and OXPHOS can function in simultaneous if not in the same cancer cell at least in the same tumour, in which metabolic symbiosis can be established[137]. Hensley *et al.* also described that glucose oxidation via PDH, and TCA cycle were higher in NSCLC tumours compared to adjacent normal lung[137]. In addition, they demonstrated that NSCLC tumours oxidize multiple types of nutrients *in vivo* besides glucose, such as lactate, fatty acids and amino acids, and this was, in part, dependent on the perfusion status[137]. Notably, further studies reported the importance of glucose-derived lactate as a carbon source for the TCA cycle in tumours from NSCLC patients[174,175]. Despite commonalities in metabolic reprogramming, growing evidence suggest that metabolic changes in NSCLC tumours are highly heterogeneous[137,176-178].

Given the high mutation burden of lung cancer[21,22], its genetic heterogeneity can also influence lung cancer metabolic diversity, since oncogenic signalling is tightly linked to metabolic reprogramming[109]. Indeed, some lung cancer driver mutations have been already described to produce metabolic alterations in these tumours[179,180]. In specific, NSCLC tumours are often driven by activation of tyrosine kinase signalling, by *EGFR* and *KRAS* mutations for instance[9,13]. Remarkably, activating mutations in *EGFR* gene promote metabolic rewiring in NSCLC by increasing aerobic glycolysis, PPP pathway, altered pyrimidine biosynthesis and redox metabolism[181,182]. Moreover, it was demonstrated that PI3K/AKT signalling plays an important role in regulation of metabolic activities in *EGFR*-mutant lung adenocarcinoma cells, by facilitating GLUT1 proper cellular localization[181].

Furthermore, *KRAS* mutations, that occur in approximately 30% of adenocarcinoma patients, have also been implicated in the metabolic reprogramming in NSCLC, including in the upregulation of glucose uptake, glutamine utilization and aerobic glycolysis[151,154,183]. For instance, a study has reported that *KRAS*-

mutated NSCLC cells express higher levels of enzymes involved in glycolysis and PPP when compared to non-malignant cells, suggesting alterations in glucose metabolism is NSCLC cells carrying mutant *KRAS* [184]. Further, Kerr *et al.* described that glycolysis is acquired as a function dependent on the number of copies of mutant *KRAS* [185]. Moreover, expression and activity of HK2 was induced by *KRAS*-mutation in NSCLC models. In particular, deletion of HK2 supressed *KRAS*-mutant lung cancer development in murine models and reduced cell growth both *in vitro* and *in vivo* by inhibiting nucleotides synthesis and glutamine-derived carbon utilization in TCA cycle[122]. In addition, *KRAS* mutations already show to enhance glutamine metabolism *in vitro* through PI3K/AKT dependent signalling in NSCLC cells[186].

Overall, the literature herein reviewed provides a strong evidence of metabolic rewiring in lung cancer. Concomitantly, the acquired heterogeneous metabolic phenotype by tumours cells to withstand the complex challenges linked to cancer progression[137,176], is still puzzling and challenging to search for more and new oncogenic alterations behind this metabolic heterogeneity.

Given the key role of RKIP in the regulation of several intracellular signalling pathways, more than just MAPK, and its recognized importance in tumours malignancy[52] in a frequency which seems to be much higher than KRAS and with a higher penetrance among solid tumours, including lung cancer[62], RKIP arises to us as one of the potential proteins behind cancer metabolic heterogeneity. To support our believe, there is on one hand, an *in silico* study reporting that RKIP is inversely correlated with genes that directly regulate cell metabolism, in clear cell renal cell carcinoma (ccRCC)[187]. By the other hand, it was demonstrated that the pro-metastatic transcription factor BACH1, which is negatively regulated by RKIP, promotes aerobic glycolysis in breast cancer[188,189]. Curiously, until now there are no studies exploiting the direct functional role of RKIP in the modulation of cancer cells metabolism.

CHAPTER 2:

Research Objectives

2. Research Objectives

The reprogramming of cellular metabolism is now widely recognised as a hallmark of cancer. Remarkably, oncogenic signalling has been linked to tumour metabolic reprogramming, being cancer cells able to maintain growth factor–independent glycolysis and survival, through the expression of oncogenic kinases. Overall, the literature provides strong evidence of an oncogenic signalling dependent metabolism in lung cancer. In fact, lung cancers exhibit a high mutation burden, but specifically NSCLC tumours, are known to be driven by oncogenic activation of tyrosine kinases, such as mutations in *KRAS* and *EGFR* genes, and many other pathways, which are well described as modulators of metabolic rewiring in lung cancer cells. Nowadays, one of the emerging themes in cancer research relies on the ability of tumours cells acquiring heterogeneous metabolic phenotypes to withstand the complex challenges linked to cancer progression, consequently, there is a demand for the search of new oncogenic alterations that can be behind this metabolic heterogeneity.

RKIP is a well-establish tumour suppressor, whose loss of expression has been observed in a plethora of solid tumours, including lung cancer. Herein, due to its important role in carcinogenesis and its master role in the regulation of several intracellular signalling pathways, more than the first described MAPK, which are known to be crucial in cancer cell metabolic rewiring, RKIP arises to us as one of the potential proteins behind lung cancer metabolic heterogeneity. Notably, recent evidence indirectly raised the suspicion that RKIP loss may be involved in tumoral metabolic processes, but without a direct functional validation. Thus, passing the redundancy, in the present study we hypothesized that RKIP could be a key player in the metabolic reprogramming of NSCLC tumours cells. To achieve our hypothesis, the present project was divided in four main aims:

The **first** was to determine whether a RKIP-associated molecular signature is correlated with metabolic alterations in lung adenocarcinoma patients. For that, an extensive *in silico* analysis was performed using TCGA database.

Secondly, it was intended to metabolic characterize the chosen NSCLC cell lines for cell proliferation, metabolic profile and RKIP expression.

In **third**, to unravel the effect of RKIP on the modulation of NSCLC cells metabolism, we pursued to positively and negatively modulate its expression and determine the cells metabolic behaviour as well as its expression patterns of metabolic proteins, upon transfection.

Finally, in **fourth**, it was planned to carry out a focused *in silico* analysis to validate and understand the pertinent obtained results.

CHAPTER 3:

Materials and Methods

3.1 Cell lines and cell culture

In this study, a panel of different NSCLC cell lines were used (Table 1). All cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen) supplemented with 10% of Fetal Bovine Serum (FBS, GIBCO, Invitrogen) and 1% penicillin/streptomycin (GIBCO, Invitrogen). For normoxia conditions, cells were incubated in a humidified atmosphere of 21% O_2, 5% CO_2 and 74% N_2 at 37°C. For hypoxia conditions, cells were placed in airtight chamber with hypoxic gas mixture 1% O_2, 5% CO_2 and 95% N_2 and incubated at 37°C for a specific period time, according to the assay carried out.

Table 1: Non-small cell lung cancer cell lines used in this study.

Cell line	Histological type	Relevant alterations
A549	Adenocarcinoma	*KRAS* mutant
HCC827	Adenocarcinoma	*EGFR* mutant
H292	Mucoepidermoid Carcinoma	*EGFR* and *KRAS* WT

WT: Wild-type

3.2 Drugs

2-Deoxyglucose and metformin were purchased from Sigma-Aldrich and MedChemExpress (MCE), respectively. Both drugs were prepared in stock solutions of 200mM by dilution in sterile water and stored at -20°C. In all experimental conditions the drugs were diluted in culture medium to a final concentration of 1.5mM. Cell culture medium was also used as the vehicle control in all experiments.

3.3 *In silico* analysis

The cBioPortal for Cancer Genomics (http://www.cbioportal.org) is a repository of multidimensional cancer genomics datasets[190,191], that was used to determine RKIP-associated signature in lung adenocarcinoma patients. In this study, mRNA expression and protein expression data were analysed from a total of 586 samples from 584 adenocarcinoma patients from the provisional TCGA dataset: Lung Adenocarcinoma (TCGA, Firehose Legacy). According to the TCGA guidelines (http://cancergenome.nih.gov/publications/publicationguidelines), this dataset has no limitations or restrictions. Enrichment analysis were performed to determine the expression profiles of genes that were positively or negatively associated to *PEBP1* gene (encoding RKIP) and specific correlations between *PEBP1* gene and other genes of interest were determined by spearmen correlation. Significant alterations in mRNA expression (RNA Seq V2 RSEM) and protein expression (RPPA) were determined by Z-score threshold of ±2.

Data from the enrichment analysis (mRNA and/or protein data) that were positively or inversely correlated with RKIP expression were retrieved for functional protein association network analysis that was done using STRING (https://string-db.org/), a database that integrates publicly available evidence, computational prediction data and textmining information into comprehensive protein-protein interaction networks[192]. The end date of the analysis was 30th of September 2020.

3.4 *In vitro* RKIP genetic modulation

For generation of stable RKIP knockout (KO), H292 and HCC827 were previously knocked out using the CRISPR/Cas9 technology, a well-established genome editing tool[193]. For that, the CRISPR/Cas9 knockout kit from Santa Cruz Biotechnology (CRISPR/Cas9 KO Plasmid - sc-401270-KO-2 and HDR Plasmid – sc-401270-HDR-2) was used[193]. Cells transfected with both plasmids for RKIP knockout were so called as RKIP KO cells, while control cells transfected only with HDR plasmid were so called as CTR.

For generation of stable RKIP overexpressing (OE) cell lines, we used a pcDNA3 vector containing the full cDNA of RKIP in H292 and HCC827. This overexpression is based on a transfection with a pcDNA vector that contains a multiple cloning site in which the full cDNA of RKIP is inserted, and also a Geneticin (G418) resistance gene that will be useful for selection of the successfully transfected cells. The cells were transfected with the empty vector for control (so called as CTR cells), and with RKIP full cDNA containing vector to overexpressed it (so called as RKIP OE cells). The cells were plated into 6-well plates at a density of $5x10^5$ cells per well, allowed to adhere overnight and transfected in serum free Opti-MEM media for 24 hours. The transfection was done using the FUGENE HD reagent (Roche) according to the manufacturer's protocol, with 2µg of plasmid at a ratio of 6:2 (reagent:plasmid). Then the stable transfectants were selected with varying concentrations of G418 (H292=800 µg/ml; HCC827=300 µg/ml).

3.5 Cell viability assay

The sulforhodamine B (SRB) assay was used to assess total protein (expressed as total biomass) overtime or in a specific time point. The cells were seeded into 48-well plates in triplicate at a density of $3x10^4$ (A459 and H292) and $6x10^4$ (HCC827) cells per well and allowed to adhere overnight. In the following day the cells were submitted to different conditions of oxygen availability (normoxia and hypoxia), glucose concentrations (1g/L, 2.5g/L and 4.5g/L glucose) or drug treatment (1.5mM of 2-deoxyglucose or metformin) depending on the assay to be carried out. The day of conditions imposition is considered the 0h time point in the overtime assays. At the 0,24,48 or 72 hours, the cells were fixed with cold 10%

trichloroacetic acid for at least 1 hour at 4°C and stained with Sulforhodamine B (Sigma-Aldrich) for 30 minutes. Next, cells were washed with 1% acetic acid to remove the excess of dye and the protein-bound dye was dissolved in 10mM of Tris-Base solution (pH=10.5) for absorbance measurement at 490nm using the Thermo-Scientific Varioskan Flash SkanIt software (Thermo-Scientific). The results were then calibrated to the starting value (time 0 h, considered as 100% of viability) and expressed as the mean ± SD. The assays were done in triplicate at least three times.

3.6 Extracellular glucose and lactate measurements

Glucose consumption and lactate production were determined through extracellular glucose and lactate measurements, respectively. The cells were seeded into 48-well plates at a density of $3x10^4$ (A459 and H292) and $6x10^4$ (HCC827) cells per well and allowed to adhere overnight. In the following day the cells were submitted to different conditions of oxygen availability (normoxia and hypoxia), glucose concentrations (1g/L, 2.5g/L and 4.5g/L glucose) or drug treatment (1.5mM of 2-deoxyglucose or metformin) for 24, 48, or 72 hours depending on the assay to be carried out. Extracellular glucose and lactate content were analysed in cell culture medium at the respective time points using commercial kits (Spinreact), in which 100µL of the commercial reagent is added to 2µL of the supernatant sample. After incubation time, the absorbance was measured at 490nm in the Varioskan Flash reader using the SkanIt software (Thermo-Scientific). Glucose and lactate quantity were calculated through a standard curve and normalized to total biomass determined by SRB assay, as previously described[129,159]. Results are expressed as mean mg/total biomass. The assays were done in triplicate at least three times.

3.7 Western blot analysis

The cells were seeded in 6-well plates at a density of $1x10^6$ cells per well and allowed to adhere overnight. The cells were then incubated for 24 hours in different conditions of oxygen availability (normoxia and hypoxia) and glucose concentrations (1g/L and 4.5g/L glucose) depending on the assay to be carried out. Afterwards, cells were washed and scrapped in cold PBS and lysed in lysis buffer containing phosphatases and proteases inhibitors (Roche). After a centrifugation at 13000 rpm for 15 minutes at 4°C, total protein was quantified using the Bradford reagent (Sigma-Aldrich). Aliquots of 40µg of total protein from each sample were prepared and separated on a standard 12% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (100V) and transferred onto a nitrocellulose membrane (Amersham Biosciences) using the Trans-Blot Turbo Transfer System (Bio-Rad) (25V, 1A for 30 min). Next, the membranes were blocked with 5% milk in Tris-Buffered Saline/0.1% Tween (TBS-

Tween) for 1 hour at RT and incubated overnight with the primary antibodies at 4°C (Table 2). After washing in TBS-Tween, the membranes were incubated with the respective secondary antibodies coupled with horseradish peroxidase (1:2500, Cell Signalling) for 1 hour at RT. Tubulin was used as loading control. Blots detection was done by chemiluminescence (Supersignal West Femto kit, Pierce, Thermo Scientific) using the Sapphire Biomolecular Imager (Azure Biosystems).

Table 2: Details of the primary antibodies used for western blot.

Protein	Reference	Dilution (Secondary Antibodies)
HK2	C64G5 (CS)	1:1000 (Rabbit)
PFKP	D4B2 (CS)	1:1000 (Rabbit)
PKM2	D78A4 (CS)	1:1000 (Rabbit)
LDHA	C4B5 (CS)	1:1000 (Rabbit)
p-GSK3β (Ser9)	D85E12 (CS)	1:1000 (Rabbit)
PDH	C54G1 (CS)	1:1000 (Rabbit)
ACO2	D6D9 (CS)	1:1000 (Rabbit)
SDHA	D6J9M (CS)	1:1000 (Rabbit)
DLST	D22B1 (CS)	1:1000 (Rabbit)
RKIP	D42F3 (CS)	1:1000 (Rabbit)
α-Tubulin	SC-73242	1:5000 (Mouse)

CS: Cell Signalling Technology; SC: Santa Cruz Biotechnology;

3.8 Immunofluorescence analysis

The cells were seeded on glass cover slips placed into 12-well plates until 60% of confluence and allowed to adhere overnight. Cells were incubated in normoxic or hypoxic conditions for 24 hours. Afterwards, the cells were fixed and permeabilized in cooled methanol for 10 minutes and blocked with 5% bovine serum albumin for 30 minutes. Slides were incubated overnight at RT with the primary antibodies (Table 3). After washing in Phosphate Buffered Saline (PBS), the TRITC (anti-rabbit) and FITC (anti-mouse) Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Invitrogen) were applied at a dilution of 1:500 for one hour at RT protected from the light. Finally, after washing in PBS, the nucleus was stained with 4',6-diamino-2-phenylindone (DAPI) (Sigma) and slides were covered using Vectashield Mounting Media. Images were obtained with a fluorescence microscope (Olympus BX61) at 200X magnification, using Cell P software.

Table 3: Details of the primary antibodies used for immunofluorescence.

Protein	Reference	Dilution (Secondary Antibodies)
MCT4	SC-376465	1:100 (Mouse)
MCT1	Ab35944	1:100 (Rabbit)
EMMPRIN (CD147)	SC-21746	1:100 (Mouse)
GLUT1	D3J3A (CS)	1:100 (Rabbit)
PDH	C54G1 (CS)	1:100 (Rabbit)

Ab: Abcam; SC: Santa Cruz Biotechnology; CS: Cell Signalling Technology

3.9 Statistical analysis

For *in vitro* assays, statistical analysis was performed using GraphPad Prism 6 version. To analyse the *in vitro* assays, single comparisons between the different conditions studied was done using Student's t-test, and differences between groups were tested using the two-way ANOVA test. The level of significance in all statistical analysis was set at $p<0.05$.

CHAPTER 4:

Results

4.1 RKIP and lung cancer: *In silico* analysis

Recently, Kapoor *et al.*, by analysing data from The Cancer Genome Atlas (TCGA) and others, demonstrated that RKIP-displayed associations during clear renal cell carcinoma (ccRCC) progression are correlated with reductions in fatty acid degradation at the mitochondrial level, pyruvate metabolism and other metabolic changes[187]. One of the many advantages of using TCGA data is the possibility to stablish correlations, both at mRNA and protein level, between our protein of interest and a large number of other genes. Hence, we investigated whether RKIP plays a role in the regulation of lung cancer metabolism by using TCGA provisional available data at cBioPortal for Cancer Genomics database (www.cbioportal.org). To do so, we used the Lung Adenocarcinoma (TCGA, Firehose Legacy) dataset, with a total of 586 samples from 584 adenocarcinoma patients.

Initially, by using mRNA data (RNA Seq V2 RSEM), we performed an enrichment analysis to determine the positively and negatively regulated genes associated with RKIP expression. The Top 50 enriched genes found in the set of patients with RKIP mRNA upregulation and downregulation (Table S1) were retrieved for functional protein association network analysis in STRING online tool (https://string-db.org/). When we plot the list of 50 genes enriched in the cases where RKIP is upregulated, we observed that, although different, a big portion of these genes are mitochondrial and are significantly related to some metabolic processes. For instance, this functional enrichment analysis shows genes that are implicated in pyruvate metabolism (*LDHD*, *ACAT* and *ALDH2*), electron transport chain, oxidative phosphorylation (*COX6A1*, *COX14*, *COX4I1* and *FMC1*), and energetic metabolism (*PRKAB1*). Besides that, the presence of GSK3 associated genes (*FRAT1* and *FRAT2*) was pointed, which is in line with the described role of RKIP as a modulator of GSK3β signalling. Regarding, the network composed of the genes negatively associated with RKIP expression no biological or molecular functions were significantly related to this group of genes, which can be due to the lack of common functions and interactions between the genes retrieved from the enrichment analysis. However, the presence of two important genes related to glucose metabolism is highlighted, being them the *SLC16A3* gene, that encodes MCT4, and the *SLC2A1* gene, that encodes GLUT1.

Given the well-described role of RKIP as an important modulator of signalling molecules, more than the mRNA correlations, it is important to study the RKIP associated proteins in lung adenocarcinoma. Unfortunately, protein expression data (RPPA) in these datasets is still limited when compared to mRNA expression data and, the RKIP protein expression data is not available yet. Despite this, we were able to do an enrichment analysis, by selecting the group of genes that were significantly upregulated at the protein level when RKIP was up or downregulated at the mRNA level (Table 4 and Figure 7).

Table 4: Top25 genes positively and negatively co-expressed (at protein level) with RKIP mRNA expression in lung adenocarcinoma patients. TCGA provisional data (www.cbioportal.org).

RKIP mRNA upregulation				RKIP mRNA downregulation			
Protein	Cytoband	p-value	q-value	Protein	Cytoband	p-value	q-value
SRSF1	17q22	5.71e-7	1.181e-4	FN1	2q35	2.915e-6	3.017e-4
AR	Xq12	6.771e-5	3.504e-3	SERPINE1	7q22.1	2.126e-5	1.467e-3
CLDN7	17p13.1	1.559e-4	5.181e-3	ERRFI1	1p36.23	1.208e-4	5.001e-3
PECAM1	17q23.3	2.683e-4	6.941e-3	INPP4B	4q31.21	1.752e-4	5.181e-3
PDCD4	10q25.2	3.836e-4	7.635e-3	EGFR	7p11.2	3.837e-4	7.635e-3
BCL2	18q21.33	4.057e-4	7.635e-3	ANXA1	9q21.13	3.412e-3	0.0353
FOXO3	6q21	4.738e-4	7.696e-3	RPS6KB1	17q23.1	5.076e-3	0.0478
PGR	11q22.1	5.006e-4	7.696e-3	EGFR_PY1068	7q31	6.190e-3	0.0493
SCD	10q24.31	5.205e-4	7.696e-3	ERBB2_PY1248	6q25.1	7.698e-3	0.0551
DIABLO	12q24.31	7.692e-4	0.0106	DVL3	3q27.1	7.718e-3	0.0551
FOXO3_PS318_S321	5q21-q22	9.985e-4	0.0129	NFKB1_PS536	16p11.2	9.428e-3	0.0630
KIT	4q12	1.097e-3	0.0134	CCNE1	19q12	0.0106	0.0662
NKX2-1	14q13.3	1.803e-3	0.0207	ITGA2	5q11.2	0.0111	0.0675
SMAD4	18q21.2	3.210e-3	0.0350	MAP2K1	15q22.31	0.0136	0.0804
CDH1	16q22.1	4.515e-3	0.0445	GAPDH	12p13.31	0.0227	0.122
YWHAE	17p13.3	5.552e-3	0.0493	HSPA1A	6p21.33	0.0236	0.122
CDKN1B	12p13.1	5.780e-3	0.0493	STK11	19p13.3	0.0273	0.134
ESR1_PS118	11q24.2	6.016e-3	0.0493	EGFR_PY1173	3p25	0.0279	0.134
RPS6KB1_PT389	6q21	6.693e-3	0.0513	PEA15	1q23.2	0.0338	0.157
MAPK8_PT183	3q13.3	8.598e-3	0.0593	RB1_PS807_S811	5q21-q22	0.0342	0.157
PRKAA1	5p13.1	9.871e-3	0.0639	CCNB1	5q13.2	0.0368	0.166
RAB25	1q22	0.0210	0.121	NDRG1_PT346	22q11.21	0.0408	0.176
YWHAB	20q13.12	0.0219	0.122	PRKCA_PS657	4q24	0.0428	0.181
FASN	17q25.3	0.0235	0.122	ERBB2	17q12	0.0739	0.283
PRDX1	1p34.1	0.0263	0.133	KDR	4q12	0.0964	0.354

*p-value (derived from Student's t-test); q-value (derived from Benjamini-Hochberg procedure); p- and q-value<0.05: significant association.

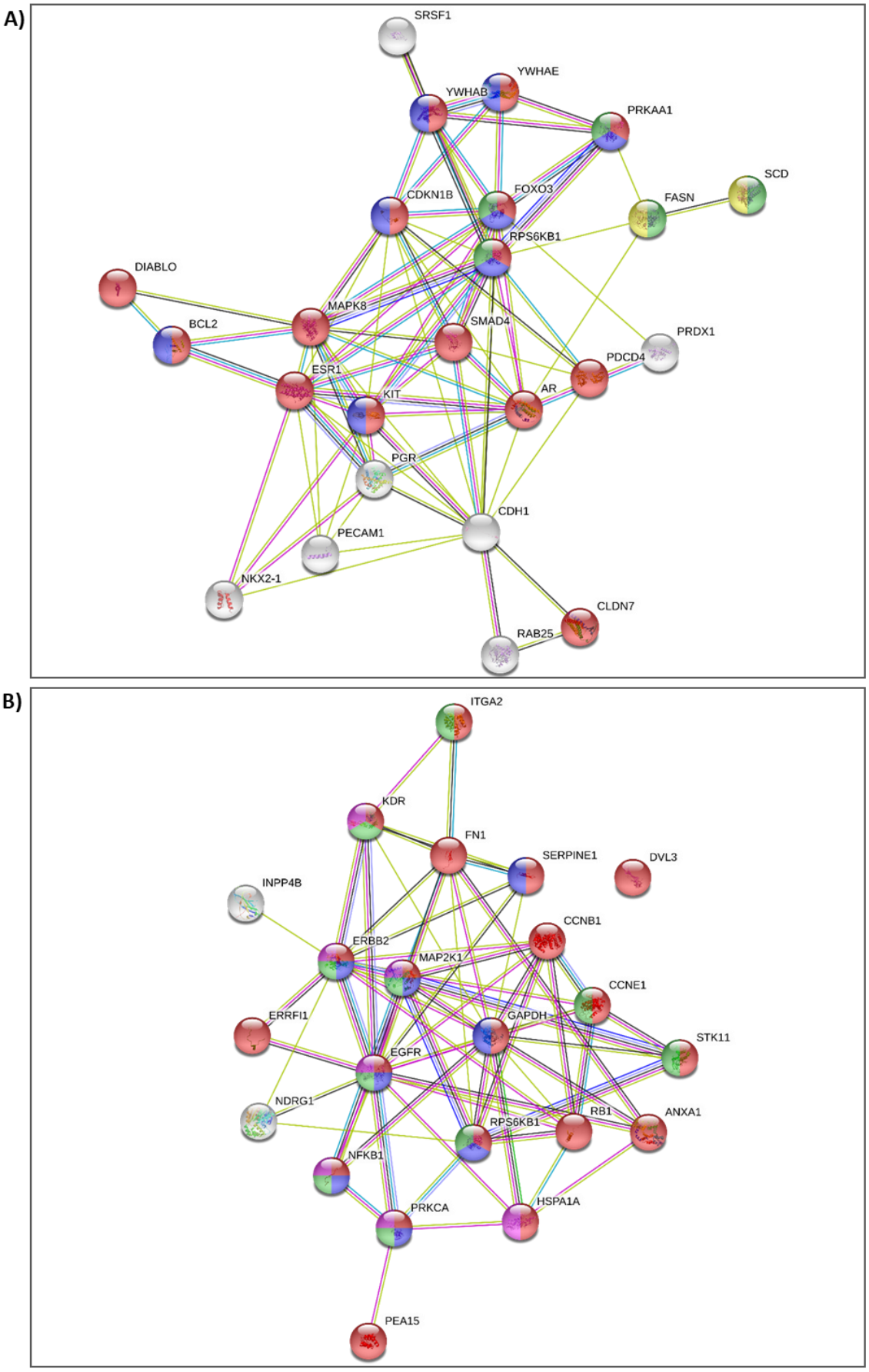

Figure 7: Functional protein association network done in STRING (https://string-db.org/). (A) Top25 proteins (Table 4) positively co-expressed with RKIP at mRNA level in lung AC. Red: regulation of programmed cell death associated genes; Blue:

PI3K/AKT signalling associated genes; Green: AMPK signalling associated genes; Yellow: fatty acid metabolism associated genes; White: others. **(B)** Top25 proteins (Table 4) negatively co-expressed with RKIP at mRNA level in lung AC. Red: regulation of metabolic processes associated genes; Blue: HIF-1α signalling associated genes; Green: PI3K/AKT signalling associated genes; Purple: MAPK signalling associated genes; White: others.

As above, the selected group of enriched genes was run on STRING, for functional protein association analysis. For the Top 25 genes that positively correlated with RKIP expression (Table 4 and Figure 7A), we can observe that, these genes are interconnected and significantly related with different biological processes (see Figure 7 legend), such as regulation of programmed cell death, regulation of signalling transduction and others. Interestingly, some of the enriched genes are associated with fatty acid metabolism and with PI3K/AKT and AMPK pathways that play an important role in the regulation of cancer metabolism[108]. Concerning the Top 25 genes that were enriched when RKIP was downregulated (Table 4 and Figure 7B), we can verify that these genes were once again significantly associated with several biological processes, being the regulation of metabolic processes, the most common function pointed in these set of genes. Some of the pathways enriched in this group were HIF-1α, PI3K/AKT, MAPK signalling, which are also involved in metabolic reprogramming regulation, especially the HIF-1α signalling[110,147].

Taking together, the *in silico* analysis pointed a strong association between the RKIP expression and metabolism associated genes expression in lung adenocarcinoma patients, which support our hypothesis that RKIP may play a role in the modulation of cancer cells metabolism in lung cancer.

4.2 Metabolic characterization of lung cancer cell lines

One of the characteristics of cancer cells is their metabolic plasticity which allows them to adapt to the reduced availability of nutrients and oxygen[103]. In that sense, and in order to study the metabolic behaviour of the *in vitro* models, we started by doing a characterization of the NSCLC cell lines used in this study, A549, H292 and HCC827.

Firstly, we evaluated the effect of the different culture conditions, such as oxygen availability (normoxia and hypoxia) and different glucose concentrations (1g/L, 2.5g/L and 4.5g/L glucose), in the proliferation rates of the cells. As it can be observed in Figure 8A, all the cell lines significantly proliferate more at normoxic conditions. We can also observe that cells decrease their proliferative rate under hypoxic conditions and mainly when incubated with low glucose concentrations (1g/L glucose). When we measured the total glucose present in the culture medium (Figure 8B), we rapidly perceived that in the

case of low glucose medium the cells consume all the glucose available in the first 24/48 hours, leading us to conclude that those cells are completely dependent on the presence of glucose to maintain an exponential proliferative rate, since in medium with high glucose concentrations the optimal cell growth is not affected (Figure 8).

Overall, from this first analysis, A549 cell line present the higher proliferative rates, followed by H292 and finally HCC827 cells, in all culture conditions (Figure 8A). In the opposite, the HCC827 cell line was the faster consuming the culture medium glucose, while H292 did it more slowly, meaning that the culture conditions affect cell proliferation, but their proliferative rates do not determine how they behave metabolically.

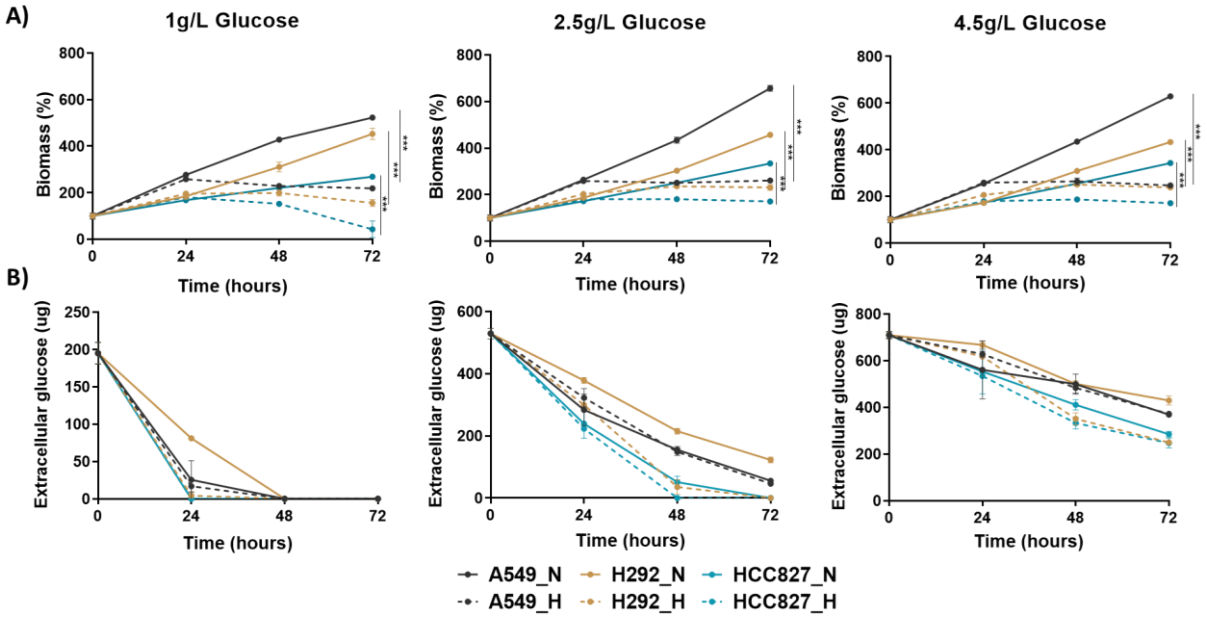

Figure 8: Effect of the culture conditions in the cell proliferation of NSCLC cell lines. Total biomass (SRB assay) **(A)** and total extracellular glucose (ug) **(B)** were measured at 24, 48 and 72 hours in A549, H292 and HCC827 cell lines maintained in normoxia (N) or hypoxia (H), and different glucose medium conditions: 1g/L, 2.5g/L and 4.5g/L glucose. Results are represented as mean ±standard deviation (***p<0.001) (N=3).

One of the metabolic hallmarks of cancer cells is their dependency on aerobic glycolysis, which is characterized by a high uptake of glucose and a consequent high lactate production[98]. Thus, to fully metabolic characterize our cell lines, we next evaluated their glucose consumption and lactate production rates overtime (Figure 9) but excluding the low glucose (1g/L) condition since it affects the proliferation rates. By analysing Figure 9, we can observe that hypoxic conditions significantly promote the glycolytic phenotype, as the cells consume more glucose and produce more lactate than in normoxic conditions, as expected. At normoxic conditions, the cells tend to stabilize the glucose consumption rates upon 24 hours (Figure 9A), while the extracellular lactate tends to decrease (Figure 9B), probably because is being

converted into pyruvate to enter in the OXPHOS at the mitochondria. It is also important to note that cells are more glycolytic under intermediate glucose concentrations (2.5g/L glucose), than when are cultured in high glucose conditions (4.5g/L glucose).

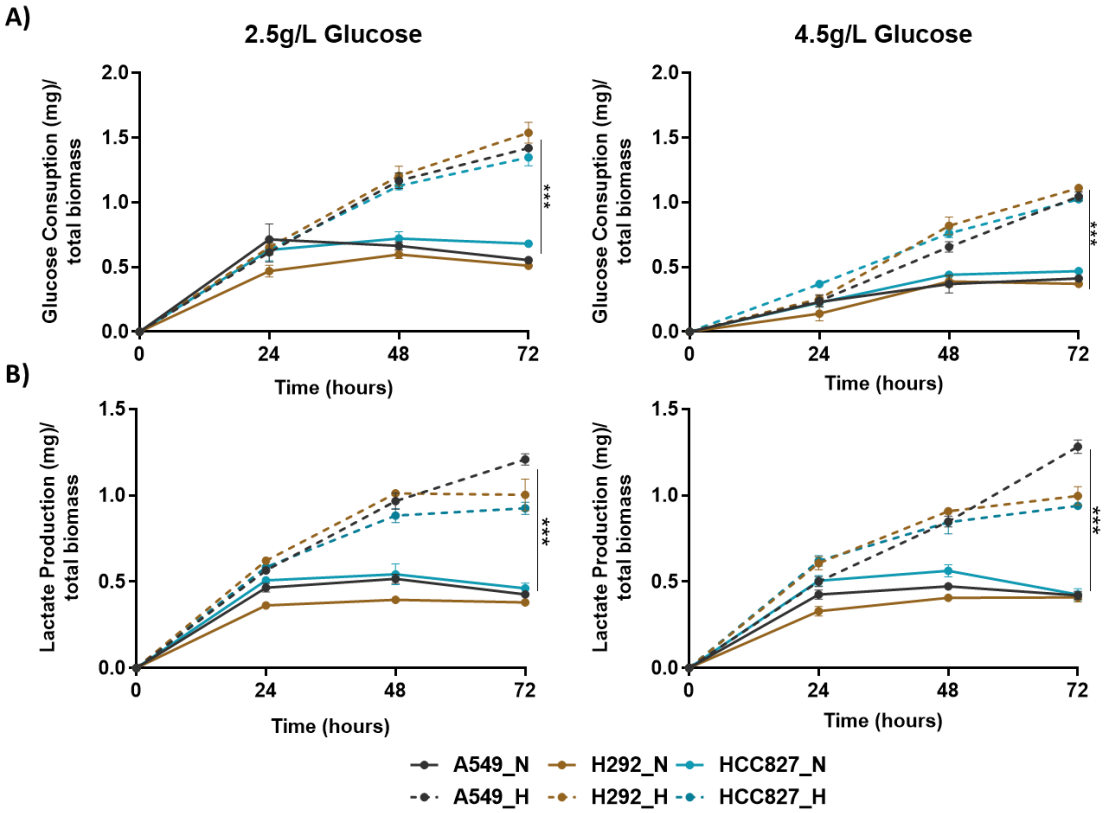

Figure 9: Effect of the culture conditions in the glucose and lactate metabolism of NSCLC cell lines. Glucose consumption **(A)** and lactate production (mg/total biomass) **(B)** was measured at 24, 48 and 72 hours under normoxic (N) or hypoxic (H) conditions, and at different glucose medium conditions (2.5g/L and 4.5g/L glucose). Results were calibrated to the total biomass, assessed by SRB, and are represented as mean ±standard deviation (***p<0.001) (N=3).

Comparing the results from the different cell lines, there are no statistically significant differences between them (Figure 9). However, even after normalizing the results to the total biomass, the tendency observed before (Figure 8B) is somehow maintained: HCC827 present higher rates of glucose consumption and lactate production overtime, mainly at normoxia and under 2.5g/L of glucose conditions, while for H292 cell line is the opposite (Figure 9). Moreover, focusing on the 24 hours we can observe that the glucose consumption levels for HCC827 cells are similar between normoxia and hypoxia conditions (Figure 9A), which indicate that these cells are already dependent of glycolysis in normoxic conditions. Whereas for H292 cells we observe an increase in both glucose consumption and lactate production levels under hypoxic conditions (Figure 9), indicating a possible switch in these cells' metabolism. Overall, it seems to us that the HCC827 cell line is more glycolytic, while H292 cell line

seems to be more dependent of OXPHOS. With that in mind and knowing that the metabolic reprogramming of cancer cells is regulated by the expression of several metabolic markers[103,113], we complete the characterization of the cell lines by assessing the expression levels of several metabolic proteins and RKIP, our molecule of interest, in the above mention culture conditions (Figure 10 and Figure 11).

Upon western blot analysis, it was possible to see that all cell lines express RKIP, although at distinct levels (Figure 10). At normoxic conditions, A549 cells present the highest expression levels, followed by H292 cells, and finally, HCC827 cells that have the lowest levels of RKIP, as our group determined before[193]. Interestingly, RKIP expression varies with the culture conditions: in A549 cell line RKIP expression decrease under hypoxic conditions, while for HCC827 increases, but in both RKIP tend to decrease under high glucose conditions. In contrast, for H292 cell line the RKIP expression slightly increase both at hypoxic conditions and with high concentrations of glucose (Figure 10).

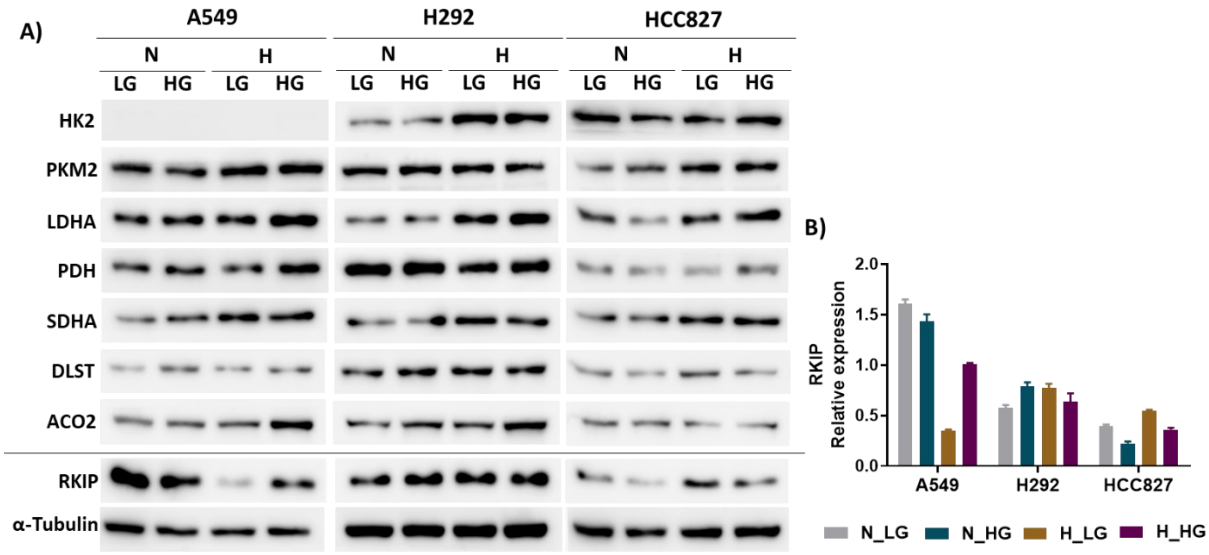

Figure 10: Metabolic proteins and RKIP expression in NSCLC cell lines. (A) Representative western blot for an assay in which cells were maintained under normoxic (N) or hypoxic (H), and low glucose (LG -1g/L) or high glucose (HG – 4.5g/L) conditions for 24 hours. The expression of several metabolic proteins was assessed: HK2, PKM2, LDHA - glycolytic metabolism; PDH and TCA cycle- SDHA, DLST and ACO2. The expression of RKIP was assessed and quantification of WB for RKIP **(B)** was performed using band densitometry analysis with Image J software. Relative protein expression results are shown as the ratio between the proteins and α-Tubulin, and represented as the mean of two independent assays.

Concerning the remain proteins assessed, it is clear that the three cell lines have very distinct metabolic expression profiles (Figure 10A and Figure 11).

For A549 cell line, HK2 expression was not detectable however higher expression levels of others glycolytic markers (PKM2 and LDHA) were observed when compared to some TCA cycle proteins (SDHA,

DLST, and ACO2) (Figure 10A and Figure 11), suggesting that A549 can still rely on glycolysis. Concerning the H292 cell line it was observed a markedly higher expression of PDH, one entry point into the TCA cycle, and other TCA cycle proteins when compared with the remaining cell lines (Figure 10A and Figure 11), indicating a preference for the oxidative metabolism. In contrast, HCC827 cell line present highest levels of HK2 expression, mainly in normoxic conditions, when compared to A549 and H292 cell lines, and also presents the lowest PDH expression levels (Figure 10A and Figure 11), reenforcing our previous observation that of all the cell lines, HCC827 is the less dependent on the oxidative metabolism.

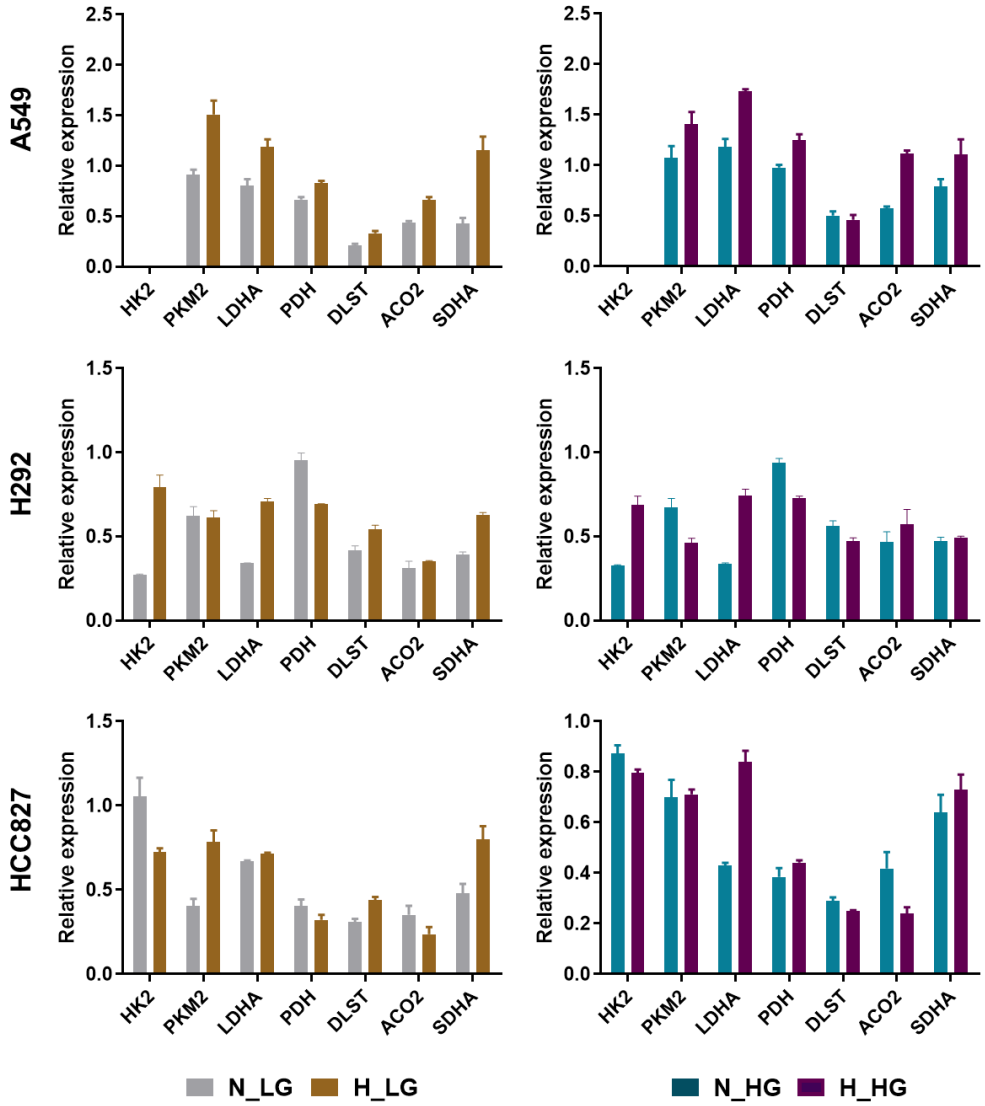

Figure 11: Quantification of the western blot presented in Figure 10, relative to metabolic associated proteins. Cells were maintained under normoxic (N) or hypoxic (H), and low glucose (LG -1g/L) or high glucose (HG – 4.5g/L) conditions for 24 hours. The expression of several metabolic proteins was assessed, and quantification of WB was performed using band densitometry analysis with Image J software. Relative protein expression results are shown as the ratio between the proteins and α-Tubulin, and represented as the mean of two independent assays.

Furthermore, it can be observed that, as expected, the different culture conditions affect the expression of the metabolic markers, being this effect more pronounced in cells that were under hypoxia. An increased expression HK2, PKM2 and LDHA was observed in cells subject to hypoxic conditions (Figure 10A and Figure 11), which is in accordance with the well-described induction of glycolytic enzymes through stabilization of HIF-1α[111,112]. For instance, H292 cell line present increased expression of HK2 and LDHA, and a decreased expression of PDH at hypoxic conditions (Figure 10A and Figure 11), suggesting a metabolic adaptation towards a more glycolytic phenotype in the absence of oxygen. Moreover, the variations on RKIP expression under different metabolic conditions, and even the difference on RKIP expression level between the above mention cell lines, emphasizes our hypothesis that RKIP expression may play a role on cancer cells metabolic reprogramming.

4.3 Effect of RKIP expression in the modulation of cancer cells metabolism

4.3.1 RKIP role on the metabolic behaviour of lung cancer cells

To pursue our hypothesis that RKIP could be a master regulator of lung cancer cells metabolism, we moved for some functional *in vitro* assays. For that, we selected two cell lines with apparently distinct metabolic phenotypes, H292 (oxidative) and HCC827 (glycolytic), and then genetically modulate RKIP expression using two different approaches: gene knockout (KO) and overexpression (OE). Taking into account our first characterizations of the WT cell lines, all the following assays were done using culture medium with 2.5 g/L of glucose, both in normoxia and hypoxia conditions.

By western blot, as it can be observed in Figure 12A, we confirmed that the transfections were successful in both cell lines. Additionally, following the same tendency of the WT experiments (Figure 10A and Figure 11), in HCC827 cell line it was observed an increase on RKIP expression in hypoxic conditions, more evident in overexpressing (OE) cell lines. In contrast, some discrepant results were found for H292 cell line between the two transfection controls, however the differences are not significant (Figure 12A).

Before we proceed to the evaluation of RKIP role on the metabolic behaviour of lung cancer cells, we first aimed to exclude the possibility of RKIP modulation be affecting cellular proliferation and compromising the following results. For that, we determined the proliferation rates of the transfected cells in the above cited culture conditions, but no significant differences were found between the clones in none of the cell lines (Figure 12B). It is important to note that the cells were not affected by the transfection process, and the distinct proliferation rates between the cell lines were even maintained (Figure 8 and 12B).

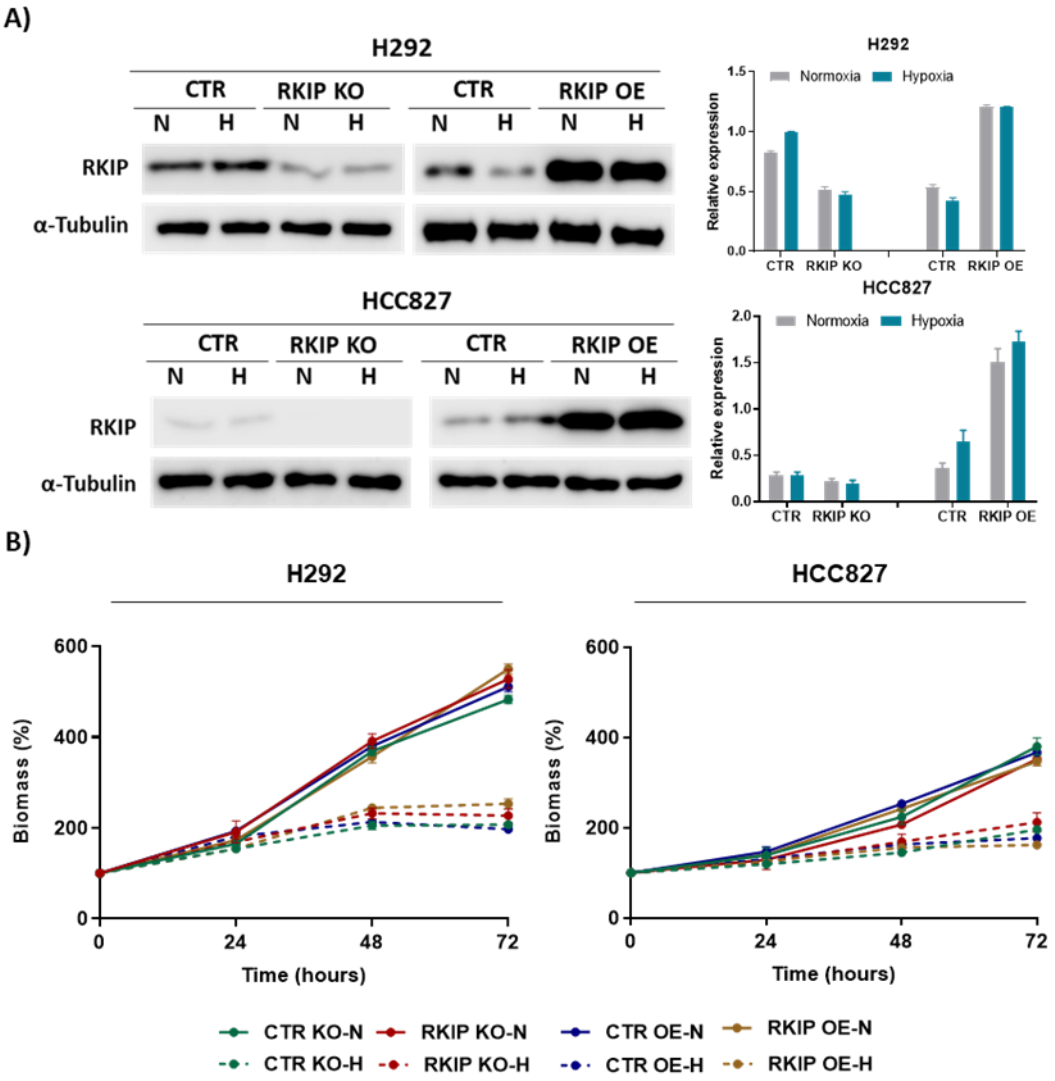

Figure 12: Effect of RKIP expression manipulation on cell proliferation. (A) Representative western blot analysis of RKIP expression for assessment of the transfection's efficiency for H292 (KO and OE) and HCC827 (KO and OE) cell lines. Quantification of WB for RKIP expression was performed using band densitometry analysis with Image J software. Relative protein expression results are shown as the ratio between the proteins and α-Tubulin, and represented as the mean of the 2 independent assays. **(B)** Total biomass was measured at 24, 48 and 72 hours by SRB assay for H292 (KO and OE) and HCC827 (KO and OE) cells maintained in normoxia (N) or hypoxia (H) in 2.5g/L glucose concentration medium. Results are represented as mean ±standard deviation (N=3).

Next, to evaluate the RKIP effect on the glycolytic metabolism of these cells, glucose consumption and lactate production rates were measured over time, under normoxia and hypoxia conditions, for the above mention transfected cell lines (Figure 13). By analysing Figure 13, we observed once again that in hypoxic conditions there is an induction of the glycolysis, which is translated in increased glucose consumption and lactate production, as expected. Additionally, it is possible to notice that in normoxic conditions there is a tendency to stabilization of both glucose consumption and lactate production over

the time, what may indicate that cells can be using alternative pathways to support their growth and proliferation, as observed for the WT cell lines (Figure 9).

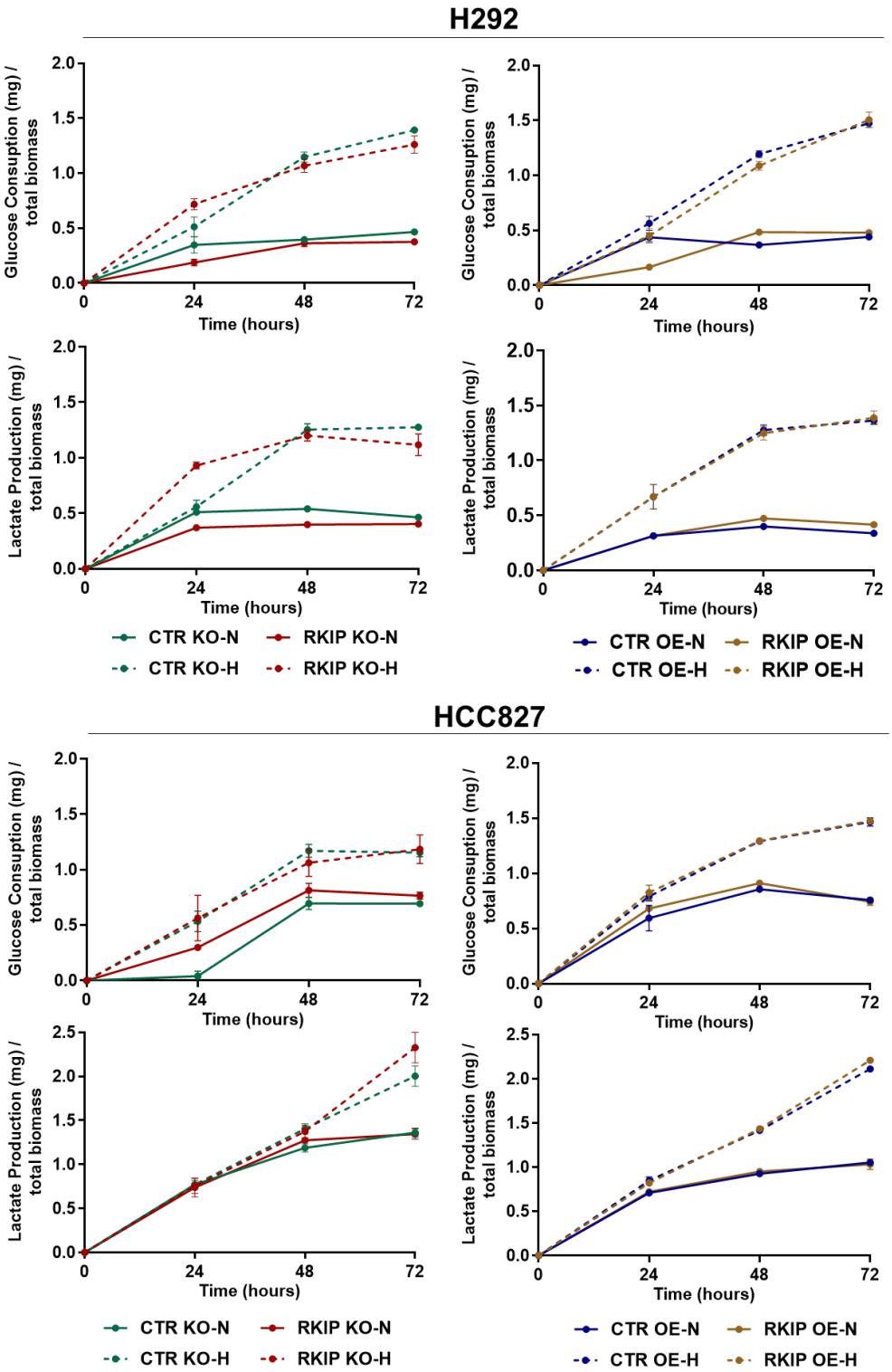

Figure 13: Effect of RKIP on cells glycolytic metabolism. Glucose consumption and lactate production was measured overtime in H292 (KO and OE) and HCC827 (KO and OE) cells maintained in normoxia (N) or hypoxia (H) in 2.5g/L glucose concentration medium. Results were calibrated for the total biomass, measured by SRB assay, and represented as mean ± standard deviation (N=3).

Unfortunately, and in contrast to what we were expecting, comparing the different manipulated cell lines for RKIP expression (KO and OE) with the respective controls, we did not observe significant differences between them overtime (Figure 13). In fact, some differences were observed for shorter time points (24 hours), but along time they tend to recover and equalize the levels of glucose consumption and lactate production.

Since the effect of RKIP expression in the modulation of the glycolytic metabolism is almost null at basal conditions and overtime, we next decided to do the assays only for 24 hours but in the presence of some glycolytic modulators to see if in the presence of a "metabolic stressor" the RKIP manipulated cells respond differentially. Thus, we assessed glucose consumption and lactate production rates upon treatment with 2-deoxyglucose (glycolysis inhibitor) and metformin (OXPHOS inhibitor). By analysing Figure 14 we can perceive that overall, the treatment with 2-DG, an HK2 inhibitor, leads to an inhibition of the glycolytic metabolism, whereas treatment with metformin, an inhibitor of mitochondrial ETC complex I, promotes it, as expected[128,165]. It is interesting to note that the influence of metformin in HCC827 cells has the same effect as hypoxia, while in H292 cells the absence of oxygen has as a stronger effect in glycolysis induction than metformin. Also, 2-DG has a stronger glycolytic inhibitory effect in HCC827 cells than in H292 cells, which can indicate that HCC827 cells rely more on glycolysis, as previously observed.

Concerning RKIP effect, again there are no statistically significant differences between the CTR and the RKIP manipulated cells (Figure 14). However, in general, there is a tendency for RKIP KO cells export more lactate in all conditions for both cell lines, even not being this tendency observed in the glucose consumption rates. Also, it is interesting to note that at least for HCC827 cell line, the opposite effect is observed, with OE cells exporting less lactate than the respective control, for all conditions (Figure 14).

In the future will be important to deepen the study with different approaches, such mitochondrial activity assays, and shorter time points since the cells seems to have a high metabolic plasticity *in vitro* over time.

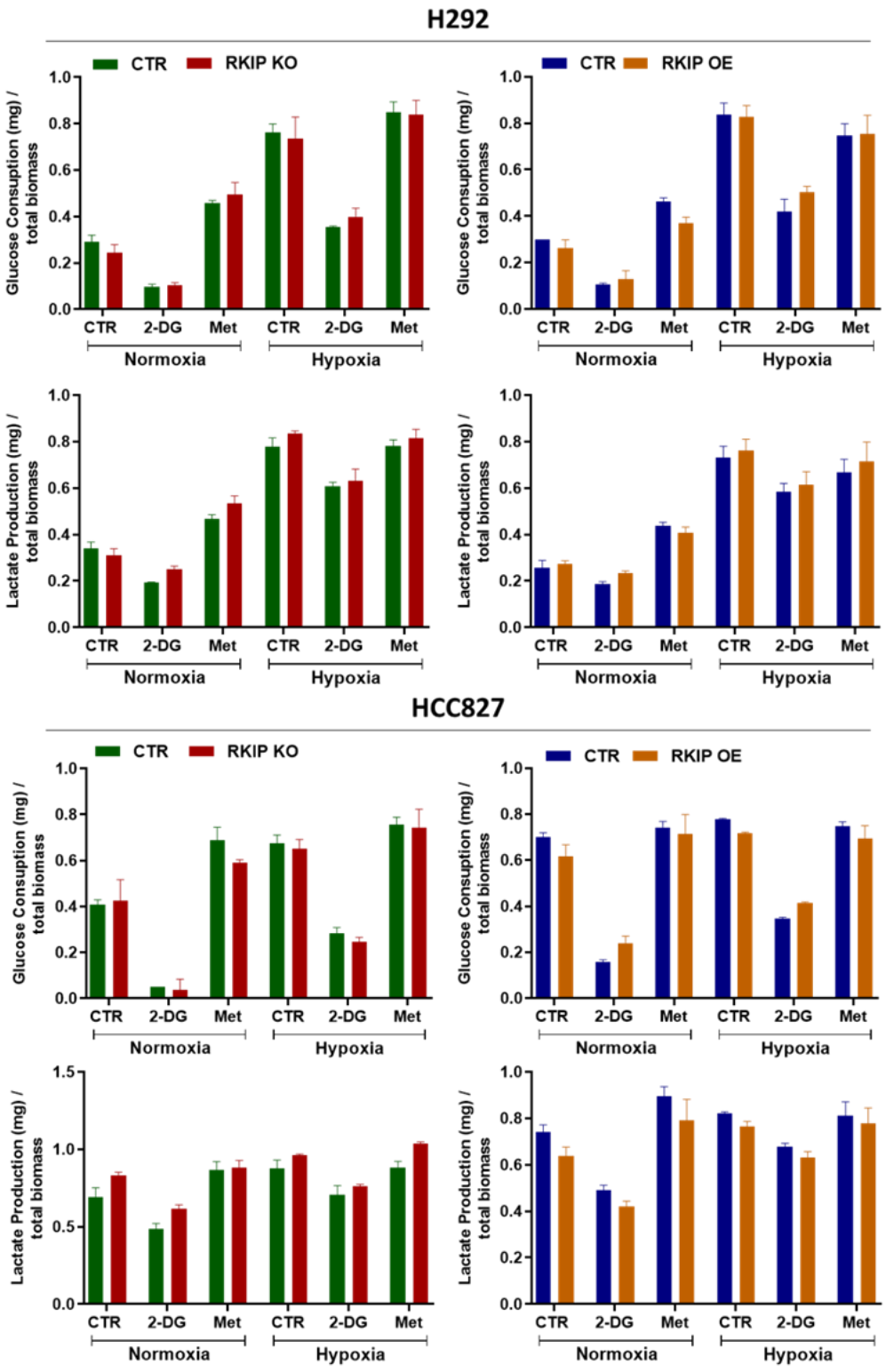

Figure 14: RKIP effect on cells glycolytic metabolism upon treatment with metabolic inhibitors. Glucose consumption and lactate production was measured overtime in H292 (KO and OE) and HCC827 (KO and OE) after treatment with 1.5mM 2-Deoxyglucose (2-DG) or metformin (Met) for 24 hours in 2.5g/L glucose concentration medium. Results are represented as mean ±standard deviation (N=3).

4.3.2 RKIP role in the modulation of metabolic proteins expression

We previously perceived from the WT cell lines characterization that metabolic differences at protein levels do not necessarily translate into differences in metabolic function, at least for the assays used in this study. Also, the *in silico* analysis showed us that patients with RKIP altered expression have alterations in the expression of metabolic-associated genes, at mRNA and protein level. Thus, in the next step we intend to assess the protein expression levels of some metabolic regulators by western blot in this genetically manipulated cell lines for RKIP expression.

In general, as it can be observed in the western blot, concordantly with what was observed for the WT cell lines, there is an upregulation of HK2 and LDHA, and downregulation of PDH upon hypoxic conditions (Figure 15 and Figure 16).

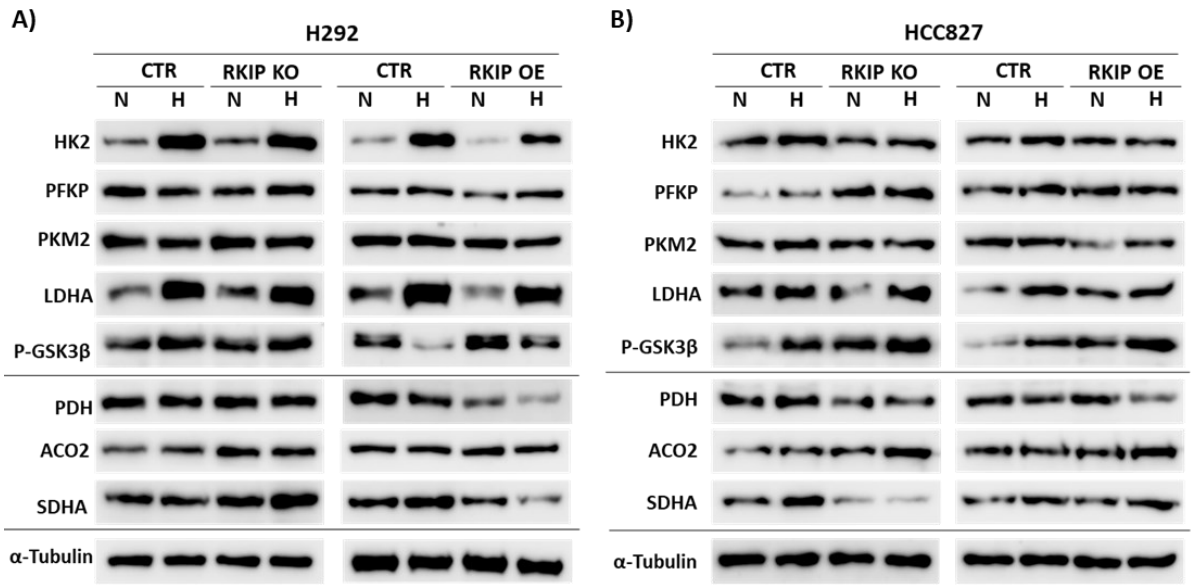

Figure 15: Effect of RKIP on the modulation of metabolic proteins expression. Representative western blot analysis for H292 **(A)** and HCC827 **(B)** cell lines under normoxia (N) and hypoxia (H), upon RKIP genetic manipulation. The expression of several metabolic proteins was assessed: HK2, PFKP, PKM2, LDHA, and p-GSK3β - glycolytic metabolism; PDH and TCA cycle- ACO2 and SDHA. Tubulin was used as loading control (N=2).

Regarding the effect of RKIP expression in metabolic-related proteins, we observed differences in some of these proteins' expression upon RKIP gain or loss of expression, for both cell lines (Figure 15 and Figure 16).

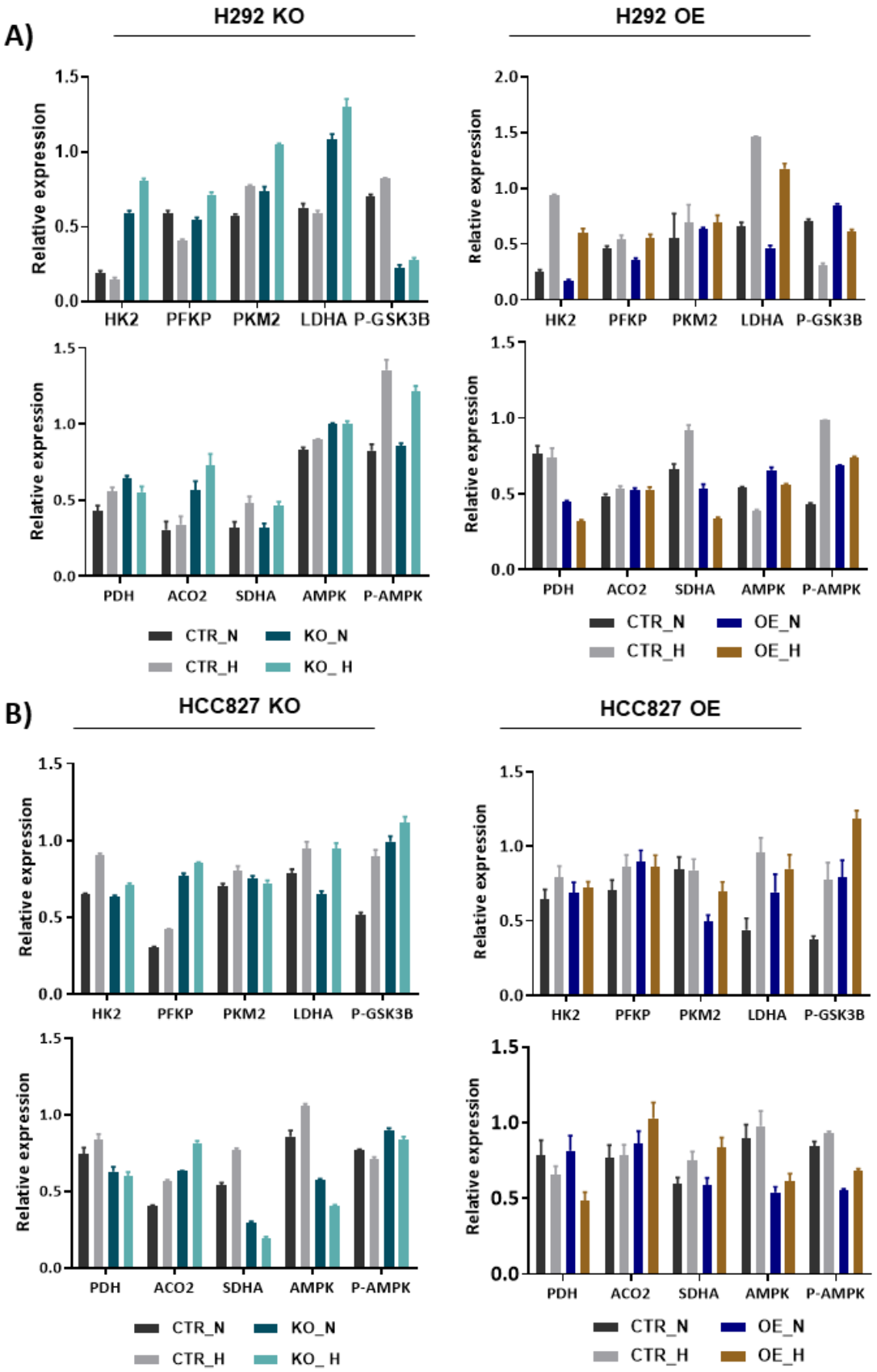

Figure 16: Quantification of the western blot presented in Figure 15. Western blot analysis for H292 **(A)** and HCC827 **(B)** cell lines under normoxia (N) and hypoxia (H), upon RKIP genetic manipulation. The expression of several metabolic proteins was assessed. The western blots quantification was performed using band densitometry analysis with Image J software. Relative

protein expression results are shown as the ratio between the proteins and α-Tubulin, and represented as the mean of the 2 independent assays.

For instance, in H292 cell line a decrease of some glycolytic proteins (HK2 and LDHA) and OXPHOS-related proteins (PDH and SDHA) in RKIP OE cells can be observed in both conditions (normoxia and hypoxia) (Figure 15A and Figure 16A), which suggests an inhibition of the glycolytic and also oxidative metabolism in these cells (Figure 19). Interestingly, we can observe an opposite tendency for RKIP KO cells, as they seem to present a slight increase on some glycolytic markers (HK2, PKM2 and LDHA) and increase expression of the TCA cycle enzyme ACO2. Moreover, it seems that RKIP KO and OE cells present opposite tendencies for p-GSK3β expression. In contrary to what was expected, given the role of RKIP as a positive regulator of GSK3β signalling, we can observe a reduction of p-GSK3β (inactive form) expression in RKIP OE cells and a slight increase on RKIP KO cells (Figure 15A and Figure 16A).

Concerning HCC827 cell line, RKIP KO seems to lead to the upregulation of PFKP and downregulation of PDH and SDHA, whereas in RKIP OE cells a slight downregulation of PDH in hypoxia and PKM2 is observed. Unexpectedly, an increase in p-GSK3β expression was observed in both RKIP KO and OE. Moreover, we also observe a slight increase in ACO2 expression, which goes against the downregulation of others TCA cycle enzymes, PDH and SDHA, upon RKIP KO (Figure 15B and Figure 16B). This can indicate that a knockout of RKIP expression in a cell line that has very low levels of RKIP, as is the case of HCC827, may not be efficient as an overexpression approach. Comparing the two cell lines although the differences are clearer in H292 OE than in HCC827 OE, they present the same tendency, inhibition of the glycolytic metabolism (Figure 19). However, HCC827 KO also presents the same tendencies that H292 OE for some proteins (p-GSK3β, PDH and SDHA) (Figure 15 and Figure 16), which once again raises doubts about RKIP KO in HCC827 cell line.

Furthermore, by immunofluorescence analysis (Figure 17 and Figure 18), we evaluated the expression patterns and cellular localization of other important glycolytic markers that we were unable to study by western blot (GLUT1, MCT1, MCT4, and CD147). It can be observed that both GLUT1 and MCTs, as well as their respective chaperone (CD147), are present in the plasma membrane as well in the cytoplasm (Figure 17 and Figure 18).

Concerning the effect of RKIP on the expression of these proteins, we can observe that RKIP OE affected the expression of all the proteins in the H292 cell line (Figure 17). For instance, both MCT1 and MCT4 have shown a slight decrease of expression in RKIP OE cells, being these clearer in hypoxic conditions (Figure 17). As for their chaperone, the immunofluorescence analysis showed an evident decrease of CD147 plasma membrane expression in both conditions upon RKIP overexpression (Figure

17). Moreover, GLUT1 also seems to be downregulated in RKIP OE cells when in hypoxic conditions (Figure 17). These results are in agreement with the downregulation of HK2 and LDHA observed in the western blot analysis emphasizing the theory that RKIP OE can lead to an inhibition of the glycolytic metabolism in H292 cell line (Figure 19).

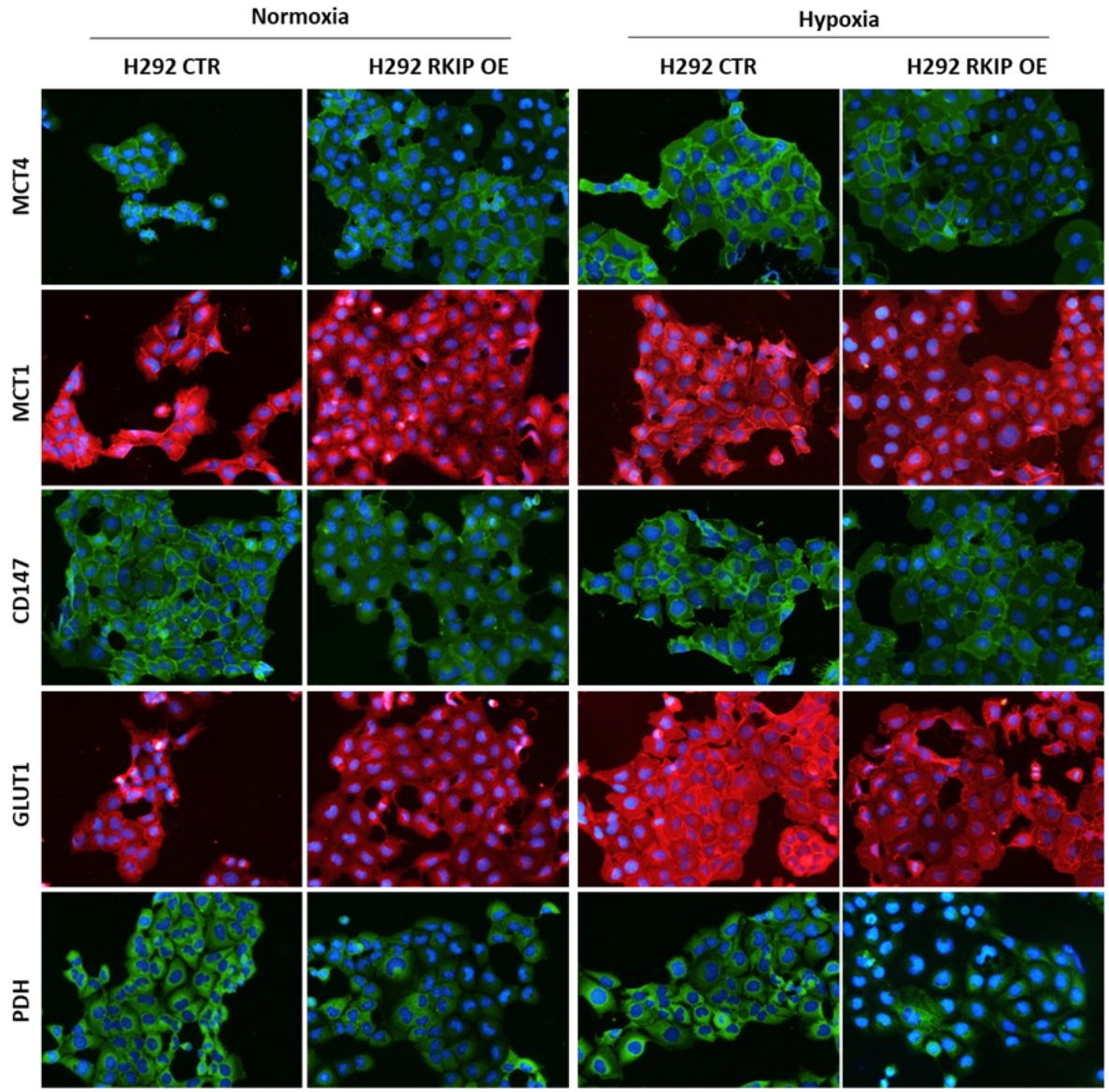

Figure 17: Immunofluorescence analysis of metabolic markers expression in H292 cell line with RKIP OE. Assessment of MCT1, MCT4, CD147, GLUT1 and PDH expression and cellular localization in H292 RKIP OE cells under normoxia or hypoxia for 24 hours (N=1). Immunofluorescence images were taken at 200x magnification (DAPI (blue) – nuclei; Green- MCT4, CD147 and PDH; Red – MCT1 and GLUT1).

Regarding the HCC827 cell line, in contrast to the H292 cells, no clear differences were observed in the expression of these glycolytic markers (Figure 18). Only a discrete increase of MCT1 expression upon RKIP OE is noted under normoxic conditions, which goes against the downregulation of MCTs expression in H292 RKIP OE cells (Figure 18).

Additionally, since RKIP have been shown to modulate PDH expression levels in all the transfections, especially in the RKIP OE of H292 cells, we attempt to validate these results. Accordantly with the western blot results (Figure 15A and Figure 16A), there is a striking reduction of PDH expression upon RKIP OE in H292 cell line (Figure 17). In contrast, even though we observed a downregulation of PDH in hypoxic conditions upon RKIP OE in the western blot analysis (Figure 15B and Figure 16B), the differences of PDH expression for HCC827 turn to be not so clear in the immunofluorescence (Figure 18).

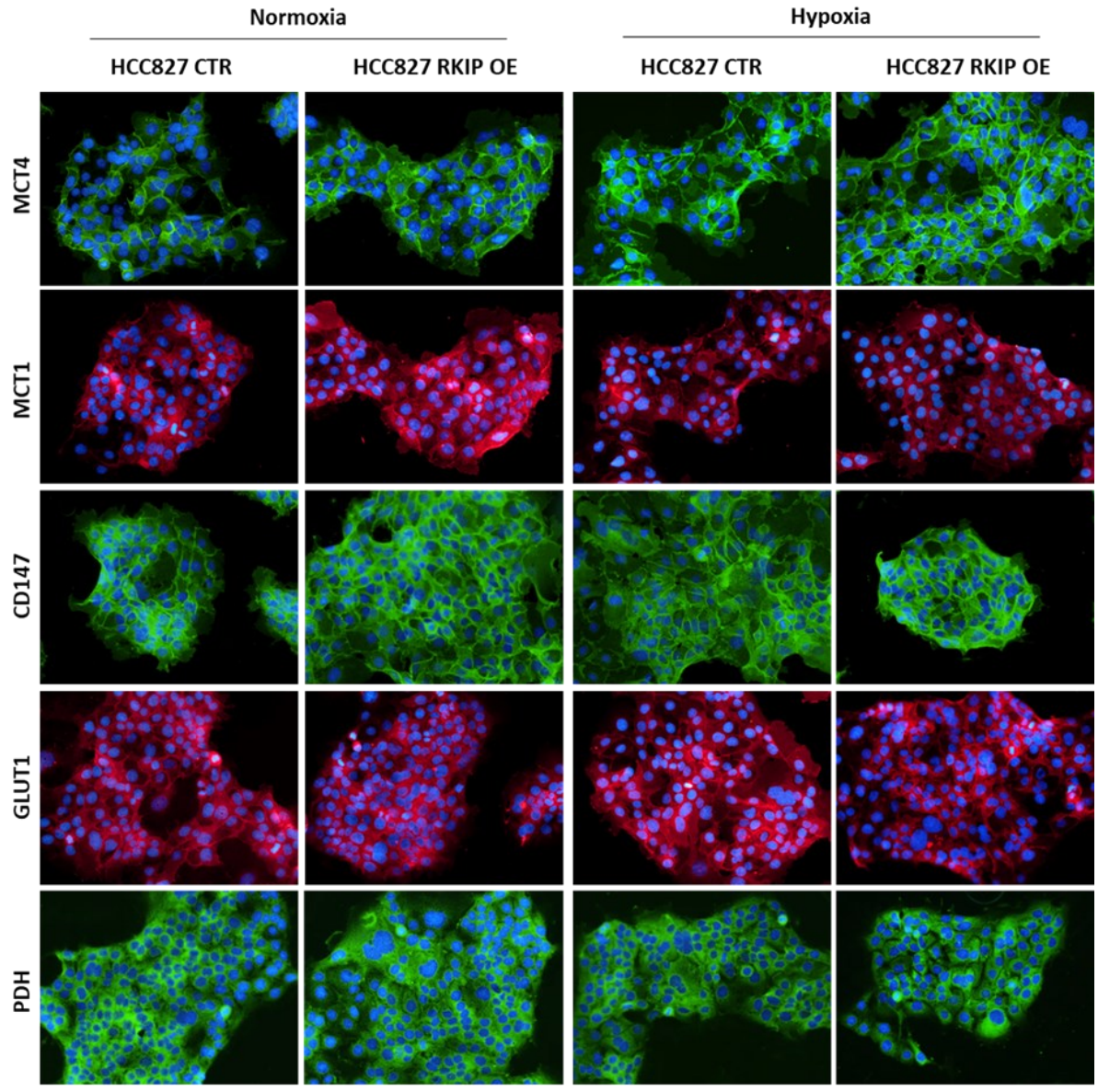

Figure 18: Immunofluorescence analysis of metabolic markers expression in HCC827 cell line with RKIP OE. Assessment of MCT1, MCT4, CD147, GLUT1 and PDH expression and cellular localization in HCC827 RKIP OE cells under normoxia or hypoxia for 24 hours (N=1). Immunofluorescence images were taken at 200x magnification (DAPI (blue) – nuclei; Green- MCT4, CD147 and PDH; Red – MCT1 and GLUT1).

To conclude, the genetic modulation of RKIP expression resulted in distinct metabolic proteins alterations in the two selected cell lines, being the RKIP OE in H292 cell line the transfection with more promising and consistent results (Figure 19). Therefore, is necessary to corroborate these results by studying how the RKIP-associated changes can influence the metabolic behaviour of lung cancer cells.

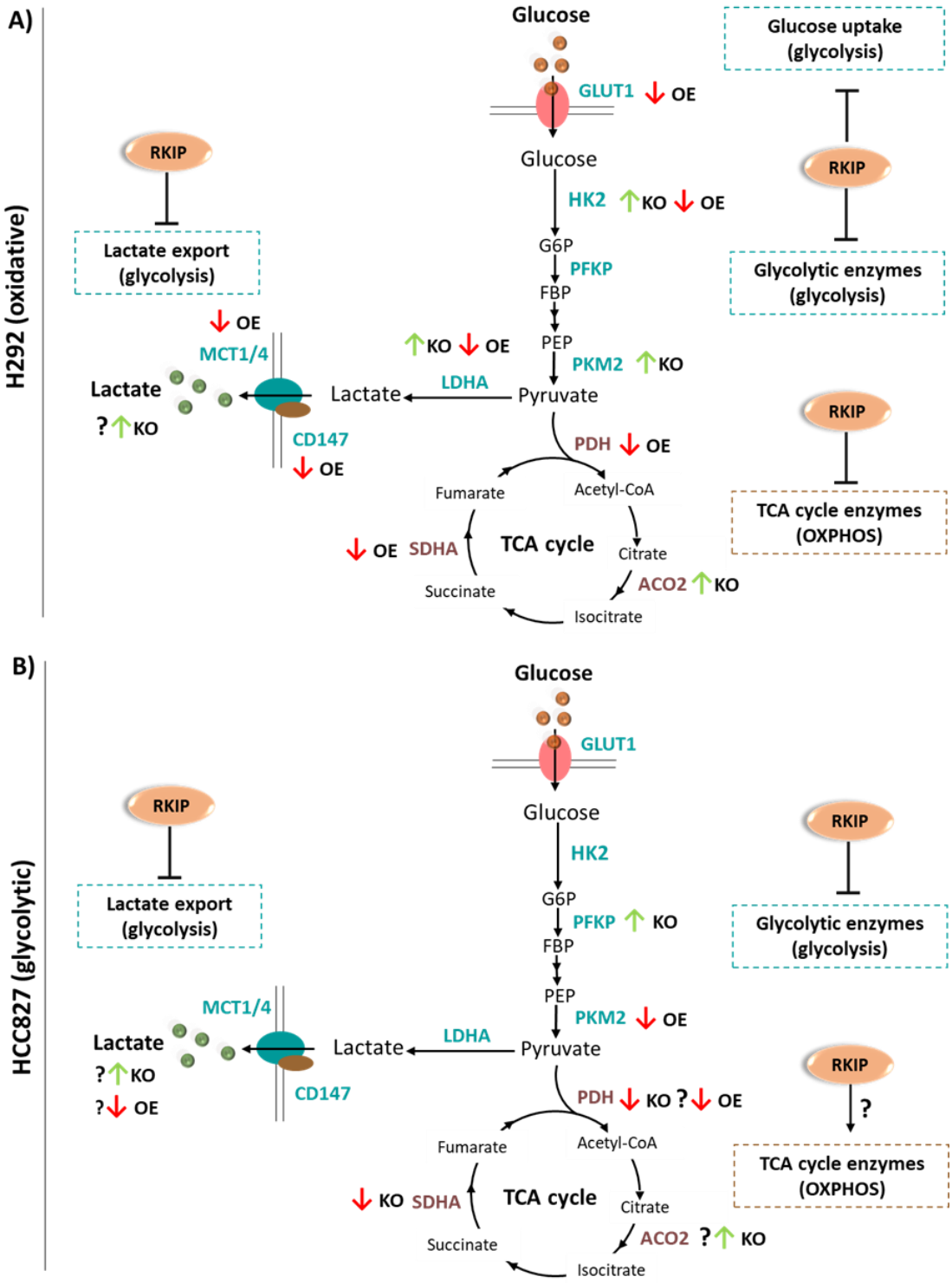

Figure 19: Schematic representation of the main results obtained throughout the thesis in what concerns the comparisons between lung cancer cells metabolism with or without RKIP expression. (A) For H292 cell line (oxidative), we observed a

tendency in increased lactate production upon RKIP KO and a decrease expression of proteins involved in lactate export (MCT1/4, CD147) upon RKIP OE, which indicates that RKIP might be blocking lactate export. Also, we observe that RKIP KO lead to upregulation of some glycolytic enzymes, while RKIP OE lead to a downregulation of glycolytic enzymes and GLUT1, which can that RKIP inhibits glucose uptake and glycolysis. Moreover, we observe a decreased expression on some TCA cycle related proteins upon RKIP OE cells and the opposite for RKIP KO cells. Together these results indicate that RKIP can possibly lead to inhibition of both glycolytic and oxidative metabolism in this cell line. **(B)** For HCC827 cell line (glycolytic) we observed a tendency in increased lactate production upon RKIP KO, and the opposite upon RKIP OE, which once again suggests that RKIP can be blocking lactate export. Although less clear differences in enzymes expression were observe upon RKIP genetic manipulation for these cell line, we still observe some differences, that indicate that RKIP can be blocking glycolysis and possible enhancing OXPHOS on these cells. KO: knockout; OE: overexpression; "?": tendency.

4.4 *In silico* validation of the results

Finally, in order to better understand the main results achieved in this thesis, we decided to go back to the TCGA database, now with specific questions.

In this study, we found evidence that RKIP expression can be modulated by the oxygen/nutrient culture conditions. HIF-1α is a master regulator of cell response and adaptation to hypoxia, which regulates the expression of numerous genes, leading to alterations in cancer cell metabolism in order to enable tumour proliferation, migration and survival[112]. Herein, to explore whether RKIP expression modulate cells adaptation to hypoxia, we studied the correlation between RKIP and HIF-1α expression in lung adenocarcinoma patients and found that they are statistical significantly inversely correlated at mRNA level (Figure 20A). Additionally, we also observed an enrichment of HIF-1α mRNA expression in patients with RKIP downregulation (Figure 20B), which is in line with what was observed on the *in silico* analysis previously performed (Figure 7B).

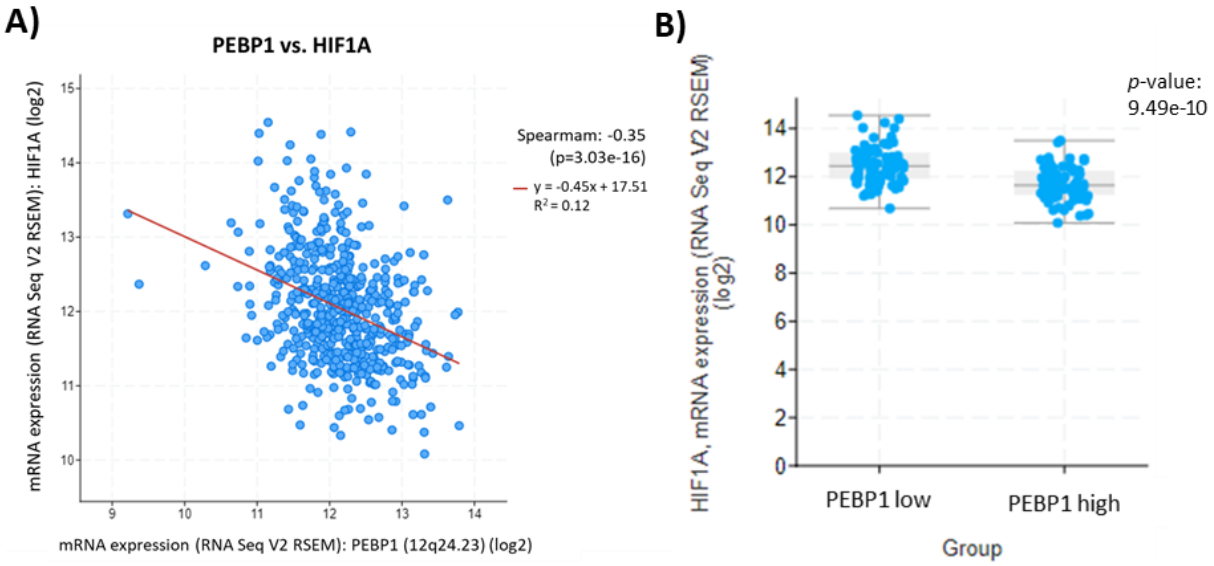

Figure 20: *In silico* correlation between RKIP and HIF-1α in adenocarcinoma patients. (A) RNA Seq V2 data for AC patients (584 patients), showing the RKIP (*PEBP1*) and HIF-1α (*HIF1A*) mRNA expression is inversely correlated. **(B)** Data from enrichment analysis in the same AC patients, showing HIF-1α is upregulated in the set of samples with RKIP mRNA downregulation. All data belongs to the Lung Adenocarcinoma (TCGA, Firehose Legacy) provisional database and is available at www.cbioportal.org.

Moreover, RKIP showed to modulate the expression of several metabolic proteins (Figure 15, Figure 16 and Figure 17). Therefore, to validate the *in vitro* results, we explored the specific correlation between the metabolic proteins addressed in this study with RKIP mRNA expression in lung adenocarcinoma patients. Analysing Table 5, we can perceive that RKIP is inversely correlated with glycolysis associated genes, which is in accordance with the downregulation of some glycolytic proteins and consequent inhibition of glycolysis observed in RKIP OE in H292 cell line (Figure 19). Regarding TCA cycle associated genes, we observed a positive correlation of these genes with RKIP mRNA expression. However, at the protein level *in vitro*, RKIP showed to downregulate some of these proteins such as PDH and SDHA (Figure 15 and Figure 16). Additionally, to better understand the role of RKIP in the mitochondrial metabolism, namely in OXPHOS, we also determine the RKIP correlation with a set of electron transport chain (ETC) associated genes and found a significantly positive correlation with all these genes (Table 5).

Table 5: *In silico* correlation between RKIP and metabolism associated proteins in lung adenocarcinoma patients. Spearman's correlations of mRNA data (RNA Seq V2 RSEM) for AC patients (584 patients), showing that RKIP is inversely correlated with glycolysis associated genes, and positive correlated with TCA cycle and ETC associated genes. All data belongs to the Lung Adenocarcinoma (TCGA, Firehose Legacy) provisional database and is available at www.cbioportal.org.

Protein	Cytoband	Spearman's Correlation	*p*-value	Associated metabolic process
GLUT1	1p34.2	-0.424	5.57e-24	**Glycolysis**
HK2	2p12	-0.329	1.58e-14	**Glycolysis**
PFKP	10p15.2	-0.265	9.89e-10	**Glycolysis**
PKM	15q23	-0.173	7.729e-5	**Glycolysis**
LDHA	11p15.1	-0.203	3.413e-6	**Glycolysis**
MCT1	1p13.2	-0.277	1.46e-10	**Glycolysis**
MCT4	17q25.3	-0.452	2.17e-27	**Glycolysis**
PDHA1	Xp22.12	0.332	9.13e-15	**TCA cycle**
PDHB	3p14.3	0.355	7.91e-17	**TCA cycle**
IDH2	15q26.1	0.264	1.01e-9	**TCA cycle**
ACO2	22q13.2	0.0945	0.0317	**TCA cycle**
SDHA	5p15.33	0.0692	0.116	**TCA cycle**
ATP5MC2	12q13.13	0.397	5.73e-21	**ETC**
UQCRC1	3p21.31	0.232	9.23e-8	**ETC**
UQCR10	22q12.2	0.248	1.09e-8	**ETC**
ATP5MG	11q23.3	0.294	9.43e-12	**ETC**
NDUFS3	11p11.2	0.315	2.41e-13	**ETC**
COX17	3q13.33	0.256	3.50e-9	**ETC**
ATP5PF	21q21.3	0.238	4.05e-8	**ETC**
NDUFB4	3q13.33	0.170	1.003e-4	**ETC**
NDUFB6	9p21.1	0.122	5.290e-3	**ETC**
COX7C	5q14.3	0.379	4.47e-19	**ETC**
ATP5MC1	17q21.32	0.273	2.82e-10	**ETC**
ATP6V0B	1p34.1	0.223	3.07e-7	**ETC**
COX7A2	6q14.1	0.258	2.78e-9	**ETC**
NDUFA8	9q33.2	0.224	2.56e-7	**ETC**

Altogether, this *in silico* analysis indicate us that RKIP is inversely correlated with the glycolytic metabolism in lung adenocarcinoma patients, validating some of our results. Along with that, we observed a positive correlation between RKIP and TCA cycle or ETC associated genes, which once again suggests that RKIP can play a role in distinct metabolic processes.

To conclude, these results provide us a strong evidence for a role of RKIP in lung cancer metabolism and will be very useful in the future to deepen our work

CHAPTER 5:

General Discussion

5. General discussion

Altered glucose metabolism is a widespread characteristic of several solid tumours and is often associated with tumour aggressiveness, metastasis, poor prognosis as well as therapy resistance[103,131,157,194,195]. While most cancers undergo metabolic rewiring, mounting evidence suggests that tumour metabolism signatures are context dependent, being influenced by a wide range of factors including, tissue of origin, tumour grade, microenvironment and oncogenic signalling[137,153,154,196]. As example, given that lung cancers exhibit a high mutation burden[13,21,22], it is possible that their genetic heterogeneity is also reflected at the metabolic level, particularly since oncogene activation and loss of tumour suppressors can alter cellular metabolism[109,154,197]. Lung cancer cells often harbour mutations in genes and pathways, such as the PI3K/AKT pathway, the oncogene *MYC*, and the tumour suppressor genes *TP53* and *STK11*[13,176,179]. Specifically, in NSCLC tumours, known to be driven by oncogenic activation of tyrosine kinases, such as mutations in *KRAS* and *EGFR* genes, that were already described as modulators of metabolic rewiring in tumour cells[154,179,180,197,198]. These cell signalling pathways are implicated in cell metabolism by securely regulating the capacity of cells to obtain access to nutrients and subsequently process these compounds[179]. Moreover, oncogenic signalling supports the cells ability to maintain a growth factor–independent glycolysis and survival through the expression of oncogenic kinases[95,107,108]. Overall, the literature provides strong evidence of metabolic rewiring in lung cancer.

Thus, nowadays, one of the emerging themes of cancer research rely on the acquired heterogeneous metabolic phenotype by tumours cells to withstand the complex challenges linked to cancer progression[137,176], being puzzling to search for new oncogenic alterations behind this metabolic heterogeneity. RKIP protein arises to us as a potential modulator of cancer cells metabolism. Given the key role of RKIP in the regulation of several intracellular signalling pathways that have been described to be crucial to cancer cells metabolic rewiring, more than MAPK, and its involvement in many malignancies[52], including NSCLC[62], in this study we hypothesized that RKIP could be a key player in the metabolic reprogramming of tumours cells. Supporting our hypothesis, Rosner and Lee recently demonstrated the importance of the pro-metastatic transcription factor BACH1, that is negatively regulated by RKIP, in promoting aerobic glycolysis in breast cancer[188,189]. In addition, Kapoor *et al.* recently reported for the first time a link between RKIP downregulation and genes that directly regulate cell metabolism, namely fatty acid degradation and pyruvate metabolism, by analysing TCGA data for ccRCC patients[187].

Keeping this in mind, our first approach was to perform an extensive *in silico* analysis to determine the RKIP-associated signature in lung adenocarcinoma patients, using the TCGA database. It was very interesting to verify that at the mRNA level, RKIP expression showed to be positively correlated with mitochondrial genes implicated in pyruvate and energy metabolism, electron transport chain and oxidative phosphorylation, and inversely correlated with genes related to glucose metabolism, indicating a potential negative association between RKIP and Warburg effect. At the protein level we found that RKIP expression is significantly correlated with genes involved in cancer signalling transduction and related with different biological processes, which is in accordance with the well-described role of RKIP as an important modulator of signalling molecules in cancer[34,35,62]. Importantly, RKIP showed to be correlated with players of MAPK signalling, which was expected considering RKIP role as an endogenous inhibitor of MAPK, as well as PI3K/AKT, AMPK and HIF1-α signalling, all described to play a crucial role in tumours metabolic reprogramming[110,112,123,147,199,200]. It is important to notice that, despite both enrichment analyses showed an association between RKIP and metabolic processes, no common genes were pointed. Probably because the protein data in TCGA datasets is still limited when compared to mRNA data, being protein expression data for RKIP and several metabolic proteins not available, or just because they are not included in the Top25 most related proteins.

Interestingly, as cited above[188,189], Lee *et al.* have reported also using TCGA database, that BACH1 gene expression is inversely correlated with ETC gene expression and OXPHOS mainly in patients with breast cancer, but also in other tumour types, including lung cancer[188]. In that study, the results were biologically validated at protein level, suggesting that mitochondrial metabolism can be exploited by targeting BACH1 to sensitize breast cancer cells to mitochondrial inhibitors[188]. In the same year, and following Lee *et al.* work[188], other group reported the importance of BACH1 in driving lung tumours metastasis, suggesting that Heme oxygenase 1 (HO-1) inhibitors represent an effective therapeutic strategy to prevent lung cancer metastasis[201]. Altogether, given that BACH1 is a downstream target of RKIP, as well as a negative regulator of RKIP expression[68,76,189], we are speculating a positive association between RKIP and ETC genes expression or OXPHOS induction. However, an association between BACH1 function and RKIP was never explored in lung malignancies. Moreover, besides all the above speculation, the direct and functional role of RKIP protein on cancer cells metabolism is completely unknown, being ours, the first study exploiting its role in the field.

Thus, we moved to a more practical approach, starting by doing a metabolic characterization of the NSCLC cell line models to be used in the study. Given the fact that both nutrient and oxygen conditions, as well as the genomic context, play a crucial role in determining cell metabolic

behaviour[103,176,196], three cell lines with different genetic backgrounds were selected and characterized in different culture conditions. First, we determined the effects of different glucose concentrations and oxygen conditions in the proliferation rates of the cells and observed that cells under stress conditions (hypoxia and low glucose concentration: 1g/L glucose) significantly proliferate less. This can be explained by a possible increased dependency of these cells on glucose, since it is described that cancer cells under hypoxia enhanced glycolysis to sustain the bioenergetic and biosynthetic requirements associated to cancer cells high-proliferative rates[112]. Also, the cells proliferative rates do not determine their metabolic behaviour, which is consistent with a previous study in NSCLC cell lines[176]. Finally, we observed that hypoxic conditions significantly promote glycolysis, which was translated in increased rates of glucose consumption and lactate production/export, as expected based on the well-documented induction of glycolysis in hypoxia[111,112]. Whereas, for normoxic conditions, a stabilization in glucose consumption and a tendency to a decrease in extracellular lactate content overtime was observed in some cases. We believe that this might be due to the metabolic flexibility of cancer cells that allows them to adapt to changes in the nutrient availability, therefore as the levels of extracellular glucose drop and extracellular lactate increases, cells can use lactate as fuel for the TCA cycle and the oxidative phosphorylation. Indeed, it has been described for different *in vitro* models, including A549 cell line, that exposure to lactic acid can reverse cancer cells phenotype, causing a shift from Warburg effect to OXPHOS[202,203]. Supporting this, some *in vivo* studies reported that lactate is metabolized in human lung tumours, and more importantly that glucose contribution to the TCA cycle occurs mostly indirectly, through circulating lactate[174,175].

A recent study demonstrated that NSCLC cells are remarkably diverse in the rates of glucose uptake and utilization, as well as in the pathways by which the glucose is metabolized[176]. Herein, we found that HCC827 cells presented the most glycolytic phenotype, followed by A549, and lastly by H292, a result that was further confirmed by the differences on the metabolic proteins expression profile by western blot. We noticed that H292 was the one that presents higher levels of TCA cycle enzymes, in particularly PDH, the enzyme responsible for the flux of glucose to TCA cycle[140,141], whereas HCC827 present the lowest PDH expression of all the cell lines, suggesting that HCC827 cell line is more glycolytic and H292 more oxidative. In addition, it was possible to observe that the different conditions affect the expression of several metabolic markers as expected, being the effects more pronounced in cells that were subject to hypoxia, which was accordance with the well-described effect of HIF-1α stabilization in the induction of several metabolic genes[111,112,142,147]. Looking to what is described in the literature, some studies have reported that A549 cells rely mainly on OXPHOS rather than in glycolysis in normoxia[128,204]. Moreover, Chen *et al.* recently demonstrated that HCC827 cells present a higher ratio of lactate secretion/glucose consumption

than A549, meaning that HCC827 cell line has a preference for aerobic glycolysis[176], supporting our believe that HCC827 cells are the most glycolytic. There are no studies on H292 cell line, hampering comparisons.

Furthermore, it was very interesting to observe that the different glucose and oxygen culture conditions affected RKIP expression levels. Several studies have focused on the mechanisms by which hypoxia and activation of HIF-1-dependent signalling promotes metastasis through regulation of metabolic reprogramming, invasion, angiogenesis, immune suppression, epithelial-mesenchymal plasticity and resistance to apoptosis[111,112,147,150]. Given the role of RKIP as a metastasis suppressor, the possibility of RKIP also playing a role in the modulation of the cellular plasticity response to tumour hypoxic stress is not surprising. It was already demonstrated, in H1299 NSCLC cell line, that hypoxia-induced Notch1 activation was significantly inhibited by RKIP overexpression and stimulated by RKIP knockdown, supporting the notion that hypoxia-induced EMT can be functionally linked to RKIP expression, which subsequently modulates Notch signalling during metastasis[49]. Additionally, a recent computational study of protein-protein docking between HIF-1α and RKIP, suggested that RKIP can act as a negative regulator of HIF-1α, and in fact, cobalt chloride treatment (mimics hypoxic conditions) resulted in RKIP dissociation from HIF-1α in prostate cancer cell lines[205]. Thus, despite the inconsistent results we found between our *in vitro* models, which deserve further exploitation, the variations on RKIP expression under different metabolic conditions suggest that RKIP expression alterations could be a possible mechanism behind cells metabolic reprogramming.

Following, to pursue the main question of this work, which is to functionally determine the role of RKIP on the modulation of cancer cells metabolism, we selected the two lung cancer cell lines with distinct metabolic and molecular phenotypes, HCC827 (glycolytic – *EGFR* mutant) and H292 (oxidative – WT for *EGFR*), and then genetically modulate RKIP expression to stable knockout (KO) and overexpress (OE) its gene.

Firstly, and as a transfection control, we determined whether RKIP expression manipulation affects the proliferation of lung cancer cell lines. For both the cell lines, no significant differences in the proliferation rates were observed, which lead us to conclude that neither RKIP expression nor the transfection process affected these cell lines proliferation capacity, and thus will not be a confounding indicator in the further studies. However, as a well-described tumour suppressor, RKIP acts as a multifunctional protein in carcinogenesis regulating several processes such as cellular growth, proliferation, migration, invasion and metastization[36,43,52,63], thus we were expecting to see proliferation differences after RKIP KO or OE. Concerning NSCLC, no clear exploration of RKIP's role in such processes

was done yet, being only demonstrated that NSCLC metastasis are inversely correlated with RKIP expression levels[46]. However, previous unpublished data from our group, also demonstrated no significant differences in cellular viability and migration rates between cells with and without RKIP in H292 cell line, although changes were seen in the expression of EMT proteins, which confirmed the presence of a RKIP phenotype[193]. The biological role of RKIP in lung cancer is yet being exploited in our group, but there were other tumour types for which an absence of RKIP impact in cells proliferation was found *in vitro* [58].

Moving on to the dissection of RKIP role in metabolism modulation, we started by comparing the overtime glucose consumption and lactate export rates in the RKIP manipulated cell lines (H292 and HCC827), both in normoxia and hypoxia conditions. Overall, the metabolic behaviour of the two cell lines followed the same tendency observed with parental cells, meaning that they were not altered with transfection. When comparing the RKIP KO/OE cells with the respective controls, in both cell lines, no statistically significant differences were observed overtime. However, for shorter time points (24 hours) some differences were observed, but along time they tend to recover and equalize the levels of glucose consumption and lactate production.

To circumvent this, we next did the same analysis but only for the shorter time point (24 hours), and upon treatment with metabolic inhibitors, to see whether RKIP manipulated cells would respond differently under stress conditions. From this analysis we observed that 2-deoxyglucose, a glycolysis inhibitor[158], lead to a significant decrease of the glycolytic rates, while treatment with metformin, a OXPHOS inhibitor, result in glycolysis induction, as expected[206,207]. Moreover, it was also evident that the two cell lines respond differently to these inhibitors. Treatment with metformin, lead to a significant induction of glycolysis on H292 cells, suggesting that was able to inhibit the oxidative metabolism and cause a shift to glycolytic metabolism on these cells. For HCC827 cells, the metformin effect was not so evident, which can be explained by a supposed lack of oxidative metabolism dependence on these cells. Consistently, the opposite was observed after 2-deoxyglucose treatment, where glycolysis inhibition was more evident for HCC827 cells, indicating a higher dependency of these cells on glycolysis. Together these results corroborate the tendency observed in the metabolic characterization of the cells before transfection, where H292 cells seemed to rely more in the oxidative metabolism, whereas HCC827 is more dependent on glycolytic metabolism.

Concerning the hypothetic role of RKIP expression on metabolism modulation, once again no statistically significant differences between the CTR and the RKIP manipulated cells were found, even when subjected to metabolic modulators. However, it was observed a tendency for RKIP KO cells export more lactate in both conditions and cell lines, which is in accordance with our hypothesis of glycolytic

phenotype induction upon RKIP loss. Interestingly, the opposite tendency was observed for RKIP overexpression in HCC827 cells. This tendency was not followed by the glucose consumption rates, to whom no differences were observed. Upon these results a few limitations warrant mention, such as the conditions where these assays were performed, and the limited sensitivity of the assays we used. Here we have to take in consideration that we are measuring extracellular levels of metabolites that can be constantly interconverted and used to fuel other metabolic pathways which can be a possible explanation for lactate levels not following the same tendency of glucose levels. For instance, besides the use of lactate to fuel TCA cycle, lactate has also been shown to serve as a gluconeogenic source for glucose generation in lung cancer cells grown with low glucose *in vitro* [208]. Moreover, the presence of other nutrients in the culture medium can also influence the results. For instance, many reports point towards the essentiality of glutamine for NSCLC lines *in vitro* [151,186,209], thus we cannot discard the possibility of glutamine being interfering with our results.

Intriguingly, that lack of significant metabolic differences, in the RKIP manipulated cell lines, was contradictory with the differences found on metabolic proteins expression, evaluated by western blot. For H292 cell line, some interesting things were spotted. Upon RKIP overexpression, we observed a decreased expression of some glycolytic markers, such as HK2 and LDHA, and also by immunofluorescence analysis a slight decreased expression of MCT1, MCT4, CD147, and GLUT1 was observed. This was consistent with the negative correlation that was found between RKIP and the genes that encode MCT4 and GLUT1 in the *in silico* analysis. In accordance, an increase in some glycolytic markers' expression, HK2, PKM2 and LDHA was observed upon RKIP loss. Although at the first sight these results seem to be in accordance with our initial hypothesis of RKIP negative regulation of Warburg effect, when we look to TCA cycle enzymes expression, we also observed a decrease in some of these proteins expression in RKIP overexpressing cells. Indeed, the reduction on PDH expression, the enzyme that control one of the TCA entries points, was very evident in both western blot and immunofluorescence analysis. These results suggest that RKIP overexpression leads to inhibition of both glycolysis and OXPHOS in this cell line. Although being the Warburg effect the most well-described phenotype of cancer cells, most tumours acquire the ability to undergo enhanced aerobic glycolysis while maintaining the mitochondrial metabolism, as has been reported for lung tumours[137,151].

Concerning HCC827 cell line, the results were not so clear, yet some differences were spotted. We observed an upregulation of the glycolytic enzyme PFKP and a downregulation of the TCA cycle enzymes, PDH and SDHA, which can indicate on one hand, an enhancement of glycolysis and on the other hand a decrease in OXPHOS, therefore a Warburg-like phenotype upon RKIP loss, as we initially hypothesized.

Looking at RKIP OE results, the differences were less pronounced and seem to be dependent on oxygen conditions, which can be due to the fact that in this cell line RKIP expression showed to be affect by hypoxia. We were still able to see a downregulation of PKM2 enzyme for both conditions, indicating a reduction in glycolysis, which is consistent with what was observed in H292 RKIP OE. Curiously, a slight downregulation of PDH in hypoxic conditions was also observed in RKIP OE cells, thus presenting the same tendency that RKIP KO cells. Moreover, in contrast to H292 cell line, we did not find significant differences in glycolytic markers by immunofluorescence analysis upon RKIP overexpression.

Regarding the existent literature for some of these metabolic proteins expression in lung cancer, it has been described that NSCLC tumours exhibited enhanced PDH activity when compared to benign lung samples[137,151] and that PDH activity is associated with EMT and drug resistance in A549 and HCC827 cells[210]. In addition, analysis of tumour samples revealed that low expression of PDK4, one enzyme that inhibits PDH activity, as a predictor of poor prognosis in lung cancer[210]. Curiously, looking at Kapoor *et al.* results they also reported that PDK2 downregulation was associated to RKIP loss in ccRCC[187]. Therefore, the downregulation of PDH could be associated to an upregulation of PDK upon RKIP overexpression.

Finally, faced with some unexpected results, we further performed an *in silico* analysis to understand and validate some of our results, using the TCGA database. Firstly, we confirmed that RKIP expression is inversely correlated with HIF-1α expression, being the later enriched in patients with RKIP downregulated, which is in line with the first *in silico* analysis that showed us an enrichment on HIF signalling associated genes upon RKIP downregulation. Unfortunately, we were not able to detect HIF-1α expression, due to the actual lack of a good antibody in the Lab, herein in the future it would be interesting to study the effect of RKIP expression on HIF-1α expression, and vice-versa, in the models used in this study. Additionally, we found that RKIP mRNA expression was inversely correlated with glycolysis-related genes and positively correlated OXPHOS-related genes in a significant way, which is not consistent with the negative correlation we found *in vitro* between RKIP and some TCA cycle proteins, mainly in H292 cell line.

At this point, it is important to note that the differences and ambiguities found between RKIP's effect in the two cell lines, and apparent lower impact of RKIP in HCC827 cell line metabolic modulation, could be somehow explained due to its metabolic heterogeneity and plasticity, that naturally occurs inside the tumours[137], but which is even more pronounced and less controlled in "artificial" *in vitro* models with immortalized cell lines. Despite the limitations of that kind of studies, the differences could be also somehow due to their different genetic backgrounds. HCC827 is *EGFR* mutated, and it was already demonstrated that *EGFR* mutations can lead to metabolic rewiring towards aerobic glycolysis, due to the

constitutive activation of EGFR-related downstream pathways, namely PI3K/AKT[181]. Also, we observed in the *in silico* analysis that RKIP downregulation is significantly associated with EGFR overactivation, a finding that we already pursue in our group, in which was reported that EGFR can influence RKIP expression, and vice-versa, through "negative feedback" regulation in lung cancer cell lines[193]. Therefore, HCC827 cell line has low levels of RKIP expression, which means higher levels of EGFR activation and exacerbated aerobic glycolysis, which may be masking RKIP activity on these cells metabolic reprogramming, even after RKIP OE, due to constitutive activation of compensatory downstream pathways. In the future it will be important to dissect whether EGFR interferes or is a mediator of RKIP modulation of cancer metabolism.

Overall, even with a few papers supporting our initial hypothesis, even our supporting *in silico* data and actual knowledge that RKIP direct or indirectly interferes with metabolic proteins expression, being ours the first functional study, the role of RKIP in cancer cells metabolic rewiring was unpredictable. Despite this, from our initial hypothesis, we were expecting to find a more significant impact of RKIP in the modulation of lung cancer cells metabolism, due to its well described biological function and role of as signalling modulator.

In that sense, in the present work we also assessed p-GSK3β expression levels, since it has been described that RKIP stabilizes GSK3β expression by preventing its phosphorylation[50], and this kinase regulates negatively the synthesis of glycogen from glucose, a mechanism of survival implemented by cancer cells[51,211]. It was described that, depletion of RKIP inactivates GSK3β by allowing its phosphorylation at the inhibitory T390 residue in colon cancer[50]. In contrary to what was expected, we observed a reduction and an increase of its inactive form (p-GSK3β) expression in RKIP KO and OE H292 cells, respectively. The same tendency was observed upon RKIP OE in HCC827 cells, but not for HCC827 KO cells where RKIP KO induces p-GSK3β expression. Upon these, it is important to mention that the antibody used form p-GSK3β is specific for the phosphorylation at Ser9 residue and not T390 residue, what can possibly explain the unexpected results, herein in the future it would be important to validate our results using antibodies specific for other phosphorylated forms, such as the one in T390 residue. In what concerns the metabolic expectations, given that GSK3β inhibits glycogen synthesis from glucose[51], RKIP KO should increase glycogen synthesis by phosphorylating GSK3β. Since in H292 cells, we found the opposite it could be expectable that H292 KO cells will synthetize less glycogen, and the OE cells the other way around. Curiously, looking back to the tendencies observed for lactate export, there is the possibility of glucose be accumulated in form of glycogen. For instance, in HCC827 cell line a tendency for a reduction of lactate export upon RKIP OE was observed, which could be possibly caused by a

deviation of glucose to glycogen synthesis. However, this is only a conjecture, and despite the discrepancies in the literature in what concerns glycogen synthesis effect in cancer cell metabolism, it would be interesting in the future to study whether RKIP can in fact modulate glycogen synthesis.

Remarkably, mounting evidence suggests that RKIP can be somehow mediating oxidative stress response in cancer cells[50,84,212]. The first association between RKIP and oxidative stress was established by Al-Mulla *et al.* that reported an increase in both general and mitochondrial reactive oxygen species (ROS) levels upon RKIP downregulation, and the opposite upon RKIP overexpression[50]. In complement to their findings that RKIP somehow reduces ROS levels Al-Mulla *et al.* sought to investigate the link between RKIP and Nrf2, a master regulator of the antioxidant response, and its repressor protein KEAP1[84]. Notably, they proved that the loss of RKIP in HEK-499 cells was associated to Nrf2 addiction and resistance to high physiological levels of hydrogen peroxide and cisplatin via upregulation of Nrf2 effector genes, suggesting that RKIP could be regulating oxidative stress response via Nfr2 regulation[84]. Given that mitochondrial ETC is one of the main sources of cellular ROS[139,213], and our *in silico* results showed a positive correlation between RKIP and ETC related genes, is possible that RKIP can regulate ROS production directly by interacting with ETC components, which will be interesting to dissect in the near future.

Furthermore, RKIP is a regulator of signalling cascades[62], that in turn have been somehow associated to regulation of tumour cell metabolism, such as the ones above mention, namely, EGFR and Notch1 related signalling. Notably, in the recent years growing evidence suggest that STAT3, that has been described to be negatively regulated by RKIP in NSCLC[46], may also play a pivotal role in metabolism regulation[214]. For instance, it was reported that STAT3 regulates the mitochondrial metabolism, when located at the mitochondria, supporting Ras-dependent malignant transformation, by sustaining an high glycolytic and ETC activity in cancer cells[215]. Thus, in the future, it would be important to evaluate if RKIP expression affects ETC components activity and expression, and also investigate whether RKIP modulation of lung cancer cell metabolism is dependent on STAT3 signalling. Moreover, Snail, other target of RKIP in lung cancer[92], have been described as a regulator of glucose flux toward PPP through repression of the glycolytic enzyme PFKP, allowing in this way cancer cell survival under metabolic stress[216]. Herein, it would be interesting to evaluate if RKIP can also modulate EMT in lung cancer by blocking the biosynthetic pathways, such as PPP, through Snail.

To conclude, the existent literature in what concerns to RKIP effect in lung cancer strongly supports a potential role of RKIP as a modulator of lung cancer metabolism, as we hypothesized, being our study the first providing functional insights in the matter and giving us important preliminary data to deepen the study from now on.

CHAPTER 6:

Conclusions and Future perspectives

6. Conclusion and future perspectives

In this work, the RKIP's role in lung cancer metabolism was evaluated for the first time. Through *in silico* analysis we found that RKIP-associated molecular signature in lung adenocarcinoma patients was significantly correlated with alterations in metabolism related genes and signalling pathways involved in tumour cell metabolic reprogramming, both at mRNA and protein level. Furthermore, RKIP was inversely correlated with HIF-1α and glycolysis related genes, and positively correlated with OXPHOS related genes in adenocarcinoma patients. Finally, although just some tendencies were observed in what concerns the RKIP role in glucose and lactate levels modulation, we found alterations in the pattern of metabolic proteins' expression upon RKIP genetic modulation *in vitro*. Overall, and although there were dissimilarities among the cell lines used, the results indicated that RKIP could be inhibiting the glycolytic metabolism in NSCLC cell lines.

Concluding, the results provide a pioneer evidence for a role of RKIP in lung cancer cell metabolism modulation. Taking in consideration those preliminary results, and the available literature, it were already identified some candidate RKIP-modulated proteins and pathways that could be behind RKIP role as a metabolic modulator in lung cancer. This is one of the most important research line to be following studied and unravel, since the identification of the crucial pathways that regulate this "new" RKIP function, if confirmed, may in the future be used as therapeutic targets to revert the aggressive phenotype mediated by RIKP in lung cancer. To corroborate and validate our findings, and mainly to determine through which metabolic process and signalling pathway RKIP modulates cancer metabolism, in the future we intend:

- Deepen the *in vitro* study, in order to corroborate and validate our findings, and determine exactly how the RKIP-associated changes influence the metabolic behaviour of tumours cells. For that, it will be important to test different culture conditions and shorten time points, and also assess the metabolism of other nutrients, such as glutamine, as well as study RKIP effect in other metabolic pathways, such as biosynthetic pathways (e.g., glycogen, nucleotide and fatty acid synthesis).

- Resort to more sensitive and complementary assays, such glucose uptake assessment by flow cytometry (2-NBDG probe), ROS production and mitochondrial activity assays, seahorse analysis, metabolomics profiling and metabolite tracing analysis.

- Extend the study to other cell lines with different genetic backgrounds and also from other tumour models.

- Validate the findings in human samples through the expression assessment of the found altered metabolic proteins and further correlation with RKIP levels and moving to *in vivo* assays.

CHAPTER 7:

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7. References

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APPENDIX I:

Supplementary data

Supplementary Tables

Table S1: Top50 genes positively and negatively co-expressed (at mRNA level) with RKIP mRNA in lung adenocarcinoma. TCGA provisional data (www.cbioportal.org).

RKIP upregulation				RKIP downregulation			
Gene	Cytoband	p-value	q-value	Gene	Cytoband	p-value	q-value
ERP29	12q24.13	3.36e-20	2.24e-16	AVL9	7p14.3	4.86e-22	4.87e-18
MMAB	12q24.11	6.23e-20	2.93e-16	OSBPL3	7p15.3	7.31e-20	2.93e-16
ISCU	12q23.3	1.39e-19	4.64e-16	GNA12	7p22.3-p22.2	8.01e-19	2.00e-15
COQ8A	1q42.13	2.65e-19	7.57e-16	CD109	6q13	2.67e-18	5.95e-15
AMBP	9q32	3.78e-18	7.57e-15	ANLN	7p14.2	6.33e-18	9.74e-15
LDHD	16q23.1	4.54e-18	8.19e-15	GREB1L	18q11.1-q11.2	1.30e-17	1.64e-14
TMED6	16q22.1	4.91e-18	8.19e-15	SLC16A3	17q25.3	1.31e-17	1.64e-14
ADI1	2p25.3	1.21e-17	1.64e-14	C1GALT1	7p22.1-p21.3	2.11e-17	2.34e-14
PRKAB1	12q24.23	2.17e-17	2.34e-14	IL1RAP	3q28	2.29e-17	2.34e-14
SNX22	15q22.31	2.56e-17	2.34e-14	FAM220A	7p22.1	2.45e-17	2.34e-14
SLC48A1	12q13.11	2.58e-17	2.34e-14	ELK3	12q23.1	3.74e-17	3.12e-14
FAM104B	Xp11.21	2.82e-17	2.45e-14	ITGAV	2q32.1	6.10e-17	4.88e-14
KLF15	3q21.3	8.59e-17	6.61e-14	ADGRF4	6p12.3	1.08e-16	7.75e-14
RANGRF	17p13.1	1.06e-16	7.75e-14	COLGALT1	19p13.11	1.27e-16	8.47e-14
ACAT1	11q22.3	1.15e-16	7.94e-14	PRDM8	4q21.21	1.63e-16	1.02e-13
DACT2	6q27	1.41e-16	9.07e-14	SPATS2L	2q33.1	1.87e-16	1.10e-13
AIFM1	Xq26.1	1.83e-16	1.10e-13	TTYH3	7p22.3	2.47e-16	1.42e-13
SMDT1	22q13.2	3.18e-16	1.77e-13	MMD	17q22	3.44e-16	1.86e-13
DRAIC	15q23	7.91e-16	4.06e-13	LAMC2	1q25.3	6.00e-16	3.16e-13
GSTA1	6p12.2	9.33e-16	4.56e-13	BCL10	1p22.3	9.03e-16	4.52e-13
MARC1	1q41	1.88e-15	8.19e-13	TPBG	6q14.1	1.01e-15	4.83e-13
NR0B2	1p36.11	2.47e-15	1.03e-12	GPRIN1	5q35.2	1.19e-15	5.54e-13
CDK2AP2	11q13.2	2.82e-15	1.14e-12	CERS6	2q24.3	1.27e-15	5.78e-13
BORCS7	10q24.32	2.84e-15	1.14e-12	BICD1	12p11.21	1.86e-15	8.19e-13
AQP7	9p13.3	3.90e-15	1.53e-12	ERO1A	14q22.1	2.47e-15	1.03e-12
UNC13B	9p13.3	6.40e-15	2.17e-12	PTPRH	19q13.42	3.98e-15	1.53e-12
COX6A1	12q24.31|12q24.2	6.40e-15	2.17e-12	STEAP1	7q21.13	4.28e-15	1.62e-12
COX14	12q13.12	7.14e-15	2.36e-12	GPR87	3q25.1	4.41e-15	1.64e-12
C15ORF61	15q23	1.56e-14	4.88e-12	PPP1R18	6p21.33	4.59e-15	1.67e-12
MTERF2	12q23.3	1.66e-14	5.03e-12	ZYX	7q34	5.23e-15	1.87e-12
POP5	12q24.31	1.79e-14	5.27e-12	CALU	7q32.1	6.39e-15	2.17e-12
GFRA3	5q31.2	1.79e-14	5.27e-12	TGFBI	5q31.1	7.20e-15	2.36e-12
FRAT1	10q24.1	2.28e-14	6.42e-12	STEAP1B	7p15.3	7.50e-15	2.42e-12
COX4I1	16q24.1	2.61e-14	7.16e-12	RASAL2	1q25.2	9.05e-15	2.87e-12
PLA2G4F	15q15.1	4.00e-14	1.08e-11	SPHK1	17q25.1	1.65e-14	5.03e-12
SIRT4	12q24.23-q24.31	5.84e-14	1.52e-11	NCS1	9q34.11	2.00e-14	5.79e-12
SLC25A38	3p22.1	6.44e-14	1.65e-11	NABP1	2q32.3	2.10e-14	6.00e-12
PCBD1	10q22.1	7.87e-14	1.97e-11	PLAUR	19q13.31	2.43e-14	6.76e-12
FBXO25	8p23.3	8.61e-14	2.10e-11	SLC2A1	1p34.2	4.42e-14	1.18e-11
TMEM116	12q24.12-q24.13	1.14e-13	2.71e-11	FKBP14	7p14.3	5.22e-14	1.37e-11
FCSK	16q22.1	1.33e-13	3.13e-11	YWHAG	7q11.23	6.62e-14	1.68e-11
FMC1	7q34	1.67e-13	3.71e-11	ANKIB1	7q21.2	8.08e-14	2.00e-11
ALDH2	12q24.12	1.85e-13	4.07e-11	TWISTNB	7p21.1	8.98e-14	2.17e-11
MRPS36	5q13.2	2.01e-13	4.32e-11	SEPTIN7	7p14.2	1.36e-13	3.17e-11
FRAT2	10q24.1	2.14e-13	4.48e-11	PLIN3	19p13.3	1.39e-13	3.20e-11
LRRC27	10q26.3	2.22e-13	4.59e-11	OSMR	5p13.1	1.54e-13	3.50e-11
PLPPR1	9q31.1	2.36e-13	4.83e-11	PCDH7	4p15.1	1.56e-13	3.52e-11
LYRM9	17q11.2	2.45e-13	4.95e-11	CBLB	3q13.11	1.93e-13	4.21e-11
SEC11C	18q21.32	2.71e-13	5.21e-11	FSCN1	7p22.1	2.10e-13	4.47e-11
GSTP1	11q13.2	2.82e-13	5.35e-11	ZNF267	16p11.2	2.15e-13	4.48e-11

*p-value (derived from Student's t-test); q-value (derived from Benjamini-Hochberg procedure); p- and q-value<0.05: significant association.