Universidade do Minho
Escola de Medicina

Ana Raquel Ferreira Cunha

Exploiting RKIP protein as a novel predictor of EGFR targeted therapies response in lung cancer

julho de 2019

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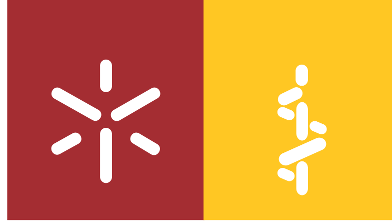

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Exploiting RKIP protein as a novel predictor of EGFR targeted therapies response in lung cancer

Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Olga Catarina Lopes Martinho

julho de 2019

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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.

Resumo

O cancro do pulmão (CP) é um dos mais fatais em todo o mundo, com uma expectativa de sobrevivência que raramente atinge os cinco anos. As terapias moleculares dirigidas têm revolucionado o tratamento do CP, sendo os inibidores do recetor do fator de crescimento epidermal (EGFR) os mais promissores e eficazes. Contudo, os pacientes tendem com o tempo a desenvolver resistência aos fármacos, daí ser necessário explorar novos preditores de resposta e mecanismos de resistência para estas terapias. Neste sentido, surge a proteína inibidora da cinase Raf (RKIP), uma reguladora importante de vias de sinalização celular, como a via de sinalização MAPK. Sendo considerada uma supressora de metástases, a subexpressão da RKIP tem sido associada a mau prognóstico em vários tipos tumorais, incluindo o CP. Neste trabalho, pretendeu-se primeiramente determinar o papel da RKIP na tumorigénese e *outcome* clinico de CP e, de seguida, explorar o papel da sua subexpressão na modulação da resposta a terapias anti-EGFR.

Assim, uma revisão completa da literatura sobre RKIP em CP foi elaborada e análises *in silico*, usando a base de dados do TCGA, foram também realizadas tanto para validação da literatura quanto para validação dos nossos próprios resultados. A seguir, o *knockout* de RKIP foi realizado pela tecnologia *CRISPR/Cas9* em quatro linhas celulares CP, e a influência da sua perda de expressão foi avaliada tanto a nível biológico como de resposta a inibidores de EGFR, recorrendo a ensaios *in vitro* (migração, viabilidade e clonogenicidade) e *in vivo* (CAM e xenotransplantes subcutâneos em ratinhos). Adicionalmente, o western blot foi a técnica escolhida para avaliar sempre que necessário a expressão e/ou ativação da RKIP e seus alvos, bem como das vias de sinalização celular.

De forma geral, conseguimos demonstrar que a perda da RKIP está associada com maior agressividade em CP, aumentando a migração e viabilidade celular, e, mais importante, validamos *in vivo* que células sem RKIP têm uma capacidade aumentada de formar tumores. Por outro lado, identificámos que a linha celular PC9, que é mutante para *EGFR*, quando *knocked out* para RKIP, se torna menos responsiva a inibidores de EGFR (*in vitro* e *in vivo*), e também que essa resistência aparente pode ser devida à modulação da via de sinalização AKT. Tendo em consideração a "assinatura molecular" associada à RKIP que determinamos por análise *in silico*, seria de maior interesse no futuro explorar ainda outras vias identificadas que possam estar por detrás do ganho de resistência observado aos inibidores de EGFR em CP.

Assim, os resultados sugeriram que a RKIP é potencialmente uma preditora negativa da resposta a terapias direcionadas ao EGFR em CP, particularmente em pacientes mutantes para *EGFR*.

Palavras-chave: Biomarcador preditivo, Cancro do pulmão, Prognóstico, RKIP, Terapia em cancro

Abstract

Lung cancer (LC) is one of the fatal cancers in the world with a life expectancy that rarely reach five years. Molecular targeted therapies have revolutionized LC treatment, with EGFR inhibitors outstanding. Unfortunately, patients end up developing drug resistance, hence, it is essential to discover novel response predictors as well as mechanisms of resistance. In this sense, Raf kinase inhibitory protein (RKIP) arises, as it is an important regulator of relevant intracellular pathways, such as MAPK signalling. Considered to be a metastasis suppressor, RKIP downregulation was associated with tumour malignancy and poor prognosis, in several tumour types, including LC. Herein, it was first aimed to explore the RKIP role in LC clinical outcome and tumorigenesis, and secondly to dissect its implication in the modulation of tumour cells response to anti-EGFR therapies.

Thus, a complete review of the literature regarding RKIP in LC was written and, using the TCGA database, *in silico* analysis were also done both for validation of the literature and validation of our own results. Following, RKIP knockout was performed by CRISPR/Cas9 technology in four LC cell lines, and the influence of RKIP's expression loss was evaluated at biological and EGFR inhibitors response levels, by *in vitro* (migration, viability and clonogenecity) and *in vivo* assays (CAM and subcutaneous xenograft mouse model). Additionally, western blot was the technique of choice to assess always that necessary the expression and/or activation of RKIP and its targets as well as of the signalling pathways.

In general, we were able to demonstrate that RKIP loss is implicated in tumorigenic processes in LC, such as higher migration and viability capacity, and importantly, we validate *in vivo* that cells without RKIP have a higher capacity to form tumours. On the other hand, we found that PC9 cell line, which is *EGFR* mutant, upon RKIP KO, became less responsive to EGFR inhibitors (*in vitro* and *in vivo*), and also that this apparently resistance might be due the RKIP modulation of AKT signalling pathway. Having in consideration the molecular signature of RKIP that we determined by *in silico* analysis, it would be of upmost interest in the future to further explore other pathways identified that can be behind the observed gain of resistance to EGFR inhibitors in LC.

Thus, our results suggested RKIP as a potential protein to be further explored as a novel negative predictor of EGFR targeted therapies response in LC, particularly in *EGFR* mutant patients.

Key words: Cancer therapy, Lung cancer, Predictive biomarker, Prognosis, RKIP

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List of abbreviations

A

AC- Adenocarcinoma

AKT- Protein kinase B

ALK- Anaplastic Lymphoma Kinase

ATP- Adenosine triphosphate

C

CAM- Chick Chorioallantonic Membrane

CNA- Copy number alterations

CSC- Cancer Stem Cell

D

DFS- Disease-Free Survival

DMEM- Dulbecco's Modified Eagle's

DMSO- Dimethyl Sulfoxide

DSB- Double-Strand Break

E

EGF- Epidermal Growth Factor

EGFR- Epidermal Growth Factor Receptor

EGFRi- Epidermal Growth Factor Receptor Inhibitor

EMT- Epithelial-Mesenchymal Transition

ERK1/2- Extracellular Signal-Regulated Kinase ½

F

FBS- Fetal Bovine Serum

FDA- Food and Drug Administration

G

GIST- Gastrointestinal Stromal Tumour

GPCR- G Protein–Coupled Receptors

GSK3β- Glycogen Synthase Kinase 3 Beta

H

HDR- Homology Direct Repair

HER- Human Epidermal Receptor

I

IC_{50}- Half Maximal Inhibitory Concentration

IHC- Immunohistochemistry

IKB- Inhibitory Kinase B

J

JAKs- Janus Kinases

JNK- c-Jun N-terminal Kinase

K

KO- Knockout

KD- Knockdown

L

LC- Lung Cancer

M

MAPK- Mitogen-Activated Protein Kinase

MEK1/2- Extracellular Signal-regulated kinase kinase ½

MET- Mesenchymal–Epithelial Transition

miRNAS- MicroRNAS

N

NFKβ- Nuclear Fator Kappa Beta

NSCLC- Non-Small Cell Lung Cancer

NSG mouse- NOD scid gamma mouse

O

OS- Overall Survival

P

P-AKT- Phosphorylated Protein kinase B

P-cRAF- Phosphorylated cRAF

PEBP1-Phosphatidylethanolamine-binding protein 1

P-EGFR- Phosphorylated Epidermal Growth Factor Receptor

P-HER2- Phosphorylated Human Epidermal Receptor 2

PI3K- Phosphoinositide 3-kinases

PKC- Protein Kinase C

P-MEK- Phosphorylated MEK

P-P90RSK- Phosphorylated P90RSK

P-RKIP- Phosphorylated Raf kinase Inhibitor Protein

R

RKIP- Raf Kinase Inhibitor Protein

RT- Room Temperature

RTK- Receptor tyrosine kinase

S

SCLC- Small Cell Lung Cancer

SCC- Squamous Cell Carcinoma

Shh- Sonic hedgehog

T

TCGA- The Cancer Genome Atlas

TK- Tyrosine Kinase

TKI- Tyrosine Kinase Inhibitor

TMA- Tissue Microarray

W

WB- Western Blot

Y

YY1- Yin Yang 1

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“Discovery is seeing what everybody else has seen,
and thinking what nobody else has thought”

Albert Szent-Gyorgyi

CHAPTER 1:

General Introduction

1.1 Cancer

Cancer is a disease characterized by abnormal cell growth which may possibly spread to other parts of the body and is among the leading causes of death worldwide, affecting countries regardless of their income levels[1,2]. It is expected that the number of reported cases and deaths associated to this disease will increase mainly due to the growth, aging and the adoption of cancer risk lifestyle behaviours of the general population[2].

Hanahan and Weinberg proposed, in 2000, that neoplastic diseases had specific hallmarks enabling the organization and rationalization of the complexities of these diseases. These hallmarks are driven by oncogenic mutations and they include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[3]. Later, two more hallmarks were recognized as so, namely the capability to reprogram the energy metabolism and the ability to evade immune destruction[4] (Figure 1). Biochemical and biomechanical properties of the extracellular matrix surrounding can also deeply influence the developing tumour[5].

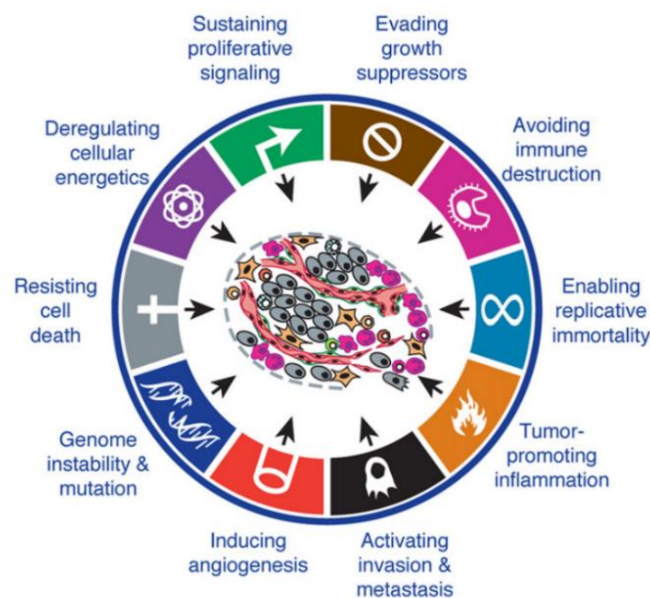

Figure 1- Hallmarks of cancer. Schematic illustration of the characteristics acquired by tumour cells enabling their growth and metastatic development. Adapted from [4].

1.1.1 Lung cancer

Of the several existing types of cancer, lung cancer is the most common and the most fatal, in both men and women[1], and it was estimated that 1.8 million new cases and 1.6 million deaths will account every year[2]. The main risk factor pointed is tobacco use and, although the number of new cases registered

is high, it is believed that, in western countries, lung cancer's epidemiology peak was already reached[6], as the incidence and death rates are decreasing. This decrease is in correspondence with the reduction of tobacco consumption observed in population[7], due to the tobacco control measures applied in the last three decades. In the last years, the incidence rates of lung cancer declined much faster in men than in women and this may be a reflection of the historical differences in tobacco use[8,9].

For clinico-pathological reasons, the various types of lung cancer are usually divided in two main categories: small cell lung cancer (SCLC, 15% of all lung cancers) and non-small cell lung cancer (NSCLC, 85% of all lung cancers). The latter is further divided into three major types, squamous cell carcinoma (SCC), adenocarcinomas and large cell carcinomas[6]. Although the number of new cases is stabilizing, it has been observed alterations in the frequencies of the different histological types of lung cancer, in western countries. All the principal histological types are associated with tobacco consumption, being this association stronger in SCLC and SCC, although it was registered an increase of this incidence in adenocarcinomas[10]. It is believed that this increase is due to the changes in smoking behaviour and cigarette manufacturing. Filter cigarettes and 'light' cigarettes allow the smoker to take a deeper aspiration enabling the smoke to reach deeper parts of the bronchi and alveoli, leading to adenocarcinomas[11].

Regarding lung adenocarcinomas, they are considered the most common type of lung cancer and has a poor survival rate (only 10% of the cases have a life expectancy of five years and those that are not treated, in average, live four months), which is usually aggravated by its late detection and lack of treatments in advanced stages[12,13]. This form of cancer is most common[14] in never smoking patients[i], having its incidence considerably increased globally[10], suggesting that, factors like environmental and occupational exposure to carcinogens, including second-hand smoke, radon, asbestos, arsenic, metals, fibres, dust, organic compounds, and air pollution also contribute to the increase of lung cancer occurrences[12].

Epidemiologic and comprehensive molecular characterization studies have resulted in the identification of novel molecular characteristics in lung cancer. These differences reside in the incidence, phenotype, genotype of tumours and also in the overall survival of the patients[6], providing evidences that these cancers arise through different molecular mechanisms. For instance, adenocarcinomas in patients that never smoked, frequently present mutations at the epidermal growth factor receptor (EGFR) level. On the other hand, smoking patients commonly harbour mutations in *KRAS* and *TP53* genes[15-17]. This is possibly explained by the exposure of smokers to highly carcinogenic agents like tobacco smoke, that might specifically induce Ras signalling pathways through mutations in *KRAS*. In never smokers, the yet

[i] Patients that, through their life, did not smoke more than 100 cigarettes.

unidentified carcinogens might target selectively the EGFR pathway by inducing mutations in EGFR[6] (Figure 2).

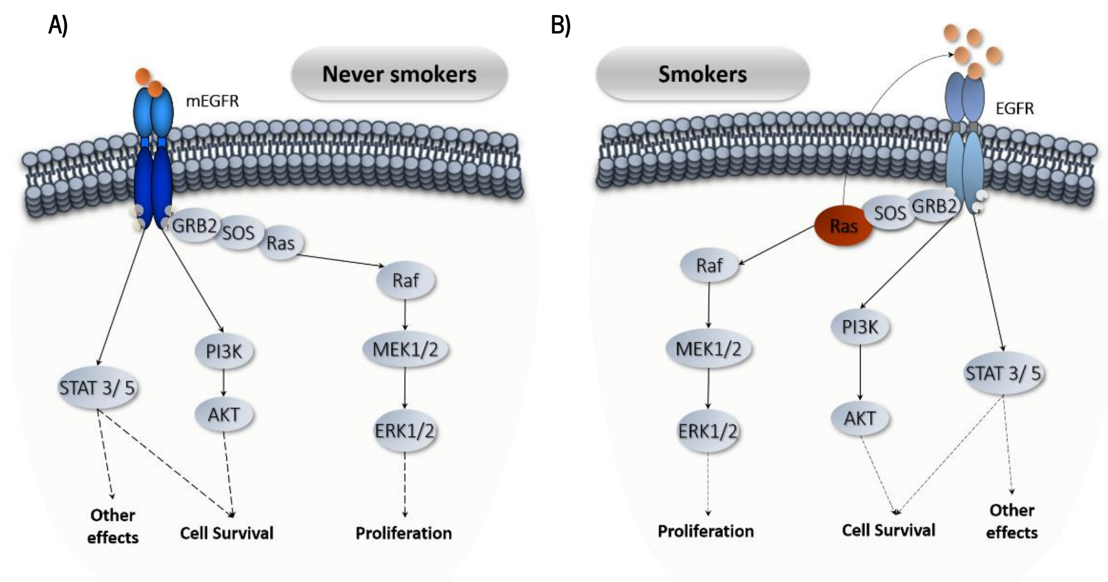

Figure 2- Two pathways that lead to adenocarcinoma. A) In never smokers the EGFR pathways is frequently activated due to mutations in the *EGFR* gene. Homo- and hetero-dimerization of the EGF receptor is induced by ligand binding, resulting in the activation of downstream effectors that leads to cell proliferation, survival and other effects associated with carcinogenesis. **B)** In smokers, often occurs mutations in *KRAS* gene, resulting in the release of growth factors, that binds to EGFR, activating its pathway. These two mutations have virtually the same consequences, and mutations in both genes in adenocarcinomas of the lung are rarely seen. Adapted from [6].

In similarity with other types of cancer, too in lung cancer, the usual first approach to treatment is surgery to remove most of the damaged tissue. Such is usually followed by chemotherapy or radiotherapy. These kind of treatments are based on a cytotoxic therapy that disrupts basic cellular processes such proliferation, maintenance, metastasis, angiogenesis and apoptosis in all cells. These are highly relevant as they continue to be the principal therapeutic method in response to most cancers[6], even though its associated limitations: lack of selectivity to tumour cells, insufficient concentrations reaching the target cells, gain of resistance to the drug and systemic toxicity[18,19]. Fortunately, in the last decade, progresses done in lung cancer expanded considerably the understanding about this disease and led to an era of targeted therapies and precision medicine. It is now a common clinical practice that patients' tumours are molecularly analysed in order for the right treatment to be applied, which leads to higher efficiency of the treatment, less secondary effects, and ultimately a better life quality to the patient. These type of therapies in lung cancer will be further addressed.

1.2 Receptor tyrosine kinases and signalling

Receptor tyrosine kinases (RTKs) are a highly conserved family of transmembrane receptors for extracellular signalling molecules, such as growth factors and hormones[20]. The molecular architecture is similar between all RTKs, with a ligand-binding domain in the extracellular region, one single transmembrane helix and a protein tyrosine kinase (TK) domain contained in the cytoplasmic region. Additionally, these receptors also have a carboxy (C-) terminal and juxtamembrane regulatory regions[20].

Upon dimerization or oligomerization, the tyrosine residues present on the TK domain auto-phosphorylate leading to conformational changes that stabilize the active state. Then, adaptor proteins are mobilized and activate several protein interactions and intracellular effectors, which include Ras proteins, members of the mitogen activated protein kinase (MAPK) family, phosphoinositide 3-kinase (PI3K) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) proteins. This ultimately results in the alteration of gene expression and protein functions, responsible for critical cellular processes like cell survival, proliferation and differentiation and also cellular functions such as metabolism and cell-cell communication[20-22] (Figure 3).

Because these receptors are involved in such critical cellular processes, it was predictable that dysregulation of RTKs was causally linked to cancers, diabetes, inflammation, severe bone disorders, arteriosclerosis and angiogenesis[20]. Specifically, in cancer, this happens mainly due to mutations, gene rearrangements or amplification of RTKs themselves or their downstream effectors, leading to oncoproteins constitutively active. Besides, silencing of suppressors of RTK signalling pathways or upregulation of key components of RTKs are induced by epigenetic alterations, leading also to oncogenic signalling[22]. Some cancer cells end up being dependent on this type of individual oncogenes, in order to sustain the malignant phenotype, developing an 'oncogene addiction'[23]. This crucial aspect is the pillar of molecularly targeted cancer therapies that, by inhibiting molecular drivers using targeted agents, allows the effective reduction of tumour growth and improvement of patient survival[24].

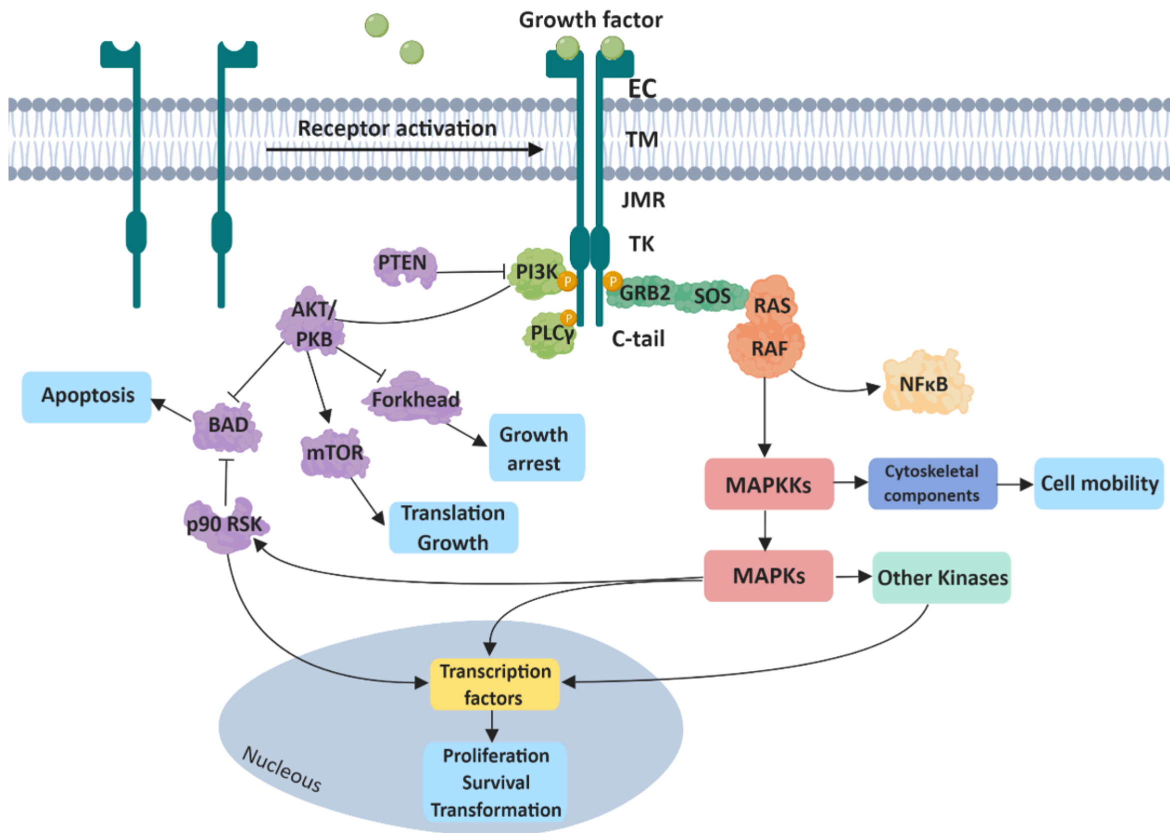

Figure 3- Schematic representation of the signalling mechanism of receptor tyrosine kinases (RTKs). The general structure of an RTK is composed by the extracellular domain (EC), transmembrane domain (TM), juxtamembrane domain (JMR) and tyrosine kinase domain (TK). Upon binding of a ligand, the receptor dimerizes, leading to the activation of the tyrosine kinase domain, phosphorylation of critical tyrosine residues in the intracellular domain and activation of downstream signalling pathways. This may lead to several events like cell proliferation, survival and transformation, apoptosis, translation growth, growth arrest or cell mortality (blue boxes). Adapted from [25]

1.2.1 Epidermal Growth Factor Receptor

Epidermal growth factor (EGF) and its receptor (EGFR) have been one of the most extensively studied cell surface receptors, not only due to their central role in developmental biology, tissue homeostasis and cancer biology but also because they present a good example to study receptor biology and specifically to further understand RTKs[26].

The family of EGFR is constituted by four members (HER1-4) that belong to the protein superfamily ErbB (ErB1-4). This family is expressed ubiquitously in epithelial, mesenchymal and neural cells and in their progenitor cells[27], and are located in the surface of the cell, where ligand-receptor and receptor-receptor interactions occur. The dimerization process of the receptor is a critical step for the activation of intrinsic tyrosine kinases which leads to the autophosphorylation of the c-terminal specific tyrosine-containing residues, which activates further downstream signalling pathways. These include

Ras/Raf/MEK/ERK, PI3K/AKT/mTOR, JAK/STAT and PLCγ/PKC that affect cell proliferation, differentiation and apoptosis, consequently exerting a critical function in many physiological processes, like organ development, survival, proliferation, regeneration, ion transportation, among others[26,28].

EGFR signalling is frequently altered in various human tumour types, like breast cancer, lung cancer, glioblastoma, head and neck cancer, among others[29], due to *EGFR* gene amplification and/or protein overexpression, which ultimately correlates with poor clinical prognosis and with the production of alfa transcription factors[30].

Classical *EGFR* mutations, pertinent to NSCLC, like the deletion of exon 19 (45% of *EGFR* mutations) and the point mutation L858R in exon 21 (40% of *EGFR* mutations), are spatially located within the ATP binding site of the kinase (Figure 4A), which decreases the affinity of the kinase to it[31,32]. The third most frequent mutations are composed of in-frame insertions within exon 20 of the kinase (5-10% of *EGFR* mutations)[31] (Figure 4B). When mutations like these happen, EGFR stays constitutively active and consequently, the signalling pathways referred before will also stay active, which leads to repercussions in the cell's metabolism, by increasing the proliferation, angiogenesis, invasion and metastasis rates and decreasing apoptosis. Mutations in *EGFR* are known to be more frequent in adenocarcinomas, in never-smokers or light smokers, in women with NSCLC and in patients with East Asian ethnicities[33]. An increase of the receptors activity can also be due to either overexpression of activating ligands or mislocalization of the receptor[26]. All this has led to the proposal of EGFR as a target for therapy against cancer, 35 years ago[34]. Also, the detection of these mutations, can be used as a predictor of therapy response to treatments with tyrosine kinase inhibitors (TKIs), as cells harbouring these mutations are more sensitive to these particular treatments[32].

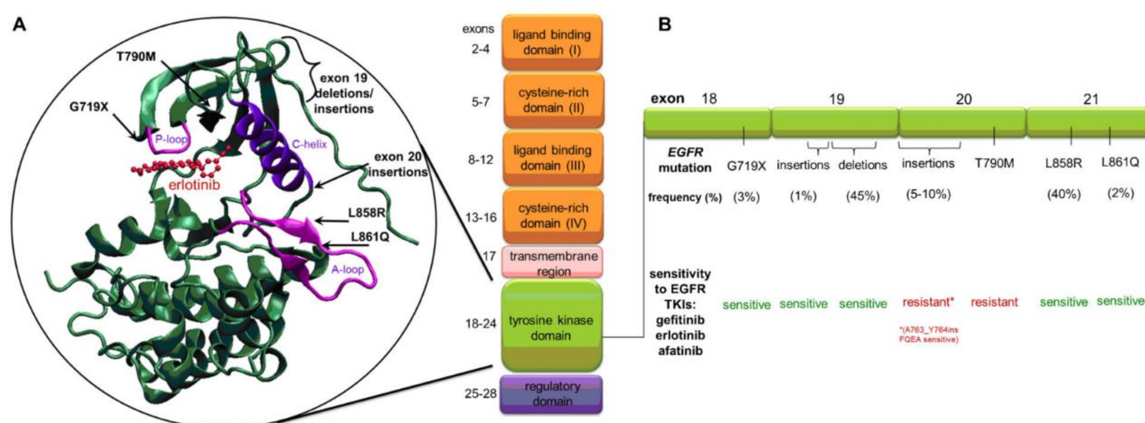

Figure 4- Mutations *EGFR* gene in non-small-cell lung cancer (NSCLC). A) Representation of the various domain of the EGFR, being the tyrosine kinase domain highlighted. The structure is complexed with the TKI erlotinib (based on Protein Data Bank [PDB] accession code 1M17) and is also pointed the localizations of the most common EGFR mutations. **B)** Exon location,

frequency and sensitivity to anti-EGFR drugs (gefitinib, erlotinib and afatinib) of the most common *EGFR* mutations in NSCLC patients. Retrieved from[26].

1.2.2 Mitogen-activated protein kinases (MAPK)

RTKs are important mediators of cell signalling, like referred before, being mitogen-activated protein kinase (MAPK) pathway one of the most critical signalling pathways regulated by them [35].

The MAPK cascade is a critical pathway responsible for the regulation of various cellular activities including proliferation, differentiation, apoptosis, survival, inflammation and innate immunity, highly conserved throughout evolution[36-38]. MAPKs are serine-threonine protein kinases, and in mammals they fall into distinct seven groups being that conventional MAPKs comprise the extracellular signal-regulated protein kinases (ERK1 and ERK2), c-Jun N-terminal kinases (JNK1, JNK2, JNK3), p38s (p38α, p38β, p38γ, p38δ), EKR5 (ERK5), ERK3s (ERK3, p97 MAPK, ERK4) and ERK7s (ERK7, ERK8)[36] (Figure 5).

Each group of MAPKs can be stimulated by, for example, growth factors or hormones, which triggers a sequential activation of a specific MAPK kinase kinase (MAPKKK) and a MAPK kinase (MAPKK) which then phosphorylates and activates their downstream MAPK[37] (Figure 5).

The protein Serine/Threonine kinase, MAPKKK, can be activated by phosphorylation and/or by interacting with RAS, a small GTP-binding protein, which is activated when RTKs stimulates the exchange of guanosine-triphosphate for guanosine-diphosphate[39]. This leads to the activation of MAPK which is followed by an activation of the MAPK by dual phosphorylation of two conserved threonine (Thr) and tyrosine (Tyr) residues (Thr-X-Tyr), within the activation loop[38]. Upon activation, MAPKs phosphorylate and control the activity of key cytoplasmic molecules and nuclear proteins, which can regulate gene expression. This means that, the gene expression regulation, will ultimately depend on the integration of the combinatorial signals given by the temporal activation of the MAPK families[37].

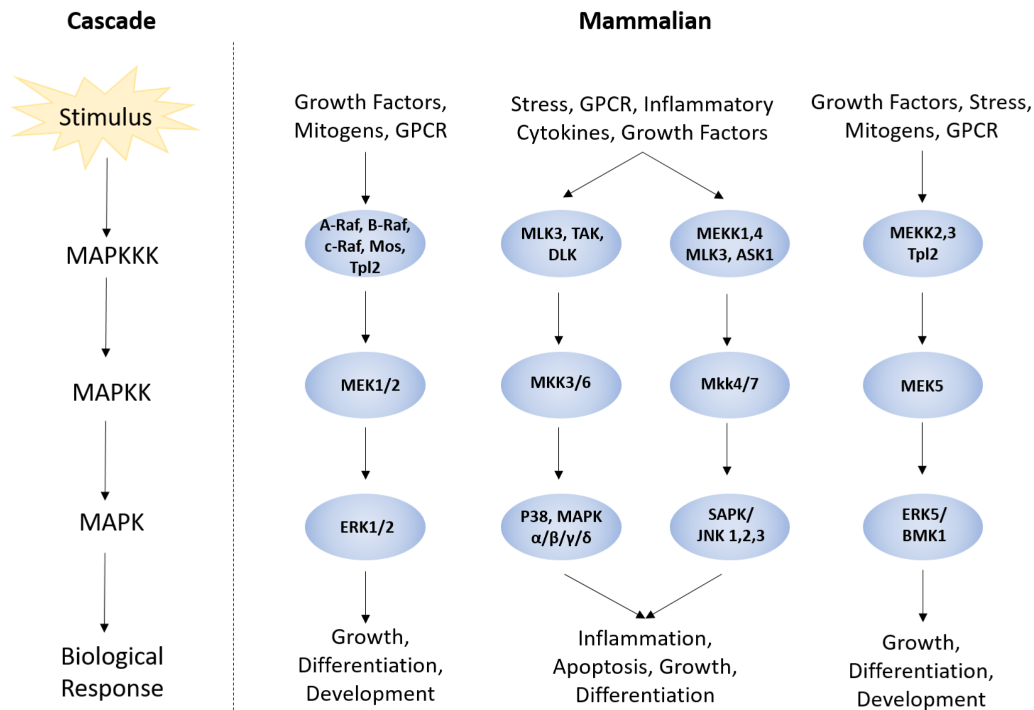

Figure 5- Conventional MAPK signalling cascade, in mammalians. On the right, the four conventional MAPKs, the respective pathway intervenient and the consequent biological response. On the left, a schematic representation of the pathway core. Adapted from [40]

ERK ½ was the first MAPK to be described in mammals[41]. They are activated by growth factors, which include platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and nerve growth factor (NGF), and in response to insulin. The ERK ½ module is involved in processes such adhesion, cell cycle, migration, cell survival, differentiation, metabolism, proliferation and transcription[35]. JNK and p38 MAPK pathways are related to stress related stimuli[40]. JNK cascade responds to growth factors, proinflammatory cytokines and environmental factors like UV and heat[39]. P38 cascade is stimulated by Tumour Necrosis Factor Alpha and Interleukine-1, regulating processes like apoptosis, cellular senesce and cell survival[40]. Finally, ERK5 is responsible for the regulation of proliferation, angiogenesis and immune response, being this MAPK the less studied until now[40].

The importance of MAPK signalling pathways is irrefutable in the regulation of cellular functions, like described before. Thus, when a deviation from the normal activities of this pathway happens, various abnormalities can arise, which might lead to human diseases, such as cancer[42]. In fact, it is well established that perturbations in MAPK are a common feature in cancer development and progression.

On one hand, activation of the MAPK pathway can occur through aberrant ERK ½ pathway activation, being this already described in one third of all human cancers[43]. Such can result from overexpression of RTKs, activating mutations in RTKs, sustained autocrine or procrine production of activation ligands, as

well as *Ras* and *B-Raf* mutations, which are ultimately responsible for the enhanced or constitutive downstream activation of Raf-MEK-ERK pathways. As consequence of this dysregulation of the ERK1/2 activation, processes such as cell proliferation, growth regulation factors and inhibition of apoptosis are enhanced[44].

Furthermore, MAPK activation can be induced by stress mediators. Cancer cells are most frequently exposed to stressful conditions such as hypoxia, inflammation and metabolic stress and even genotoxic and pharmacologic stresses due to chemo- or radiotherapy exposure[45], leading to activation of stress-activated MAPK pathways, JNKs and p38[46]. Concerning the latter, it is believed that the role of p38 in solid tumour biology may be determinant for tumour cell survival and metastasis, even though the mechanisms behind it are quite diverse and complex[47].

Additionally, it is important to emphasize that the biological outcome of MAPK activation is deeply dependent not only on the type of stimuli, but also on the strength/duration of the signal or even on dependent on the cell type/tissue specificity[44].

1.3 Molecular targeted therapies

Although conventional methods like surgery, chemotherapy and radiation are still mainly used in cancer therapy, in spite of the significant toxic effect of the latter, targeted therapies are gaining popularity, being included as component of various cancer therapies[48]. Contrary to chemotherapy, targeted cancer therapies are designed to specifically modulate pathways related to carcinogenesis and tumour growth. This is achieved by inducing apoptosis, blocking specific enzymes and growth factor receptors involved in cell proliferation, or modifying the function of proteins that regulate gene expression and other cellular functions, only in carcinogenic cells[48].

There are two main classifications of targeted therapies. The first one consists in using therapeutic monoclonal antibodies (mAbs) which target specific antigens found in the surface of the cell, like transmembrane receptor or extracellular growth factors. It can also be attached to them a radioactive substance or a poisonous chemical, allowing specific delivery to cancer cells[49]. Examples of monoclonal antibodies FDA approved for the treatment of solid tumours are cetuximab, panitumumab and transtuzumab which are naked antibody drugs that target RTK of the cell membrane[48].

The second main targeted therapy deals with small molecule drugs. These are usually organic compounds with low molecule weight (<800 Da) which are able to penetrate the cell membrane and are specifically designed to act on targets found inside the cell or to interfere with signalling pathways[50]. Their designation includes a suffix "-ib", meaning that the agent has protein inhibitory properties[50]. A great

amount of these small molecules is developed as tyrosine kinase inhibitors (TKIs), being afatinib, erlotinib, gefitinib and lapatinib well known examples.

Even though the advances achieved in targeted therapies are significant, there are still some limitations associated. On one hand, patients treated with these therapies usually become resistant to them. Resistance can occur due to changes through mutations on the target, so the target therapy no longer interacts with it, and/or the tumour finds a new pathway, independent from the target, to continue growing[51]. On the other hand, there is some difficulty associated with the development of drugs for some target's structure and/or the way its function is regulated in the cell. Ras protein is one example, being mutated in as many as one-quarter of all cancers, and to date, no inhibitors of Ras signalling were developed with success[51].

1.3.1 Targeting RTKs in Lung Cancer

To treat early-stage non-small-cell lung cancer (NCLSC) patients, surgery is usually the first line of therapy[52]. However, for advanced stage NCLSC patients such strategy cannot be applied. Fortunately, there has been a considerable increase in the understanding of the molecular characteristics of these types of tumours, specially concerning alterations in RTKs, and, also great investment was made by pharmaceutical companies to develop new molecular therapies for these specific tumours. This means that patients who have tumours with specific genomic aberrations may benefit from molecular targeted therapies recently developed[53].

Nowadays, most of the molecular targeted cancer therapies produced are inhibitors of RTK. These type of drugs have been approved by the FDA and are used in the clinic for the treatment of NSCLC. In Table 1 is shown the most common drug targets, the prevalence of its alterations, the associated patients characteristics and the agent used in their treatment. Patient's tumour with mutations in *KRAS*, which represent 30% of cases of adenocarcinoma, are often predictive of a lack of benefit of TKIs and are associated with poorer overall survival[54].

One of the most important targets for personalized therapy against NSCLC is EGFR due to its overexpression in this type of tumours, being associated mutations used in clinic as a biomarker/preditor of treatment response[55]. The first TKIs against EGFR to be used in clinic, called first generation TKI, were gefitinib and erlotinib, which bind reversely to the ATP binding site, inhibiting the phosphorylation and tyrosine kinase activity of the receptor. When gefitinib was in early phase III trials, patients were not selected according to their EGFR mutations, but smaller subsequent trials showed that the presence of *EGFR*-activating mutations was a stronger predictor of clinical benefit for these TKIs, when compared to

Table 1- Molecular targets in lung adenocarcinomas, its prevalence, patient characteristics and the targeted therapies applied (FDA approved)[56].

Molecular target	Prevalence (%)	Commonly Associated Patient Characteristics	Agent Used in Targeted Therapy
RAS	30	Former/current smokers	None
EGFR	10-18 (Caucasian) 40-55 (Asian)	East Asian, female, never smokers	Afatinib, Dacomitinib, Erlotinib, Geftinib, Necitumumab, Osimertinib,
ALK	3-7	Young, never smokers	Alectinib, Ceritinib, Crizotinib,
ROS	1-2	Young, never smokers	Brigatinib, Crizotinib,
RET	1-2	Never smokers	Vandetanib, Cabozantinib
BRAF	3-5	Former /current smokers	Dabrafenib
HER2	1-4	Female, never smokers	Afatinib, Trastuzumab
MET	11	Mutually exclusive with EGFR mutations	Crizotinib, Onartuzumab

treatment with standard chemotherapy[57]. These mutations, frequently exon 19 deletion and single-point substitution mutation L859R in exon 21 (classical *EGFR* mutations), are characteristically located near the ATP cleft of the TK-domain. They lead to a stabilization of the interaction with ATP and stimulate phosphorylation of tyrosine residues, causing intracellular transduction activation in an aberrant way[58]. Mirsudomi *et al.* also demonstrated that patients under the same mutation patron displayed longer progression-free survival when treated with gefitinib than when treated with cisplatin chemotherapy[59]. Studies done with erlotinib also indicated better survival when compared with chemotherapy, reinforcing the prognostic value of EGFR mutations, in NSCLC patients[60,61].

Although promising, NSCLC tumours with EGFR-activating mutations treated with first generation TKIs inevitably develop resistances. Several resistance mechanisms have been described, being the most frequent one (described in 50-60% of patients) the development of a T790M missense mutation in exon 20[62.] Other mechanisms include alterations related to the MET receptor[63] and HER3[64].

In an attempt to circumvent the gain of resistances, a second generation of TKIs were developed, being these characterized by irreversibly inhibiting the tyrosine kinase activity of EGFR, HER2 and HER4 by forming covalent bonds with the receptors[65]. Afatinib and Dacomitinib are examples of these kind of molecules, being its irreversible inhibition more potent and prolonged than the reversible first-generation EGFR-TKIs[65,66]. Another TKI with potential is AST1306 (allitinib), analogue of lapatinib, which after phase I trials, showed promising anticancer activity, and ability to inhibit the growth of cells with T790M mutation in EGFR in NSCLC cells. The lower side effects, when compared with afatinib and dacomitinib, makes

AST1306 worthy to be further investigated[67]. Our group (Silva-Oliveira *et al.*) took on the largest *in vitro* assessment of allitinib cytotoxicity done to date, in which was identified the cancer types that could potentially benefit from this drug, being NSCLC one of them. In this study it is also suggested that prevalent *KRAS* mutations constitute a potential negative predictive biomarker for allitinib response[68].

There are now being explored a third generation of EGFR TKIs, which are more selective to T790M mutations, clinically more potent and less toxic than the current EGFR TKIs. Preliminary results of CO-1686 and AZD9291 were very promising with improved tolerability and clinical activity. Patients with NSCLC, which reported resistant mutations T790M and treated with these TKIs, reported an overall response rate (ORR) of 58% and 64%, respectively[69,70]. Of both, only AZD9291 was FDA approved, under the commercial name of Osimertinib, in April 2018, for first-line treatment of patients with metastatic NSCLC whose tumours have classical EGFR mutations (exon 19 deletions or exon 21 L858R mutations)[71]. Kiura *et al.* suggested that EGFR-T790M-positive status of a NSCLC can predict a clinical benefit with Osimertinib treatment[72].

1.3.2 Predictive Biomarkers to anti-EGFR therapy response

Currently, most patients with NSCLC, particularly the ones with AC, go through a biopsy for molecular profiling of the tumour. The improvement of genomic sequencing techniques enabled and promoted this molecular analysis, which provides the molecular and histological characteristics of the disease, so the appropriate strategy of treatment is applied[55]. In these analysis, the samples are usually tested for specific mutations in genes encoding tyrosine kinases, like *EGFR* and *ALK*, signal transducers, like RAS, or downstream signalling pathways, including the MAPK and PI3K/AKT, which are crucial for cell survival and proliferation[73] (Figure 6). Those mutations, in particular in adenocarcinomas, have been demonstrated to predict drug sensitivity[74].

The first approach to determine if tumour cells are going to present primary resistance to the treatment is by determining mutations in the driver oncogene *KRAS*. The most frequent mutations in this gene occurs in exon 2 at codon 12 (less frequently at codon 13 (3-5%)) and, more rarely at exon 3 codon 61 (<1%), causing the loss of KRAS GTPase activity. This renders KRAS protein constitutively GTPbound (active), leading to the stimulation of effector proteins, independently of the upstream growth factor receptor activity[75]. As referred before, *KRAS* mutations are correlated with tobacco smoking being its incidence the most frequent especially in non-Asian population, and usually these and *EGFR* mutations are rarely found in the same tumour[76,77]. Adenocarcinomas harbouring *KRAS* mutations are not sensitive to TKI therapy, and so, this type of patients is instead redirected for chemotherapy[54,76,78].

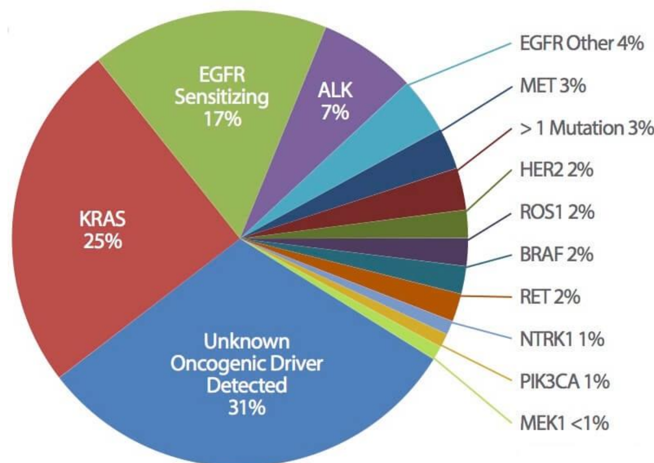

Figure 6- Pie chart summarizing the prevalence of driver oncogenes mutations in lung adenocarcinoma. Mutations in these oncogenes are screened on patient's tumours to then apply the most suitable therapy. Note that this is referred to non-Asian population, since the prevalence of EGFR mutations in Asian population has a greater weight. Retrieved from [79]

The second most incident mutations in NSCLC are the ones associated with *EGFR*, being its frequency considerably high in Asian patients (33,4%)[74]. *EGFR* mutations are defined as 'activating' and 'sensitizing' because they activate RTK independently of ligand binding, and also because they cause an increased sensitivity of EGFR for TKIs, which means, lower concentrations of drugs are needed to inhibit the receptor[80]. Currently, *EGFR* mutation is the strongest predictive biomarker for efficiency of EGFR-TKIs and so, patients harbouring them are recommended to be treated with EGFR-TKIs, providing a shorter progression-free survival (PFS) (and may prolong overall survival), when compared with first-line treatment with platinum doublet chemotherapy[81]. However, and like it was referred before, most of them end up developing resistance mutations like T790M, desensitizing cells for the therapy. The presence of T790M mutation is rarely found in tumours from untreated patients, but when is found, it is associated with a significant shorter PFS when compared to patients without detectable T790M[82]. So, detecting this particular mutation may be a useful pre-treatment biomarker to identify patients who are going to have long lasting responses to reversible EGFR-TKIs. To patients who have these resistance mutations, is recommended the treatment with second and third generation TKIs.

Patient's tumours can also be tested for ALK translocations, which are present in 3-7% of NSCLC. A fusion gene involving *ALK* and the *Echinoderm microtubule associated protein-like 4* (*EML4*) gene (*EML4-ALK*) results in constitutive activity of the ALK kinase, generating oncogenic activity[83]. This type of rearrangements is associated with younger age diagnosis and with a non-smoking history. Like with *EGFR* and *KRAS* mutations, ALK fusion seems to be mutually exclusive, and patients positive for this mutated

protein frequently present an advanced clinical stage[84]. Oral treatment with the ALK-inhibitor Crizotinib is the first line of treatment for this kind of patients. Although the initial high response rate of this therapy, patients tend to acquire resistance, usually through the development of secondary *ALK* mutations, being the most common ones L1196M (located in the gatekeeper residue) and G1269A (located in the ATP binding pocket)[85]. To overcome these resistances a second-generation of ALK TKIs have been under development, being Alectinib and Ceritinib two FDA approved examples, used in patients that no longer respond to Crizotinib[86].

Although with only a prevalence of 2-4 %, *BRAF* mutations may also be interesting as a prognostic marker for NSCLC. Mutations of this kind usually lead to constitutive activation of the protein, consequently leading to activation of the MAPK pathway[87]. The prognostic value of *BRAF* mutations, specifically V600E mutation, was already established in melanoma, and in these cases, direct inhibition of it and/or the downstream mitogen-activated protein kinase (MEK) led to prolonged survival[88]. This provided a strong rationale for validating the predictive value of BRAF V600-mutant in NSCLC. In NSCLC, *BRAF* mutations seems to be associated with tobacco consumption[89], and more specifically V600E mutation is significantly associated with female sex, representing a negative prognostic factor (shorter disease-free and OS rates)[90]. Fortunately, it was observed that this type of patients, when treated with BRAFi and MEKi, had a numerically higher ORR (63%) and longer PFS (9.7 months), comparing with BRAFi monotherapy[91].

Even though there is greater understanding of all these proteins and their value as biomarkers of therapy response, tumour plasticity and heterogeneity makes the continuous search for novel predictors and the improvement of the ones known always important and relevant. In this sense, Raf Kinase Inhibitory Protein arises (RKIP).

1.4 Raf Kinase Inhibitory Protein (RKIP)

One of the mechanisms by which the cell insures the high fidelity of signalization involves proteins modulators of signalling cascades. The depletion of these is not directly fatal to the cell, but it can, over time, lead to the accumulation of chromosomic abnormalities, which culminate in mutations and disease[92]. In this sense, the modulator Raf kinase Inhibitory protein (RKIP) arises, which is associated with the Raf/MEK/MAPK signalling cascade, its endogenous inhibitor[93,94].

Raf kinase inhibitory protein (RKIP), also known as PEBP1 (phosphatidylethanolamine-binding protein 1), is a 23kDa protein, originally purified from bovine brain, and is mainly present in the cytoplasm and in the periplasmic internal cell membrane in a great variety of tissues[93,95-97]. RKIP family is highly

conserved and does not share significant homology with other protein families. It is a multifunctional protein that is implicated in many physiological processes, such as neural development, cardiac debit and spermatogenesis a [98,99]. It is also known that RKIP's broad set of functions is correlated with its important role in different signalling pathways[100]. Thus, the deletion or downregulation of RKIP has been described and related with some human diseases, like Alzheimer's disease, diabetic nephropathy, sperm decapitation, heart failure and cancer[98,99,101-103].

1.4.1 RKIP in cell signalling

In what respects Ras/Raf/MAPK pathway, RKIP binds specifically to Raf-1 kinase and so, inhibiting the signalling pathway[94]. RKIP inhibits the kinetic activity of Raf-1 through the dissociation of Raf-1/MEK complex, acting as a competitive inhibitor for the phosphorylation of MEK, and it binds directly to the N-region of the Raf-1 kinase domain, preventing Ser338 and Tyr340/341 phosphorylation, this way inhibiting its activity[104]. The fact that RKIP is significantly more abundant in the cell than Raf-1 protein, led to the conclusion that this would not probably be its only function, and that RKIP probably has more molecular targets (Figure 7).

Additionally, it was reported that RKIP can indirectly interfere with upstream activators of Raf-1, such as G-protein coupled receptors (GPCR), being this dependent on the phosphorylation status of RKIP. Thus, upon phosphorylation at serine 153, a process mediated by protein kinase C (PKC), RKIP releases from Raf-1 and binds to G protein-coupled receptor kinase 2 (GRK2), which is an endogenous inhibitor of GPCR activation[105-107]. This leads to the dissociation of GRK2 from GPCR, allowing GPCR activation and phosphorylation of downstream targets (Figure 7).

It was then demonstrated that RKIP antagonizes the signalling of the nuclear transcription factor κB (NF-κB) along with the interaction with other kinases located upstream, which regulate the inhibitory kappa B (IκB) protein, thus, possibly being involved in apoptosis regulation[108] (Figure 7). Inactive NF-κB is bound to IκB, both located in the cytoplasm, and the latter, must be degraded so NF-κB is activated and, consequently, be translocated to the nucleus[40]. Yeung *et al.* demonstrated that RKIP has the capacity to physically interact with four kinases necessary for the activation of NF-κB pathway, blocking the phosphorylation of IκB, thus inhibiting it[108]. Further studies, carried by Tang *et al.,* revealed that RKIP could also modulate these pathways through interactions with components that act upstream of IκB kinases[109].

Besides acting as an inhibitory protein, RKIP can also act as a signalling activator in glycogen synthase kinase 3 beta (GSK3β) pathway[110,111]. RKIP physically interacts with GSK3, preventing its

phosphorylation at the inhibitory T390 residue by p38 MAPK. In a context of RKIP depletion or oxidative stress conditions, the levels of p38 are significantly increased which leads to the phosphorylation of GSK3β, activating the pathway[110]. Hence, RKIP downregulation correlates with high levels of GSK3β substrates, leading to induction of β-catenin, Snail and Slug, which may explain the aggressive invasive behaviour of cancer cells with lower RKIP expression[110].

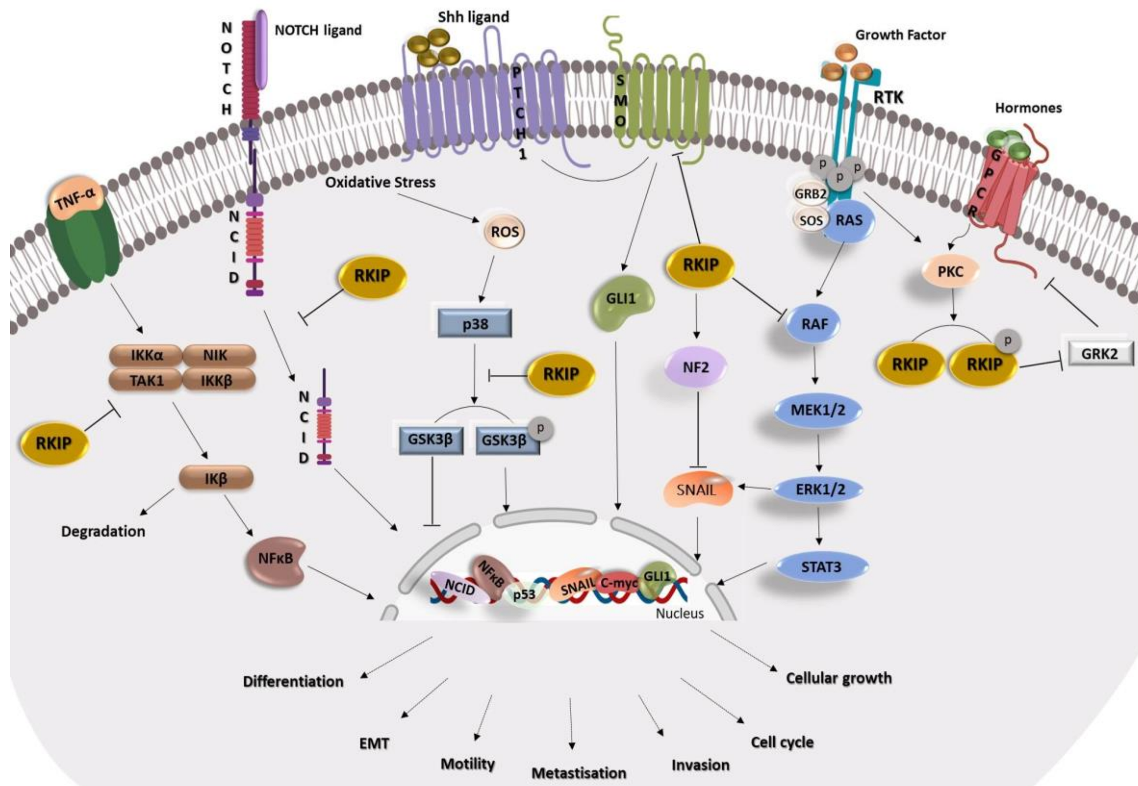

Figure 7- RKIP as a modulator of intracellular signalling cascades. On the left, RKIP binds to IKK complex preventing Ikβ phosphorylation and degradation which ultimately blocks the translocation of NFkβ to the nucleus. On the left too, RKIP binds to the Notch Intracellular Domain (NICD) preventing its proteolytic cleavage. In the Notch1 pathway, RKIP inhibits 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. Too in the middle, the depletion of RKIP enhances oxidative stress–mediated activation of the p38 MAPK, which, in turn, inactivates GSK3β by phosphorylating it at the inhibitory T390 residue, leading to transcription activation of several genes. 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. Also, 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 [112].

1.4.2 RKIP in cancer

The first association between RKIP and cancer was established in prostate cancer, in which the RKIP expression levels in the cells were below average and even lower in metastatic ones. This suggested that the expression of RKIP could be inversely associated with the invasion capacity of the cancer[113]. The same association was made in other types of cancer like breast cancer[114], colon cancer[114], gliomas[97], endometria cancer[115] and cervical cancer[116]. On the other hand, Fu *et al.* also demonstrated that, when RKIP expression was re-established in metastatic cells, the invasion capacity of them was inhibited, not affecting however the growth of the primary tumour[113]. This suggests that RKIP does not have a central role in the primary tumour, but instead, has great importance as metastasis suppressor. Therefore, *RKIP* is a tumour suppressor gene due to, when its expression is reduced, an increase in tumour aggressiveness and poor prognosis in metastatic cancers.

As a central metastasis suppressor, RKIP exerts an inhibitory effect in processes responsible for metastasis initiation like angiogenesis, epithelial to mesenchymal transition (EMT), cell migration and invasion[117]. During the EMT process, epithelial cells lose their polarity and cell-to-cell adhesion and gain motility properties, essential in the early stages of metastasis formation. This shift is characterized by the downregulation of 'epithelial markers' like E-cadherin and the overexpression of 'mesenchymal markers' such as vimentin, N-cadherin and fibronectin[118]. It has been described, in several tumours, that RKIP is able to negatively regulate this process through the NFκ-B/Snail/YY1/RKIP circuitry[119-121]. EMT induction is mediated, in part, by the constitutive activation of the Snail metastasis-inducer transcription factor. Snail is transcriptionally regulated by NF-kB and in turn, Snail represses RKIP and E-cadherin transcription[122,123]. Along this, RKIP was demonstrated to modulate NF-κB pathway, preventing its activation, and supressing EMT inducers, Snail, YY1, and upregulating E-cadherin, this way stopping the EMT process[124-126].

RKIP downregulation in a cancer context is well established and accepted, however, the mechanisms behind its alterations remain elusive[127]. Yet, until now, it is acknowledged that RKIP expression can be regulated at multiple levels. At the epigenetic level, RKIP promoter has been frequently found methylated in several cancers, such as in breast, colorectal or esophageal squamous cell carcinomas[127-129]. However, some controversy arises as to this being always the mechanism behind RKIP downregulation, because in tumours such as GIST, promotor hypermethylation was not found[130].

Concerning the regulation at the transcription level, transcription factors such as BACH1 and Snail1 are able to bind to RKIP promoter, supressing its transcription and further expression. Such is so, that the inversed expression levels of RKIP, BACH1 and Snail are considered significant prognostic markers

for metastatic-free survival for breast and prostate cancer patients[122,131]. Besides those, Sp1, cAMP, CREB and p300, which are too transcription factors, are able to regulate RKIP's promoter activity[132]. Interestingly, it was found in several tumours that RKIP expression could also be regulated at post transcriptional level, more specifically, RKIP mRNA can be targeted and supressed by several microRNA, such as miR-224[133], miR-543[134], miR27a[135] and miR-23a[136]. Finally, RKIP can be further modulated at a post-transcriptional level through phosphorylation, mediated by PKC, as referred before[105].

The implications behind RKIP's downregulation have been the focus of several *in vitro* and *in vivo* studies which have tried to understand which are the pathways modulated by this protein in tumorigenesis. However, unravelling such matter turned out to be challenging, as RKIP role seems to vary slightly between different types of cancer (Table 2). Notwithstanding, concerning the main cancer developing processes such as migration, invasion and proliferation tend to increase upon RKIP depletion across several tumour types, being the mechanisms behind this RKIP modulation still elusive in some malignancies (Table 2). Due to this involvement of RKIP and cancer, several studies went on to screen for a relation between RKIP expression and the patients' prognosis[137]. Our group and others described that loss of RKIP expression is an independent marker of poor clinical outcome in many types of metastatic and aggressive cancers, being in Table 2 specified the percentages of downregulation of RKIP (negative cases) for each type of tumour. Besides these, Martinho *et al.* also associated the absence of RKIP expression with poor survival in soft tissue sarcomas and in gastrointestinal stromal tumours (GIST), indicating that too in these types of tumours RKIP may have an important role as a prognostic marker[130,138].

Table 2- Clinical evaluation of RKIP as an independent prognostic factor.

Tumour type	Negative cases	*In vitro / in vivo* role	References
Acute myeloid leukemia	18- 24%	Proliferation	139
Amoulla of vater	33%	-	140
Bladder cancer	17%	-	141
Colorectal cancer	50- 57%	Metastasis, differentiation, proliferation, chemosensitivity	96,142,143
Esophageal Cancer	50 – 70%	-	144-146
Gallbladder carcinoma	69%	-	147
Gliomas	10 - 58%	Viability, migration, invasion	97,148,149
Hepatocellular carcinoma	86%	Proliferaton, migration, differentiation	150,151
Nasopharyngeal carcinoma	58 -78%	Invasion, metastasis, resistance to radiotherapy	152-155

Prostate cancer	48 -50%	Invasion, vascular invasion, metastasis, resistance to radio and chemotherapy	113,156-158
Renal cell carcinoma	42- 80%	Invasion	159-161

NOTE: only studies that reported RKIP protein expression were considered, those reporting RKIP mRNA and pRKIP expression were excluded for this table

Looking even further, RKIP expression has also been implicated in the regulation of tumour cell resistance to conventional therapy, chemotherapy and radiotherapy [116,157,162-165]. It seems that RKIP acts as an apoptosis inducer, by interacting in multiple ways with signalling modules known to confer a resistant phenotype to the cells. Examples of these RKIP-modulated cascades are STAT3, NF-κB, Shh and Raf1/MAPK which are interestingly also involved in metastasis regulation, suggesting that RKIP might exert a dual function in metastasis and resistance by affecting common regulatory paths[117] (Figure 8). The underlying molecular mechanisms of resistance behind these pathways are usually based on the induction of anti-apoptotic genes, thus increasing tumour resistance to apoptosis and promoting neoplastic invasion. Thus, RKIP was demonstrated to be downregulated in several tumours resistant to conventional therapies, which might emphasize the activation of tumorigenic-related pathways responsible for the resistance pathway[166]. Yousuf *et al.* confirmed the importance of RKIP in this matter by reversing breast and prostate cancer resistance to microtubule inhibitors (MTIs) through the inhibition of STAT3 activity *in vitro* and *in vivo* upon RKIP overexpression[166].

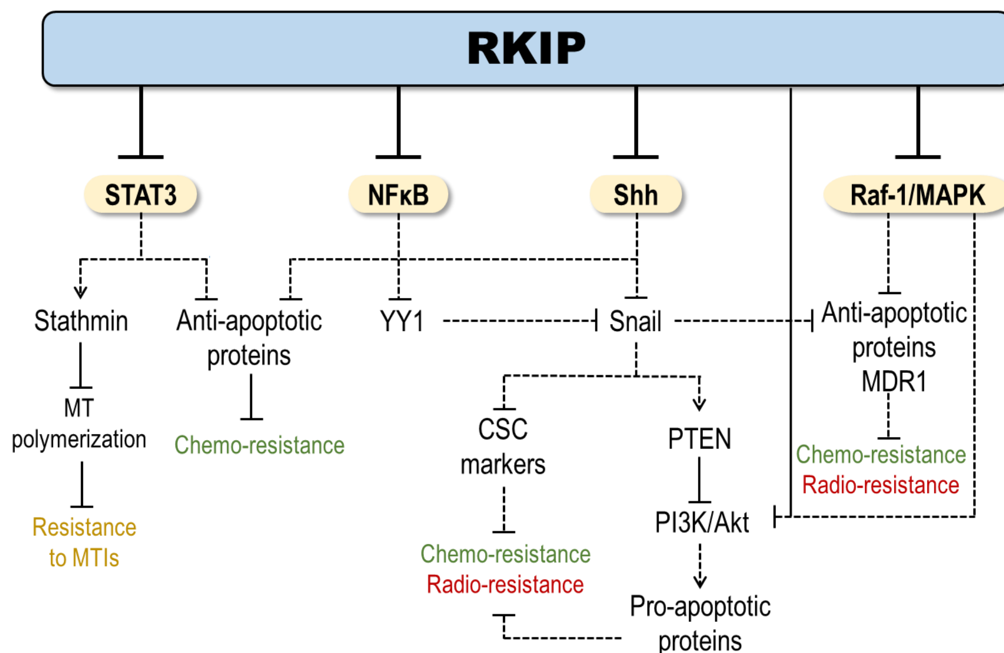

Figure 8 –Schematic representation of RKIP involvement with signalling pathways involved in the regulation of tumour resistance to therapy, namely chemo- and radiotherapy. Solid lines indicate the physiological functions of each protein on the

expression of downstream targets, whilst the dotted lines illustrate the downstream effect(s) of the referred protein of its levels by RKIP. MT: Microtubule; MTI: microtubule inhibitors; CSC: Cancer stem cells; MDR: Multi-drug resistance. Adapted from [117].

Besides all the knowledge collected in the last years, the role of RKIP in cancer is still puzzling. Although it seems clear that the different biological roles of RKIP could be mediated by its function as modulator of different intracellular signalling pathways, still, some work has yet to be done to dissect through each pathways RKIP is acting, in each particular tumour type.

CHAPTER 2:

Research Objectives

2. Research Objectives

RKIP has been the focus of several cancer-related studies. In fact, it seems that RKIP involvement in tumours malignancy occurs in a higher frequency when compared with *KRAS* or *BRAF* alterations (considered predictors of excellence in anti-EGFR therapy response) and with a higher penetrance among solid tumours. In addition, the loss of RKIP was implicated in resistance to chemo- and radiotherapy in various malignancies. Moreover, since RKIP has a recognized role in the regulation of important signalling pathways, such as the ones controlled by EGFR, and, because its downregulation is correlated with poor prognosis, we hypothesized that RKIP can be a novel predictive biomarker for therapy response in lung cancer. Regarding lung cancer in particular, although there is a fair amount of studies about RKIP, the literature is dispersed, especially when looking into its potential role as a prognostic biomarker. In this sense, we intended to tackle this project into three main goals.

The **first** main objective was to review all the available studies regarding RKIP in lung cancer, reaching important matters such as its expression levels and its value as a prognostic biomarker. Besides the importance of compiling a literature review, it was also our purpose to carry out an *in silico* analysis, to understand at what level these alterations in RKIP occur and in which particular subtypes of lung cancer.

Secondly, was aimed to further dissect the biological role of RKIP in lung cancer. For that it was used a panel of different histological types of lung cancer cell lines and the work divided into 2 tasks:

1. Characterization of RKIP and EGFR signalling in lung cancer cell lines;
2. Determine the RKIP associated phenotype through different *in vitro* assays, particularly viability, migration and colony formation and *in vivo* by using the NOD scid gamma mouse (NSG) model and Chick Chorioallantoic Membrane (CAM) assay.

Finally, in the **third** aim was intended to shed light on the specific role of RKIP in the modulation of lung cancer cells response to EGFR targeted therapies. To achieve such, the work was divided in 3 parts:

1. Assess the sensitivity of RKIP low expressing cells to EGFR inhibitors;
2. Unravel through which pathways can RKIP be modulating primary response to anti-EGFR drugs;
3. Validate results and determine the RKIP-associated signature by *in silico* analysis.

CHAPTER 3:

Materials and Methods

3.1 Literature Revision

A search of PubMed and Google Scholar was performed to identify all literature regarding RKIP in lung cancer, particularly studies exploring the expression levels, the clinical outcome, the biological and response to therapy role of this protein in this type of malignancy. To do so the terms 'Raf Kinase Inhibitory Protein', 'RKIP' and 'PEBP1' were crossed with 'Lung', 'Lung diseases', 'Lung cancer', 'NSCLC'. The search end date was 1st of March 2019, with no starting date.

3.2 *In silico* analysis

The cBioPortal for Cancer Genomics (http://www.cbioportal.org) is a repository of cancer genomic datasets that was used to analyse RKIP alterations and its clinical significance. In the present study, putative copy number alterations were analysed, as well as, mRNA expression, protein expression and survival data from a total of 4028 samples that belongs 17 studies regarding lung cancer from the TCGA dataset by March 2019. According to the TCGA guidelines (http://cancergenome.nih.gov/publications/publicationguidelines), this dataset has no limitations or restrictions. Significant alterations in copy number were obtained by GISTIC method and alterations in mRNA expression (RNA Seq V2 RSEM) and protein expression (RPPA) were determined by Z-score threshold of ± 2. Alteration on RKIP were considered for associations with overall survival using the Kaplan–Meier method with log rank testing with $p < 0.05$.

Data from genes (mRNA and/or protein) 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/), DAVID functional annotation tool (https://david.ncifcrf.gov/) and KEGG database (https://www.genome.jp/kegg/).

3.3 Cell lines and cell culture

For this work, a panel of different molecular types of NSCLC cell lines were used (Table 3). All cells were maintained and grown 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) at 37°C and 5% CO_2.

Table 3- Non-small cell lung cancer cell lines used in this work.

Cell line	Histological type	Origin	Molecular characteristics
A549	Adenocarcinoma	Primary tumour	*KRAS* mut (p.G12S)
HCC827	Adenocarcinoma	Primary tumour	*EGFR* mut (exon19del E747-A750)
H292	Mucoepidermoid Carcinoma	Metastatic tumour	*EGFR* and *KRAS* WT
PC9	Adenocarcinoma	Primary tumour	*EGFR* mut (Exon19del E746-A750)

Mut: Mutant; WT: Wild-type

3.4 Drugs

Erlotinib, Afatinib and AST1306 were obtained from Selleck-Chemicals (Houston, USA). All the drugs were prepared as stock solutions in Dimethyl Sulfoxide (DMSO) and stored at -20°C, as previous described[167]. In all experimental conditions the drugs were diluted in 0.5% FBS culture medium. The vehicle control was also used in all experiments.

3.5 *In vitro* RKIP knockout

Through CRISPR/Cas9 technology, a well-established genome editing tool, we generated a stable RKIP knockout (KO) in the NSCLC cell lines referred before. To do so, we used the CRISPR/Cas9 knockout kit from Santa Cruz Biotechnology (CRISPR/Cas9 KO Plasmid- sc-401270 and HDR Plasmid – sc-401270-HDR-2). The principle behind this kit is based on a co-transfection with two vectors, a CRISP/Cas9 KO plasmid (Cas9 plasmid, Figure 9A) and a Homology-direct repair plasmid (HDR plasmid, Figure 9B), both specific to target RKIP. Upon its encoding, Cas9 nuclease is guided to a specific site in RKIP gene thanks to a 20nt guide RNA (gRNA), this way ensuring maximum knockout efficiency. When positioned in the correct site, Cas9 induced a double strand break (DSB) in the genomic DNA. As a result of this break, it is expected that endogenous mechanism of repair are activated inserting parts of the HDR plasmid by homologous repair, as this has specific sequences (homologous arms) designed to bind RKIP sequence around the DNA breaking site. Hence, the HDR sequence is inserted into RKIP's sequence, which also contains a puromycin resistance gene, useful to later select the stable RKIP KO cells.

To perform the KO, each cell line was cultured into a 6-well plate at a density of $5x10^5$ cells per well in DMEM 10% FBS and allowed to adhere overnight. In the next day, co-transfection was done using the FUGENE HD reagent (Roche) according to the manufacturer's protocols, with 1µg of each plasmid at a ratio of 6:2 (reagent:plasmids), in serum free Opti-MEM media. Past 48h, the cells that had been successfully transfected were selected with varying concentrations of puromycin (H292=2.5 µg/ml; PC9=0.5 µg/ml; HCC827=0.75 µg/ml; A549=1 µg/ml)

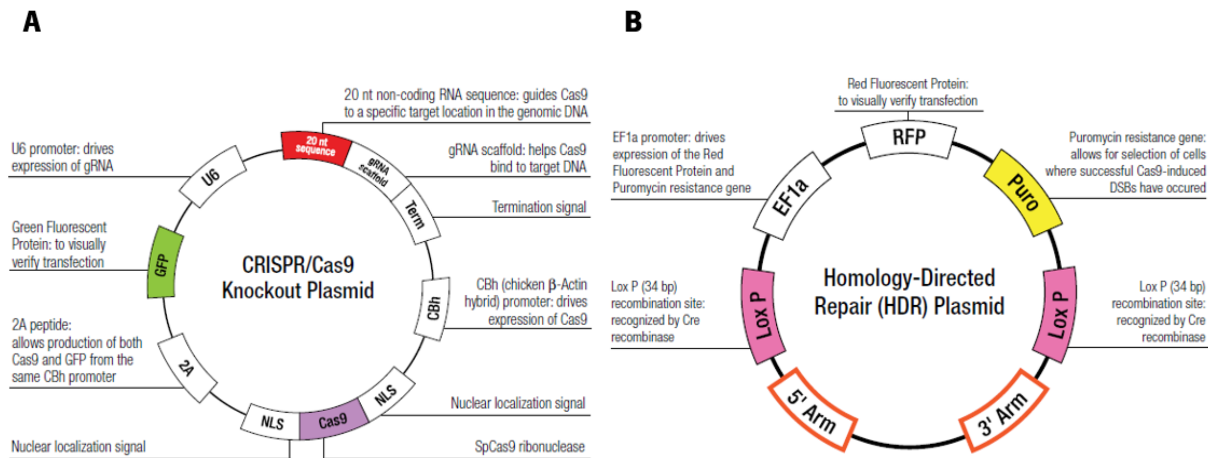

Figure 9- Vectors available in Santa Cruz Biotechnology for RKIP KO. (A) CRISP/Cas9 KO Plasmid and **(B)** Homology-Directed Repair Plasmid. Retrieved from Santa Cruz Website (https://www.scbt.com/scbt/home).

3.6 Immunofluorescence analysis

The cells were seeded on glass cover slips placed into 12-well plates until ~60% of confluence. Next, the cells were fixed and permeabilized in cooled methanol during 10 min. After blocking with 5% bovine serum albumin for 30 min, the cells were incubated overnight at room temperature (RT) with the primary antibody for RKIP (1:500, 07-137, EMD Millipore). After washing in Phosphate Buffered Saline (PBS), the TRITC Alexa Fluor-conjugated secondary antibody (Molecular Probes, Invitrogen) was used at a dilution of 1:500 for one hour at RT protected from light. Finally, after washing in PBS, cells were mounted in Vectashield Mounting Media with 4',6-diamino-2-phenylindone (Sigma) and images were obtained with a fluorescence microscope (Olympus BX61) at 200X magnification, using Cell P software.

3.7 Immunohistochemistry analysis

Histological slides with 4 µm-thick tissue sections were subjected to immunohistochemical analysis according to the streptavidin-biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation), as previously described [116,167]. Briefly, deparaffinised and rehydrated slides were submitted heat-induced antigen retrieval for 20min at 98°C with 10mM citrate buffer (pH 6.0). As primary antibodies it was used the following specific antibodies: RKIP (1:800, 07-137, EMD Millipore) and Ki-67 (Gennova, dilution 1:100). All the primary antibodies were incubated ON at 4°C. The secondary biotinylated goat anti-polyvalent antibody was applied for 10 minutes followed by incubation with the streptavidin-peroxidase complex. The immune reaction was visualized by 3,3'-Diamonobenzidine (DAB) as a chromogen. All sections were counterstained with Gill-2 haematoxylin. For negative controls, primary antibodies were omitted and also replaced by a universal negative control

antibody (CEA, rabbit anti-human, DAKO Corporation). Prostate carcinoma tissues were used as positive controls [116]. Stained slides were evaluated and then photographed under a bright field microscope Olympus BX61 at 200X magnification, using Cell P software.

3.8 Cellular viability assay

To assess cellular viability overtime, the cells were seeded into 96-well plates in triplicate at a density of $3x10^3$ cells per well and allowed to adhere overnight in DMEM 10% FBS. In the following day, the cells were submitted to two different medium conditions (DMEM 10% FBS or DMEM 0.5% FBS) and incubated for 24, 48 and 72 hours. The day of conditions imposition is considered the 0h time point. The Viable cells were quantified overtime by MTS assay- Cell Titer96 Aqueous cell proliferation assay (Promega). The results were calibrated to the starting value (time 0 h, considered as 100% of viability) and expressed as the mean ± SD. The assay was done in triplicate at least three times.

To determine the concentration at which 50% of the cell viability is inhibited by drugs treatment (half maximal inhibitory concentration - IC_{50}), the cells were seeded in 96-well plates at a density of $5x10^3$ cells per well and allowed to adhere overnight in DMEM 10% FBS. Following, the cells were treated with increasing concentrations of the drugs or with DMSO alone, both diluted in 0.5% FBS culture medium to a final concentration of 1% DMSO. After 72 hours, cell viability was quantified using the Cell Titer96 Aqueous cell proliferation assay (Promega). The results were expressed as the mean percentage ± SD of viable cells relative to the DMSO alone (considered as 100% viability). The IC_{50} was calculated by nonlinear regression analysis using GraphPad Prims software version 6.

3.9 Wound Healing Migration Assay

The cells were seeded in 6-well plates and cultured to at least 95% confluence. Monolayer cells were washed with PBS, scrapped with a plastic 1000μl pipette tip and incubated with fresh DMEM medium with either 10% FBS or 0.5% FBS. The “wounded” areas were photographed by phase contrast microscopy at specific time points: For H292 cell line it was 12, 24 and 48 hours; For PC9 cell line it were 8, 12, 24 and 32 hours. The relative migration distance was calculated by the following formula: percentage of wound closure (%) = 100 (A-B)/A, where A is the width of the cell wounds before incubation, and B is the width of cell wounds after incubation. The results are expressed as the mean ± SD. The assay was done in triplicate at least three times.

3.10 Clonogenicity assay

The cells (750 cells/well) were seeded in 12-well plated and incubated overnight to adhere. Medium was replaced for DMEM 0.5% FBS and cells incubated for 10-15 days, with medium renewal after 3 days. The colonies were stained with 5% Giemsa for 45 minutes and manually counted. Results were expressed as the mean colonies ± SD. The assay was done in triplicate at least three times.

3.11 Western Blot analysis

The cells were seeded in a 6-well plates at a density of $1x10^6$ cells per well and allowed to adhere overnight. In the next day, the cells were serum starved by 4 hours, or when necessary, by 2 hours followed by a 2 hours-treatment with drugs. Before the end of the time point, cells were also, when necessary, stimulated with 10 ng/ml of Epidermal Growth Factor (EGF) for 15 minutes. The cells were scrapped in lysis buffer containing phosphatases and proteases inhibitors, and then centrifuged at 13000 rpm for 15 minutes at 4°C. Protein quantification was performed using Bradford reagent (Sigma-Aldrich). Aliquots of 50μg of total protein from each sample were separated on 10% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (100V) and transferred onto a nitrocellulose membrane (Amersham Biosciences) in 25mM Tris-base/glycine buffer using the Trans-Blot Turbo Transfer System (25V, 1A for 30 min). The membranes were blocked with milk 5% Tris-Buffered Saline/0.1% Tween (TBS-Tween) for 1 hour at RT and incubated overnight with the primary antibodies at 4°C (Table 4). Next, after washing in TBS-Tween, the membranes were incubated with the respective secondary antibodies coupled with horseradish peroxidase (1:2500, Cell Signalling). Tubulin was used as loading control. Blots detection was done by chemiluminescence (Supersignal West Femto kit, Pierce, Thermo Scientific) using the ChemiDoc™ XRS+System (Bio-Rad).

3.12 Chick Chorioallantoic Membrane (CAM) assay

CAM assay was used to assess *in vivo* tumour growth, as previously described [167]. Fertilized chicken eggs were incubated at 37°C and 70% humidity, and on day 3 of development, a window was made into the shell, sealed with tape and the eggs were returned to the incubator. On day 9, a suspension of $1x10^6$ cells and 20μl of matrigel were injected over the CAM. On day 13, the tumours formed were photographed *in ovo* using a stereomicroscope (Olympus S2x16) at 16X magnification, and the tumours were treated with the Afatinib 10 nM for more four days. At day 17 of development the tumours were again photographed *in ovo* (16X magnification) and *ex ovo* (20X magnification). The chickens were sacrificed at

-80°C for 10 minutes, and the tumours were fixed with paraformaldehyde at 4% to further analysis. The perimeter of the tumours was measured using Image J *in ovo* at day 13 and the results were expressed as mean perimeter ± SD of the tumours from each group. Concerning tumours treated with Afatinib, the tumour perimeter was determined *in ovo*, and expressed as the difference between the tumour perimeter in day 13 and 17 of development. For blood vessel counting, photographs were taken at day 17 *ex ovo*, and the results were expressed as the mean ± SD of the vessels counted for each group of isoforms/treatments. For a blood vessel count, we used the Angio Tool, the adjustments of diameter and intensity were made, until an overlap of the markers corresponds to the blood vessels; we use default settings for all photos.

Table 4- Details of the primary antibodies used for Western Blot.

Protein target	Reference	Dilutin (Secondary Antibodies)
p-EGFR (Tyr1068)	D7A5 (CS)	1:1000 (Rabbit)
EGFR	D38B1 (CS)	1:500 (Mouse)
p-MEK1/2 (Ser217/221)	41G9 (CS)	1:1000 (Rabbit
MEK1/2	L38C12 (CS)	1:1000 (Mouse)
p-ERK1/2 (Trh202/tyr204)	D13.14.4E (CS)	1:2000 (Rabbit)
ERK1/2	137F5 (CS)	1:2000 (Rabbit)
p-AKT (Ser473)	D9E (CS)	1:2000 (Rabbit)
AKT	C67E7 (CS)	1:200 (Rabbit)
p-GSK-3β(Ser9)	D85E12 (CS)	1:1000 (Rabbit)
p-STAT3 (Tyr705)	D3A7 (CS)	1:1000 (Rabbit)
p-NF-κB p65 (Ser536)	93H1 (CS)	1:1000 (Rabbit)
RKIP	D42F3 (CS)	1:1000 (Rabbit)
p-RKIP (Ser153)	SC-135779	1:1000 (Mouse)
E-cadherin	24E10 (CS)	1:1000 (Rabbit)
Vimentin	D21H3 (CS)	1:1000 (Rabbit)
N-cadherin	D4R1H (CS)	1:1000 (Rabbit)
YY1	SC-7341	1:1000 (Mouse)
Snail	C15D3 (CS)	1:1000 (Rabbit)
SLUG	C19G7 (CS)	1:1000 (Rabbit)
α-Tubulin	SC-73242	1:5000 (Mouse)

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

3.13 Xenograft mouse model

With the purpose of evaluating the role of RKIP in tumour growth, a pilot study was done using subcutaneous NOD scid gamma mouse (NSG) model with PC9 cell line. For that, the number of cells to inject was firstly optimized. Thus, a group of 9 animals (9-10 weeks old) were used, in which three groups of randomly three animals were injected subcutaneously in its right flank with $2x10^6$ cells with Matrigel (1:1), $3x10^6$, or $5x10^6$ PC9 cells. Tumour growth was measured with a calliper twice a week and tumour volume was calculated by the following formula: Volume = 3,14 (D^2 x d)/6, where D is the major tumour axis and d is the minor tumour axis. Of this experiment, it was determined that the ideal quantity of PC9 cells to inject would be $3x10^6$ and so, we continued this pilot study using 10 animals, randomly divided into 2 groups: one was injected with PC9 control cells and the other was injected with PC9 RKIP KO cells at the quantity referred before. When tumours reached $1cm^3$ mice were sacrificed and the tumour was harvested and preserved in 4% paraformaldehyde and then included for histological analysis.

Throughout the experiments the mice were observed periodically for signs of morbidity/mortality. The animals were maintained under standard laboratory conditions, which included an artificial 12 h light/dark cycle, controlled ambient temperature (21 ± 1 °C) and a relative humidity of 50-60%. All the experiments with mice were done under approval of the institutional ethical committee (ORBEA, Portugal) and in accordance with European Union Directive 2010/63/EU.

3.14 Statistical analysis

For *in vitro* and *in vivo* assays, statistical analysis was performed using GraphPad Prism 6 version. To analyse the *in vitro* and *in vivo* 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

Part of the results presented in this chapter (section 4.1) were published in an international peer reviewed journal:

Ana Raquel-Cunha, Diana Cardoso-Carneiro, Rui M. Reis, Olga Martinho. Current Status of Raf Kinase Inhibitor Protein (RKIP) in Lung Cancer: Behind RTK Signalling. *Cells*, 2019, 8, 442.

4.1 RKIP and lung cancer: Literature Review

4.1.1 Expression and Prognostic Value

Regarding RKIP expression and its clinical significance in lung cancer, we have found that the studies available are still scarce and inconclusive, and are not concordant among them (summarized in Table 5)[164,168-172].

In general, RKIP mRNA was detected in 41.9% and 47.7% of NSCLC patients[168,169], while RKIP protein positivity has been described by immunohistochemistry (IHC) in among 49.1% to 64.5% of NSCLC patients[164,170,171]. This was independent of the histological type, as all the studies that compared AC with SCC found no statistically significant differences among them, both at the mRNA and protein levels (Table 5)[164,169,170]. Additionally, there is one study that assessed the expression levels of the inactive form of RKIP, with phosphorylation at serine 153 (pRKIP)[172]. The study is unclear because the authors do not state the percentage of positive cases, but from their survival curves we were able to estimate that the minority of patients expressed pRKIP, corresponding to 37.6% (140/372) of the samples (Table 5)[172]. Overall, the studies are not comparable due to the different techniques (RT-PCR vs. QPCR), or antibodies and methodologies (IHC), used. The highest discrepancies were found in protein studies (Table 5) as the variability inherent to IHC studies is well-known. Furthermore, the median percentage of RKIP positive cases is higher in IHC studies than in mRNA ones, which is probably also due to the variability and lower specificity of IHC (Table 5).

RKIP is a well-established metastasis suppressor[113,156,173]. It is described in several tumour types as underexpressed in primary tumours when compared to normal tissues, and significantly decreased or even absent in metastases[137]. In lung cancer, Zhu C *et al.* found a significant reduction of RKIP mRNA expression levels in tumour tissues when compared to the surrounding normal tissues, which showed 76.7% (66/86) RKIP positivity (Table 5)[168]. In accordance, even without specifying the percentage of positive samples, Wang Q *et al.* described a similar difference between normal and tumour samples by QPCR[169].

At the protein level, Huerta-Yepez S *et al.* compared the expression levels of RKIP between lung tumour and normal tissues in a large series of samples (671 lung tissues) by analysing both RKIP and pRKIP expression through an IHC approach[172]. The authors did not find differences in total RKIP expression levels between normal epithelium, primary NSCLC, or metastatic lesions, but instead described a slight statistically significant decrease in pRKIP expression in metastatic, compared to nonmalignant, lesions[172]. Consistently, they also showed that lower levels of pRKIP are correlated with poor outcome, however,

contradictorily, that was not concordant with their own findings, which showed that a higher pRKIP expression level is associated with aggressiveness markers, such as age and presence of lymph node metastases[172]. Looking deeply into the data, the results are unexpected, because, by concept, RKIP phosphorylation at serine 153 dissociates RKIP from Raf-1, reversing its inhibitory function[106]. Therefore, it is expected that expression of pRKIP should result in a poor outcome. In fact, for other tumours, such as multiple myeloma and stage II colon cancer, pRKIP may contribute positively to overall cell survival and drug resistance, and hence, tumour aggressiveness[174,175]. However, other studies in melanoma and breast cancer, such as the study by Huerta-Yepez S *et al.*[172], have also shown that low levels of pRKIP could predict poor survival in comparison with relatively higher expression[176,177]. Thus, the clinical differences of pRKIP in different tumours are worth further study.

Recently, using a different antibody for total RKIP, Wang A *et al.* observed negative or weak staining of RKIP in the majority of lung tumour tissues, compared with the intense staining of noncancerous tissues, as expected[171]. Furthermore, even though they did not analyze RKIP expression in metastatic tissues as in the Huerta-Yepez S *et al.* study[172], the authors found a statistically significant association between low total RKIP protein expression levels and higher TNM stage, and presence of lymph node and distant metastases[171], a result that is concordant with the remaining studies available, either at the mRNA[168,169] or protein level[164,170] (Table 5).

Regarding the predictive role of RKIP in the prognosis of lung cancer patients (Table 5), the results are also ambiguous. As cited above, Huerta-Yepez S *et al.*[172] found that low levels of pRKIP were an independent poor prognostic marker, while total levels of RKIP had no predictive value in their cohort of patients[172]. However, a more recent study showed that a decrease in the total levels of RKIP expression constitutes an independent poor prognostic marker in NSCLC patients, as assessed by IHC in primary tumours[164]. Interestingly, the authors also showed that the RKIP expression level was generally lower in radioresistant NSCLC tissues, pointing out its putative role in radiotherapy response modulation[164]. The difference among the two studies is most likely because the first study used tissue microarrays (TMAs), which we showed previously was not the best way to study RKIP expression because it requires the largest representative sample possible as loss of RKIP expression in primary tumours is essentially focal[97,115,116,130,178,179]. Technical problems explaining the difference are unlikely because Huerta-Yepez S *et al.*[172] used the same antibody (from Millipore, Upstate Biotechnology) that we and other authors have used without problems[97,115,116,130,178,179].

Importantly, two distinct papers showed that RKIP expression levels are associated with the expression of other cancer-related proteins in lung tumour tissues, such as positive expression of E-

Cadherin [168] and negative expression of phosphorylated STAT3[171]. The associations between RKIP, E-cadherin, and STAT3 are not novel, and are also well described in other tumour types (as reviewed in[117]), emphasizing the biological importance of studying RKIP in lung cancer, as will be reviewed in the next section.

Although many questions remain regarding the best method to reliably detect RKIP expression levels in lung cancer, most studies agree on the clear association between low expression of RKIP and higher TNM stage or presence of lymph node metastases (Table 5). More studies are still needed to validate its prognostic value, both in its active or inactive (pRKP) form.

4.1.2 RKIP Biological Role: A Modulator of Cell Signalling

The first evidence showing RKIP as a modulator of cell signalling in lung cancer came from *in vitro* experiments using a *KRAS* adenocarcinoma mutated cell line (A549)[171]. The authors demonstrated, by lentiviral overexpression, that RKIP decreases the levels of IL-6 dependent ERK and STAT3 phosphorylation (Figure 10) and, consequently, the cells migratory capacity[171].

Furthermore, it was demonstrated that RKIP acts as a physiological inhibitor of NOTCH1, a major player in EMT and metastases[180]. Using H1299 cells, transfected to overexpress RKIP, the authors demonstrated that RKIP directly interacts with the full-length of NOTCH1, preventing its proteolytic cleavage and NICD release (Figure 10), decreasing EMT markers like Vimentin, N-cadherin and Snail. As a consequence, the migratory and invasive capacity of the cells also decreased, a phenotype that was reverted *in vivo* by *RKIP* knockdown in A549 cells[180].

Signalling axes involving RKIP and microRNAs were also described in NSCLC as important modulators of EMT and metastasis[135,181]. Using the A549 cell line, it was found that, by downregulation of RKIP, miR-27a increases Vimentin expression, as well as cell invasion capacity, and decreases E-cadherin levels[135]. Furthermore, it was demonstrated that dysregulation of the miR-150-FOXO4 axis promotes EMT through modulation of the NF-κB /SNAIL/YY1/RKIP loop[181]. *In vitro* assays showed that miR-150 downregulates FOXO4, resulting in increased levels of NF-κB and its targets, SNAIL and YY, which in turn, will lead to RKIP downregulation[181].

Table 5- Raf kinase inhibitory protein (RKIP) expression and its clinical impact in lung cancer patients.

	Positive RKIP Expression (%)				Prognostic Value	Clinical Correlations	Molecule Analyzed (Technique)
	AC	SCC	AC+SCC	Nontumour			
Zhu C *et al.*, 2012 [168]	-	47.7% (41/86)	47.7 % (41/86)	76.7 % (66/86)	-	LN metastasis; TMN stage; E-Cadherin expression	mRNA (RT-PCR)
Wang Q *et al.*, 2014 [169]	49% (31/63)	32.7% (16/49)	41.9% (47/112)	Yes [†]	-	LN metastasis; TMN stage	mRNA (QPCR)
Yan H *et al.,* 2012 [170]	52.6% (30/57)	47.0% (48/102)	49.1% (78/159)	-	-	LN metastasis; TMN stage	Protein (IHC [1])
Shi-Yang X *et al.*, 2017 [164]	53% (16/30)	79% (44/63)	64.5% (60/93)	-	Yes (independent)	LN metastasis; TMN stage; Radiotherapy resistance	Protein (IHC [2])
Wang A *et al.*, 2017 [171]	-	-	51% (51/100)	Yes[†]	-	LN metastasis; TMN stage; Distant metastasis; phosphorylated STAT3	Protein (IHC [3] e WB)
Huerta-Yepez S *et al.*, 2011 [172]	-	-	37.6% (140/372) [4,*]	Yes[†]	Yes (independent) [1]	LN metastasis; Age	Protein [4] (TMA, IHC, WB)

AC: adenocarcinoma; SCC: Squamous Cell Carcinoma; LN: Lymph Node; IHC: Immunohistochemistry; WB: Western Blot; TMA: Tissue Microarray; RT-PCR: semi-quantitative PCR: QPCR: Real Time PCR. [†] Comparison of expression between surrounding healthy tissue and tumour tissue was performed and found significantly lower in tumour tissues, but percentage of RKIP positivity in nontumour tissues were not discriminated. [1] RKIP antibody not specified. [2] Antibody: ab76582, Abcam, Cambridge, MA, USA (Dilution—1:400). [3] Antibody: reference 13006, Cell signalling Technology, Inc. (Danvers, MA, USA) (Dilution: 1:200). [4] Relative to phosphorylated RKIP: Rabbit-anti-human pRKIP from Santa Cruz Biotechnology (Dilution—1:250). * Calculated from the survival curves presented in the paper.

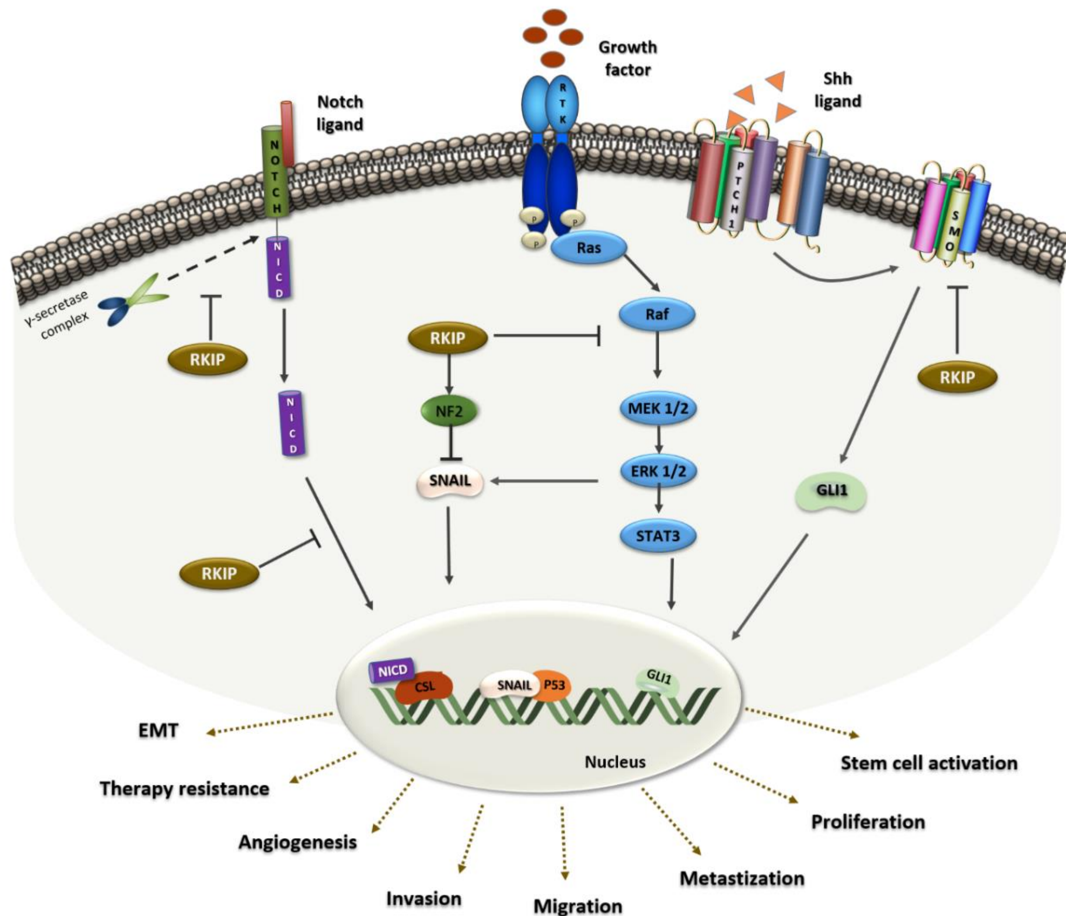

Figure 10- RKIP protein as a signalling modulator in lung cancer. On the left, RKIP binds to the Notch Intracellular Domain (NICD) preventing the proteolytic cleavage by the γ-secretase complex. Furthermore, in the Notch1 pathway, RKIP inhibits the translocation of NICD to the nucleus, which would then activate the translocation of EMT-related genes, ultimately promoting cell invasion and metastasis. In the middle, RKIP binds to Raf, preventing the phosphorylation of MEK by Raf and consequently, Raf/MEK/ERK/STAT3 signalling is inhibited. This will enhance events such as angiogenesis, proliferation and metastization. Additionally, RKIP blocks Snail through MAPK inhibition and NF2 stabilization. In the nucleus, SNAIL acts as a p53 suppressor and upon this EMT related-processes will occur. On the right, RKIP act as an inhibitor of the Shh signalling pathway. RKIP binds to the SMO receptor, keeping it inactive and preventing Gli1 transcription, and promoting therapy resistance and stem cell activation.

RKIP was also identified as a p53 modulator in malignant pleural mesothelioma (MPM), an asbestos-induced human lung cancer[182]. Using MPM and NSCLC cell lines treated with silica, an increase in ERK activation and a decrease in p53 expression levels promoted by RKIP depletion were observed. In this RKIP tumour-promoting context, MAPK signalling activation and neurofibromatosis 2 (NF2) protein inactivation triggers SNAIL expression that ultimately leads to p53 and E-cadherin inhibition (Figure 10)[182].

Furthermore, it was demonstrated that the expression levels of the signal transducer Smoothened (SMO) and Gli1, a zinc-finger transcription factor, are decreased in RKIP knockdown cells, pointing to

RKIP as an inhibitor of Sonic Hedgehog (Shh) signalling[164]. Briefly, in the activated state of the pathway, the ligand Shh binds to a transmembrane protein receptor, Patched-1 (PTC1), which loses its catalytic inhibition of SMO (Figure 10). Consequently, active SMO will trigger the transcription of the Shh target gene Gli1, which acts as a transcriptional activator of numerous genes, regulating proliferation, differentiation, extracellular matrix interactions, and cancer stem cell (CSC) activation[183]. Mechanistically, RKIP binds to SMO keeping it inactive and preventing the transcription of Gli1 (Figure 10)[164].

Finally, as referred to before, RKIP was established as a metastasis suppressor for the first time in prostate cancer, where it was reported that low RKIP expression in primary tumours increases the probability of lung metastasis development[113], a finding that was further demonstrated for other tumours of different primary sites[120,155,184,185]. Beshir *et al.*, by using a breast cancer orthotopic model injected with RKIP expressing cells, showed that tumours expressing RKIP formed less lung metastases[120]. Later on, in an attempt to understand the mechanism behind this event, Dattar *et al.* proposed that RKIP inhibits the occurrence of lung metastases through the regulation of the CCL5 protein and the reduction of macrophage lung infiltration[184]. Similarly, in the nasopharyngeal carcinomas, RKIP downregulation promotes invasion, metastasis and EMT by activating STAT3 signalling[155]. Using a different approach, it was recently shown in the hepatocellular carcinomas, that somatostatin octapeptide significantly reduced the occurrence of pulmonary metastases *in vivo* by increasing RKIP levels in the primary tumour[185].

4.1.3 RKIP Implications in Therapy Response

RKIP has been reported as an important molecular player in the modulation of tumour cells resistant to conventional therapies, however the mechanisms behind this remain largely unclear[157,186-188].

Concerning NSCLC, it has already been reported *in vitro* that A549 cells treated with the chemotherapeutic agents, adriamycin and 9NC, increased RKIP expression in a time and dose dependent manner. Activation of RKIP expression is, in the case of adriamycin, fully dependent on the p53 transcription factor, which is able to bind to RKIP's promotor at two different binding sites[186].

Moreover, gemcitabine, another chemotherapeutic drug, also induces RKIP expression not only in the A549 cell line, but also in the CALU-1, CALU-6, H23, and HCC 827 cell lines[189]. The authors demonstrated that gemcitabine and sorafenib—an oral multikinase inhibitor that decreases the kinase activity of both C-RAF and BRAF—interact with each other, resulting in potent inhibition of cell proliferation and induction of apoptosis. In this synergistic interaction, Raf inhibition, a pharmacologic effect of sorafenib, is enhanced by gemcitabine as a consequence of its ability to induce RKIP expression[189]. NF-κB activation was suggested by the authors to be a possible mechanism for gemcitabine-

mediated RKIP induction, a consequence of DNA damage induced by the same drug[189]. Later, Giovannetti *et al.* also studied the synergistic interaction between sorafenib and erlotinib, reporting that sorafenib slowed cell cycle progression and induced apoptosis, which was significantly increased with the combination of drugs[190]. Moreover, sorafenib-related reduction of AKT/ERK phosphorylation in erlotinib-resistant cells (A549 and H1975) was associated with significant RKIP upregulation, probably by NF-κB activation, a consequence of erlotinib's EGFR inhibition[190].

RKIP was further implicated in the mechanism through which miR27a regulates cisplatin resistance in the A549 cell line[135]. Li *et al.* reported, both *in vitro* and *in vivo*, that miR27a appears to be increased in cisplatin-resistant A549 cells when compared with the parental A549 cell line, while RKIP, which they report as a direct target of miR27a, appears to be decreased. RKIP knockdown in the A549 cell line decreased the sensitivity to cisplatin, while ectopic expression of RKIP, in part, rescued miR27a-mediated resistance to cisplatin[135]. Importantly, the authors were also able to demonstrate an association between miR27a and RKIP expression with chemotherapeutic resistance using clinical tumour tissue samples collected from patients with advanced lung adenocarcinoma[135].

Similarly, Xie *et al.*, through the analysis of RKIP expression in a series of human NSCLC tissues divided into radiosensitive and radioresistant, reported that RKIP expression levels were positively correlated with radiosensitivity[164]. Accordingly, the authors demonstrated *in vitro* that both the A549 and SK-MES-1 cell lines, with RKIP knockdown, showed increased resistance to different degrees of radiotherapy as well as lower radiation-induced apoptosis[164]. The modulation of the Shh pathway, specifically its activation through RKIP depletion, was one of the mechanisms proposed to explain radioresistance. The authors demonstrated that, in RKIP knockdown cells, Gli1 overexpression increased the number of CSCs, somehow explaining the observed radioresistance *in vivo* [164].

4.1.4 RKIP and Lung Cancer: *In Silico* Analysis

Recently, Zaravinos A and colleagues, by analysing RKIP mRNA expression across 37 different cancer types and using data from The Cancer Genome Atlas (TCGA) platform, showed that RKIP is downregulated compared to normal lung tissues, with lung adenocarcinoma being among the eight tumour types with the lowest RKIP expression levels[117]. Another study, using the same database, suggested that RKIP downregulation in cancer is not due to genetic or mutation events, but rather to transcriptional or post-transcriptional mechanisms[191]. Even so, breast cancers, gliomas, and NSCLCs seem to present the highest RKIP genetic heterogeneity among the 25 tumour types analysed[191].

Analysing the TCGA data with regard to lung cancer[192-200] shows that there are 17 studies available at cBioPortal database (www.cbioportal.org) that account for more than 4028 samples. We found that RKIP molecular alterations are in fact a rare event (<0.5% of altered cases among the 4028), and they occur exclusively in NSCLC (Figure 11A and Table 6). In total, there are 14 cases that depict *PEBP1* gene alterations: three missense mutations, one frameshift deletion, one fusion with *HECTD4* gene, four cases with gene amplification, and five with homozygous deletion (Table 6). Remarkably, disregarding the case with gene fusion, all other mutated cases (4/5) have hotspot mutations in known lung cancer-related genes, while those alterations were found in only 44% (4/9) of the tumours with *PEBP1* copy number alterations (CNA) (Table 6). Regarding *RKIP* mutations, all were found in exons 2 and 3 at the phosphatidylethanolamine-binding domain of the protein (Figure 11B). Interestingly, even though it is a rare event, a significant association was found between the presence of genomic alterations in the *PEBP1* gene and a poor overall survival in NSCLC (Figure 11C); knowing this, it could be interesting to include mutational and CNA analysis in the studies aiming to explore the prognostic value of RKIP in the future.

Regarding mRNA expression, relative to RNA-seq data available from the TCGA PanCancer Atlas[192-197], we observed that SCC cases present lower mean levels of RKIP mRNA when compared to AC (Figure 11D). Specifically, categorizing the patients by RKIP mRNA up and downregulation (as defined by the cBioPortal settings), mRNA upregulation was found in around 4.7% of the total cases (51/1094), including both AC (5.49%, 28/510), and SCC (4.75%, 23/484), while data for the RKIP mRNA downregulation (1%, 11/1094) was higher in SCC (1.86%, 9/484) compared to AC (0.39%, 2/510) (Figure 11E). The remaining cases (94%, 1032/1094) are described in the database as having "no altered" mRNA expression (i.e., up or downregulated), and are considered positive with normal expression levels of RKIP mRNA. No statistical associations were found between RKIP mRNA expression and patient survival, still, patients with lung AC overexpressing RKIP have a double progression-free survival time when compared with the ones with no alteration in RKIP mRNA (67.18 vs. 35.58 months) (Figure 2F).

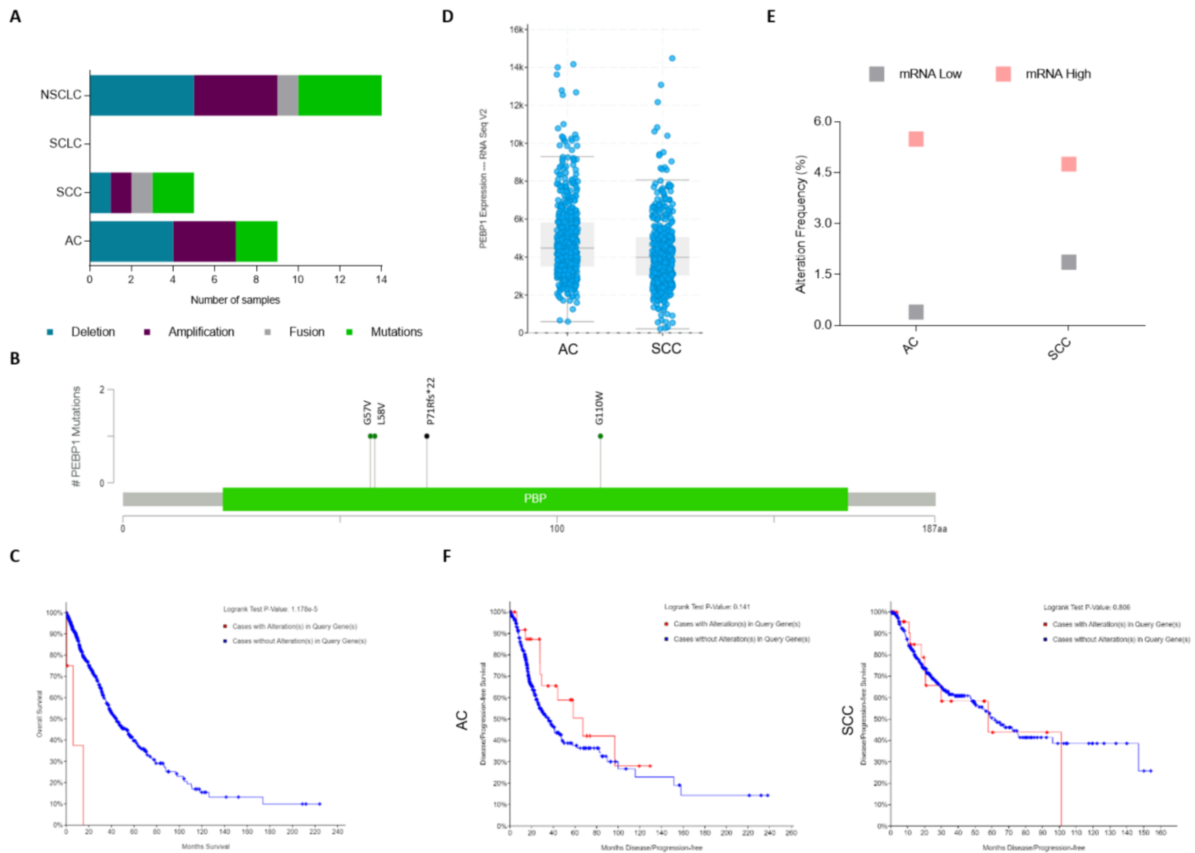

Figure 11- Lung cancer The Cancer Genome Atlas (TCGA) data for genomic alterations on the RKIP encoding gene (*PEBP1*). A) Number of cases depicting RKIP genomic alterations in the different histological types (from an analysis of 17 different studies containing 4028 samples). **B)** Scheme showing distribution of *PEBP1* mutations in the entire RKIP protein. **C)** Kaplan–Meier analysis of NSCLC patient's overall survival (OS) in months distributed by the presence (red line: 6.18 months of median OS, from 6 patients) or absence (blue line: 43.91 months of median OS, from 948 patients) of RKIP gene alterations ($p < 0.05$). **D)** RNA Seq V2 data, showing the mean of RKIP mRNA expression levels in AC (566 patients) and SCC (487 patients). **E)** Percentage of cases depicting mRNA up and downregulation in the different NSCLC histological types (refers to a total of 1094 cases). **F)** Kaplan–Meier analysis of NSCLC patient's progression-free survival in months, distributed by the presence (red line, 31 cases for AC and 27 SCC) or absence (blue line, 475 cases for AC and 348 SCC) of RKIP mRNA alterations. All data is available at www.cbioportal.org. SCLC: Small Cell Lung Carcinoma; NSCLC: Non-Small Cell Lung Carcinoma; AC: Adenocarcinoma; SCC: Squamous cell Carcinoma.

Additionally, stratifying the patients by CNA, we can unequivocally observe that RKIP mRNA expression levels vary and are wholly associated with the copy numbers of the gene in both histological types (Figure 12A), with CNA strongly associated with the 12q chromosome. Additionally, we were able to confirm for AC samples (the only samples with methylation data available) that there is a good negative correlation (Pearson = −0.33, $p = 1 \times e^{-12}$) between low mRNA expression levels and high methylation status of the gene (Figure 12B).

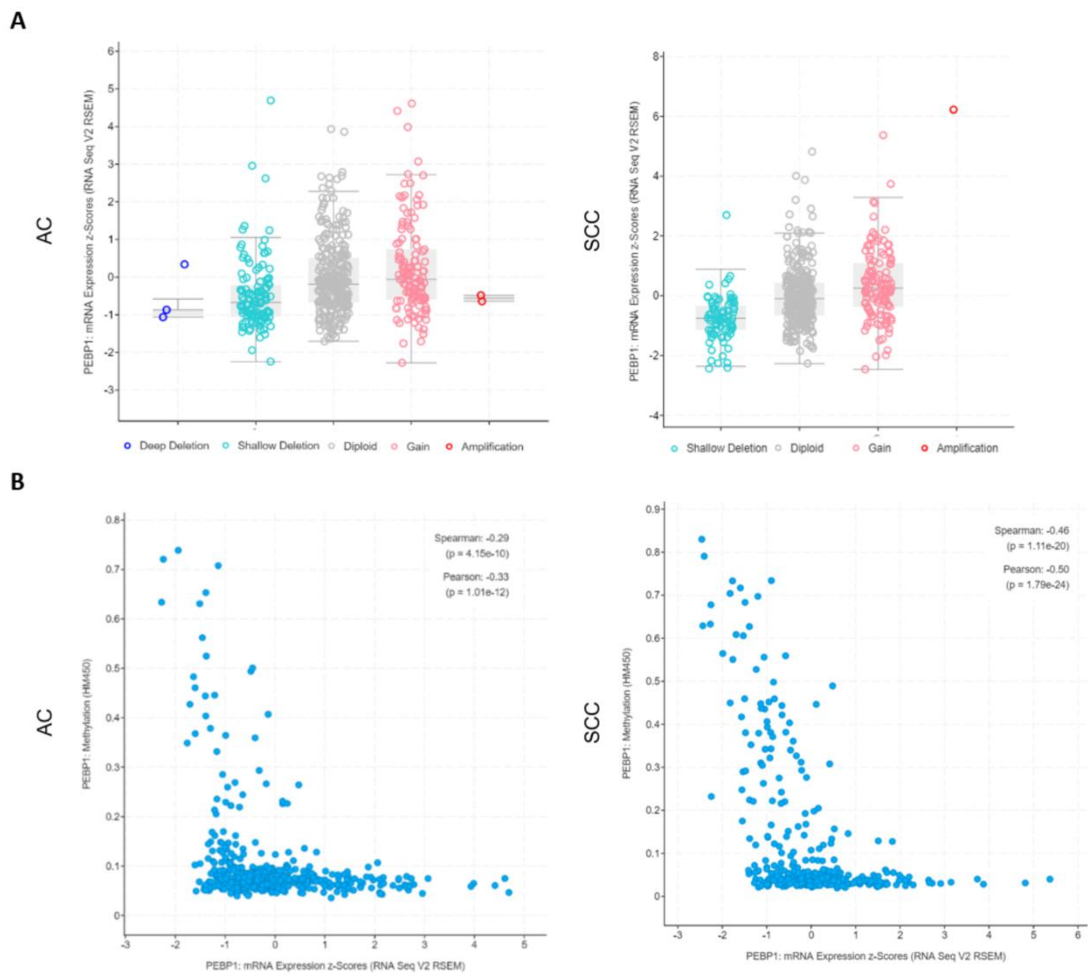

Figure 12- NSCLC TCGA data regarding RKIP encoding gene (*PEBP1*) alterations. A) RKIP mRNA expression levels according to the copy number variations status (X axis) of the *PEBP1* gene (refers to 515 AC and 501 SCC). **B)** Correlation between the levels of RKIP mRNA expression and methylation status of the *PEBP1* gene (refers to 515 AC and 501 SCC). All data is available at www.cbioportal.org. NSCLC: Non-Small Cell Lung Carcinoma; AC: Adenocarcinoma; SCC: Squamous cell Carcinoma

Table 6- Genomic alterations on PEBP1 gene in lung cancer (TCGA data) *.

Study Reference	Sample ID	Histology	PEBP1 Mutations	Mutation Type	PEBP1 CNA	Oncogenic Alterations
TCGA, Cell 2018 [193-195]	TCGA-05-4244-01	AC	G110W	Missense	no alteration	KRAS (G12C)
TCGA, Cell 2018 [193-195]	TCGA-97-7938-01	AC	L58V	Missense	no alteration	KRAS (G12C); ALK (E1299 *)
TCGA, Cell 2018 [193-195]	TCGA-34-5232-01	SCC	HECTD4-PEBP1	Fusion	no alteration	-
TCGA, Cell 2018 [193-195]	TCGA-66-2756-01	SCC	P71Rfs*22	FS del	no alteration	ROS1 (R1129S)
TCGA, Cell 2018 [193-195]	TCGA-66-2792-01	SCC	G57V	Missense	no alteration	EGFR AMP
Broad, Cell 2012 [198]	LUAD-B00416	AC	no alteration	-	AMP	-
TCGA, Provisional	TCGA-18-4083-01	SCC	no alteration	-	AMP	-
TCGA, Provisional	TCGA-44-7670-01	AC	no alteration	-	AMP	ALK (G446R)
TCGA, Nat Genet 2016 [199]	TCGA-50-5939-01	AC	no alteration	-	AMP	EGFR AMP
TCGA, Nat Genet 2016 [199]	LUAD-NYU994-Tumour	AC	no alteration	-	HOMDEL	-
TCGA, Provisional	TCGA-35-3615-01	AC	no alteration	-	HOMDEL	KRAS (G12C)
TCGA, Nature 2014 [200]	TCGA-55-6986-01	AC	no alteration	-	HOMDEL	ROS1 (Fusion)
TCGA, Nat Genet 2016 [199]	TCGA-75-6203-01	AC	no alteration	-	HOMDEL	-
TCGA, Cell 2018 [193-195]	TCGA-85-A513-01	SCC	no alteration	-	HOMDEL	-

*www.cbioportal.org; CNA: copy number alterations; AC: adenocarcinoma; SCC: Squamous Cell Carcinoma; FS del: frameshift deletion; AMP: amplification; HOMDEL: homozygous deletion.

4.2 Biological Role of RKIP in Lung Cancer

4.2.1 Characterization of RKIP and EGFR signalling in lung cancer cell lines

The second major aim of this work was to dissect the biological role of RKIP in lung cancer, and for that four molecularly different LC cell lines were used. To firstly characterize the cell lines, we started by assessing the expression levels of RKIP, as well as, the expression of one of the most important RTK in LC context, EGFR, and its main downstream signalling pathways, AKT and MAPK, all at basal and EGF stimulated conditions (Figure 13).

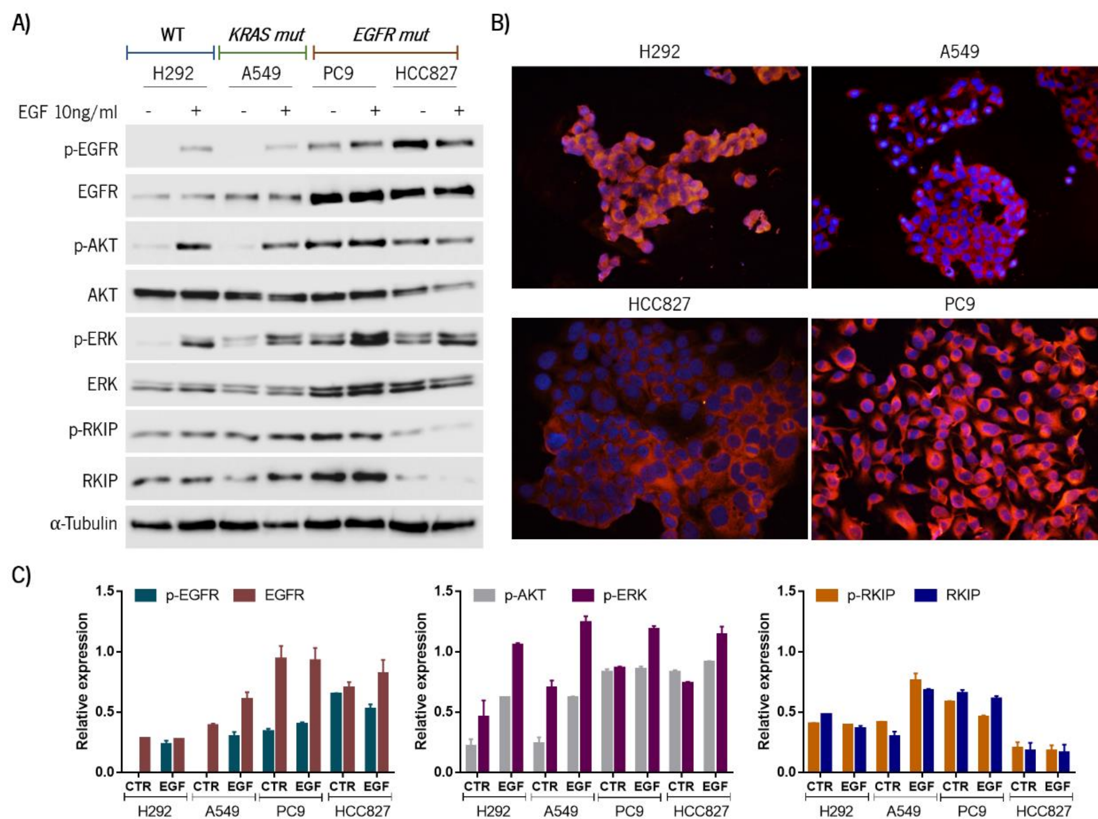

Figure 13- Characterization of RKIP and EGFR signalling proteins expression in LC cell lines. A) Analysis of EGFR, AKT, ERK, RKIP and their respective phosphorylated forms expression in lung cancer cell lines, with and without EGF stimulation, was done by Western Blot. EGF ligand was used at 10ng/ml for 15 minutes. Tubulin was used as loading control. This is a representative assay of two independent experiments. **B)** Immunofluorescence analysis of RKIP basal expression levels in LC cell lines (200x magnification). **C)** Quantification of WB results was performed using band densitometry analysis with Image J software. Relative protein expression results for p-EGFR, EGFR, RKIP and p-RKIP are shown as the ratio between the proteins and α-Tubulin, while p-AKT and p-ERK are shown as the ratio between the respective total proteins, AKT and ERK. The results are shown as the mean value achieved after quantification of the two independent assays.

Upon western blot analysis, it was possible to determine that all cell lines express RKIP, although at distinct levels: PC9 is the one with the highest expression levels, followed by H292 and A549, which have similar levels, and finally, HCC827 has the lowest levels of RKIP. This tendency is transcendent to the levels of the phosphorylated form of the same protein, the p-RKIP (Figure 13A). By immunofluorescence analysis, the differences observed before for total RKIP, turn to be not so clear (Figure 13B). Nonetheless, with this analysis we verify that RKIP is mainly located at the cytoplasm in all cell lines, with some H292 cells staining too in the nucleus. Additionally, to understand whether the expression levels of RKIP could be influenced by the activation status of EGFR, we stimulated the cells with EGF, and a slight decrease on RKIP or p-RKIP expression levels was observed upon EGFR activation in all cell lines, with exception for A549 cell line that showed increased levels RKIP upon EGF treatment (Figure 13A and 13C). However, this augmentation on RKIP was accompanied by a simultaneous increase on RKIP phosphorylated levels, meaning that the protein is mainly in its "inactive" form. In accordance, it was interesting to verify that the cell line (HCC827) with highest basal activation levels of EGFR was the one with lowest RKIP and p-RKIP expression levels (Figure 13A and 13C).

Regarding EGFR and downstream signalling, as expected, EGFR was highly expressed and activated (phosphorylated) in *EGFR* mutant cell lines, PC9 and HCC827, being the remaining less EGFR positive and activated only upon EGF stimulation (Figure 13A and 13C). Consistently, AKT and ERK were significantly activated upon EGF stimulation in H292 and A549 cell lines, demonstrating that they are also EGFR dependent, even not being as positive as the mutant ones. For PC9 and HCC827 cell lines it was verified that, as it happens for EGFR, they have constitutive activation of AKT and ERK pathway, being that last one yet responsive to EGF stimulation (Figure 13A and 13C).

4.2.2 Biological role of RKIP in lung cancer aggressiveness

4.2.2.1 Is RKIP a modulator of EMT in lung cancer?

To dissect the biological role of RKIP in lung cancer, we then forward to genetically modulate its expression by using CRISPR/Cas9 technology to knockout (KO) it in all cell lines. As it can be observed in Figure 14, KO of RKIP expression was accomplished, with higher efficiency in A549, PC9 and HCC827 cell lines, whereas in H292 cell line we just achieved a protein downregulation. The levels of expression p-RKIP followed the same tendency, as expected (Figure 14).

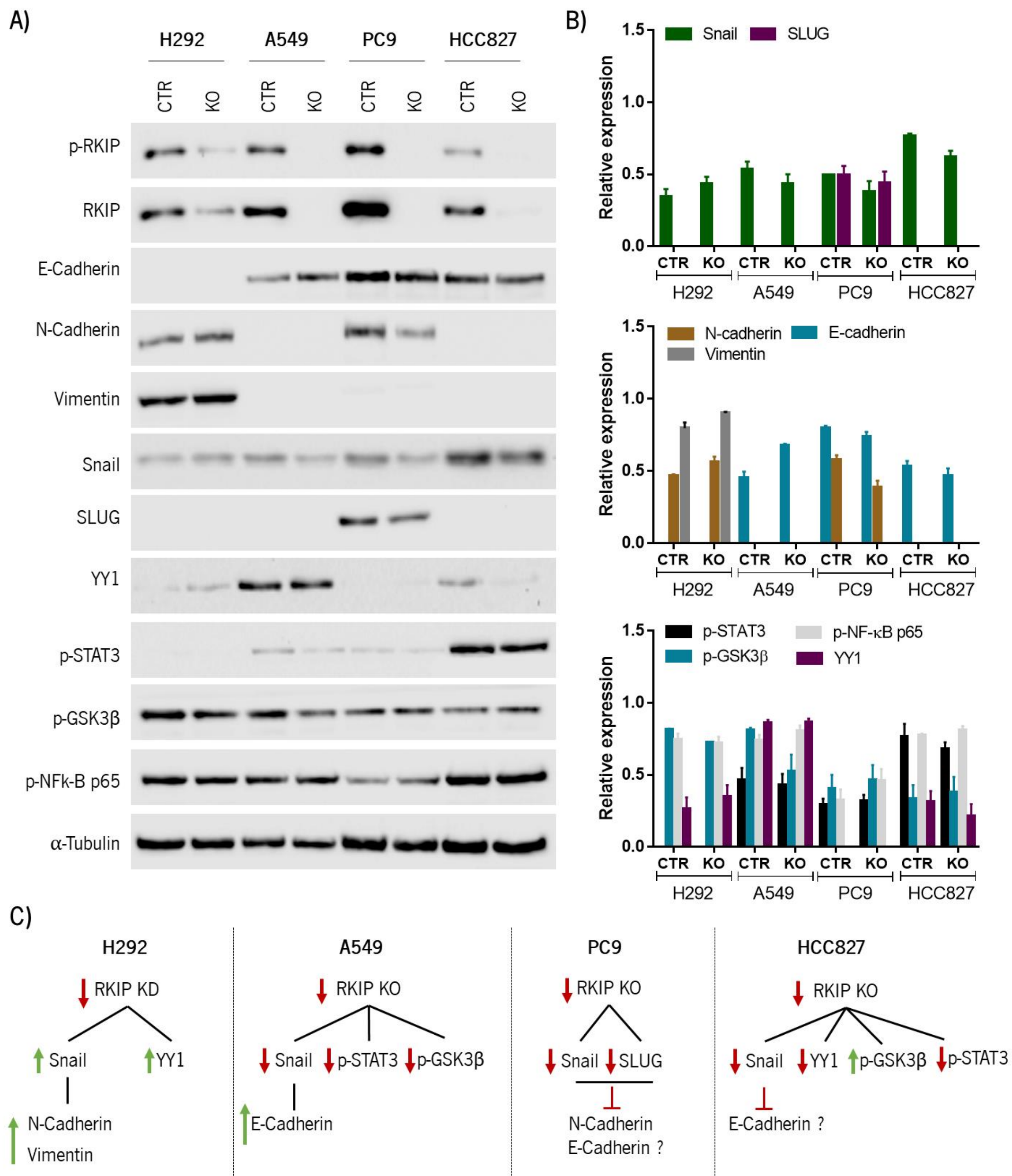

Figure 14- Western Blot analysis for expression assessment of EMT-players and known regulators/targets of RKIP in the KO LC cell lines. A) Expression of RKIP and its phosphorylated form was assessed to determine the KO efficiency. Three transcription factors were analysed: YY1, Snail and SLUG (mesenchymal markers); EMT-related proteins: E-cadherin (epithelial marker), Vimentin and N-cadherin (mesenchymal markers); and activation of STAT3, GSK3β and NF-κB p65. Tubulin was used as loading control. This a Representative assay of three independent experiments. **B)** Quantification of WB results 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 3 independent assays. **C)** Schematic representation of the major findings from WB presented in (A). KD: Knockdown; KO: Knockout.

As RKIP is as master regulator of EMT process, through the modulation of some epithelial and mesenchymal markers expression[126,201], we firstly aimed to understand whether RKIP absence affected *in*

vitro the EMT process also in lung cancer. Analysing Figure 14, it is clear that the various cell lines are distinct: H292 shows a more mesenchymal phenotype, as it expresses N-cadherin and Vimentin; A549 and HCC827 tend to be more epithelial like, expressing only E-cadherin; and in the case of PC9, it is both E-cadherin and N-cadherin positive. Additionally, all the cell lines were positive for the transcription factor SNAIL, while only PC-9 depicted positivity for SLUG, being both inducers of the mesenchymal phenotype and aggressiveness in cancer.

Regarding the effect of RKIP KO in EMT-related proteins modulation, we observed, in H292 cell line, that RKIP absence leads to upregulation of Snail and consequently of the mesenchymal markers N-cadherin and Vimentin, while in A549 cell line RKIP KO leads to downregulation of Snail and consequently a slight increase of E-cadherin. Similarly, in PC9 cell line, the RKIP KO leads to a decrease in both Snail and SLUG levels, which in turn leads to a decrease in N-cadherin expression, and unexpectedly of E-cadherin too. For HCC827 cell line it was also unexpectedly observed a decrease of E-cadherin and Snail in response to RKIP KO (Figure 14).

From this part, we can perceive that RKIP role in the modulation of EMT-related proteins in lung cancer is not as relevant as expected, since the differences are minimal. Additionally, excepting H292 cell line, the tendency observed for RKIP KO leading to downregulation of SNAIL/SLUG is in contrast with what was expected from the literature (Figure 14B). Thus, even discrete, the apparent contradictory results obtained led us to explore the expression of other proteins described to be modulated or modulators of RKIP, such YY1 and GSK3β, STAT3 or NF-κB p65 phosphorylation (Figure 14). Regarding YY1, it was seen to be upregulated upon RKIP KO in H292 cells, as expected, but its expression remained unchanged or decreased in A549 and HCC827 cell lines, respectively. STAT3 is highly activated in HCC827, however in A549, PC9 and H292 is almost inactive, being downregulated in HCC827 and A549 cell line in KO cells. Regarding the p-GSK3β, it is expressed in all cell lines, and it is slightly downactivated in A549 RKIP KO cells, being the opposite seen for HCC827 cell line. Finally, there are not striking differences upon RKIP KO in the expression levels of p-NF-κB p65, being PC9 cell line the less activated to this particular signalling pathway (Figure 14).

To conclude, the RKIP KO resulted in distinct EMT-related alterations in LC cell lines, being H292 cell line the one where an expected RKIP downregulation-associated phenotype was observed. On the remaining cell lines, with few exceptions, the EMT-related proteins are changing accordingly to the expression of EMT-related transcriptions factors (Snail and SLUG), but both in the opposite direction of was expected upon a RKIP protein KO. As the KO was complete, the existence of off-target effects cannot be excluded, thus this are results that have to be further explored.

4.2.2.2 Biological impact of RKIP downregulation in lung cancer

With the purpose of assessing the impact of RKIP absence in lung cancer aggressiveness, we performed both *in vitro* and *in vivo* assays in two of our four lung cancer cell lines (H292 and PC9). The reason behind this choice was based on its different histological and molecular types (Table 3), and different behaviours in what concerns EMT-related proteins upon RKIP downregulation (section 4.2.2.1).

Concerning H292 cell line, both migration and viability assays (Figure 15A and B, respectively) were done under two conditions, one with full access of enriched media (10% DMEM) and the other under nutrients deprivation (0.5% DMEM). No differences were found between the control and KO cells regarding the migration rate nor the viability overtime. Further, a clonogenicity assay was performed with the cells subjected to 0.5% DMEM (Figure 15C), and although no statistically significant differences were found, we can see a tendency for H292 KO cells to form more and larger colonies, when compared to its control. Next, moving on to the CAM *in vivo* assay (Figure 15D), we observed that they successfully form tumours, being the rate of its formation higher when the eggs were inoculated with RKIP KO cells (11tumours/13eggs - 85%), when compared to the control ones (12tumours/15eggs - 80%). After tumours perimeter and vessels quantification, no significant alterations were found between the tumours formed by the RKIP KO vs CTR cells, however, the ones with lower levels of RKIP seem to have grown more (Figure 15D).

Performing a similar analysis with PC9 cell line, it was possible to see clear differences in the migration rate, with KO cells migrating more when compared to its control, in nutrient-deprived medium. Although this difference was not apprehended in the graphic analysis at 10% DMEM, during each assay, when taking the pictures, it was visible that the PC9 KO cells grew more densely, resulting in a more pronounced wound closure, as it can be seen in the representative pictures in Figure 16A. Concerning the viability assay (Figure 16B), no differences were found between the control and KO cells, when growing in 0.5% DMEM, with exception for the 24 hours' time point. In contrast, PC9 KO cells showed a clear viability advantage overtime when compared to control cells (at 10% DMEM), indicating a probable proliferative advantage. Since this cells do not form colonies easier, its clonogenicity was not measured.

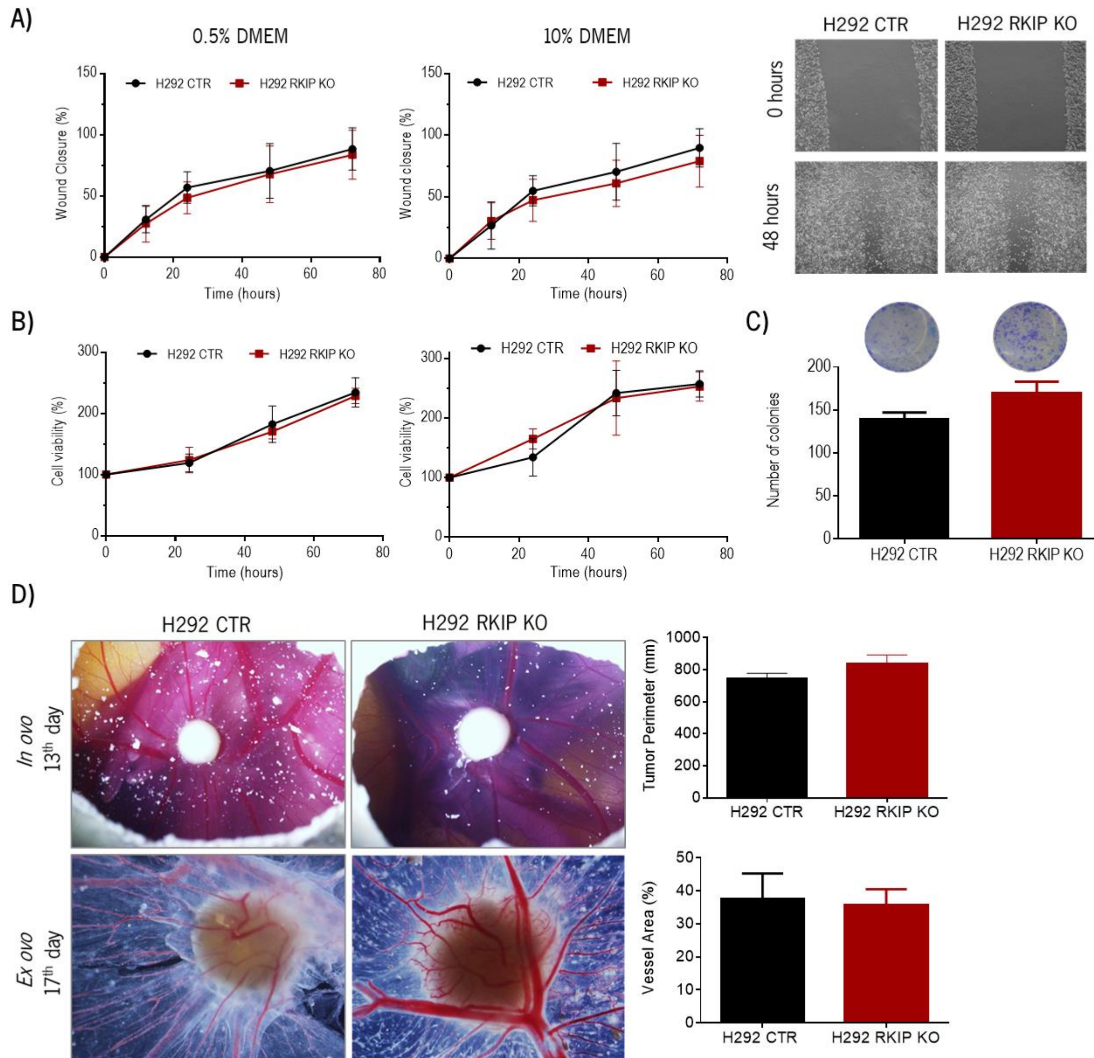

Figure 15- ***In vitro*** **and** ***in vivo*** **analysis of the biological role of RKIP in H292 transfected cell line. A)** Wound healing migration assay (N=6), where a standardized scratch (wound) was applied to monolayers, and digital images were taken at several time points (0; 12, 24; 48 and 72 hours). Representative images at 0 and 48 hours are present in the right panel. **B)** Cell viability measured at 24, 48 and 72 hours by MTS assay (N=4) at two different growth medium conditions (0.5% and 10% DMEM). **C)** Clonogenicity assay, where cells were assessed for their ability to proliferate in growth medium containing 0.5% DMEM and the formation of multicellular colonies photographed after 14 days (N=3). **D)** *In vivo* CAM assay, where tumour growth capacity was assessed between RKIP KO cells vs Control cells. Representative pictures were taken at 13th day *in ovo* (16X magnification) and *ex ovo* after 17 days (20X magnification) of development using a stereomicroscope (left panel). At the right panel is represented the tumour perimeter, measured at 13th day of development *in ovo* (CTR, N=14; KO, N=12) and percentage of blood vessels around the tumour was measured at 17th day *ex ovo* (CTR, N=5; KO, N=5) using Image J software and represented in the graphs as the mean value.

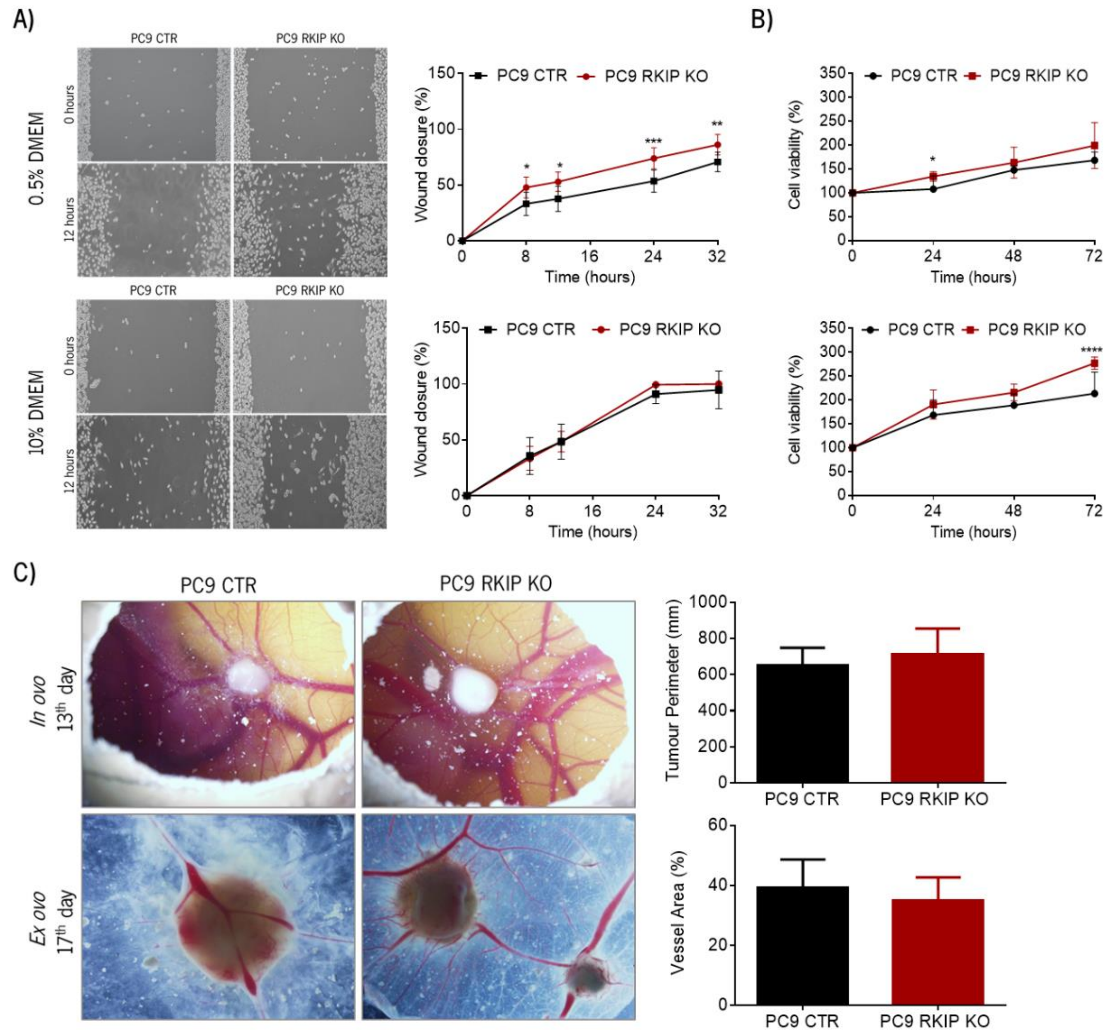

Figure 16- ***In vitro*** **and** ***in vivo*** **analysis of the biological role of RKIP in PC9 cell line. A)** Wound healing migration assay (N=4), where a standardized scratch (wound) was applied to monolayers, and digital images were taken at several time points (0; 8; 12; 24 and 32 hours). Representative images at 0 and 12 hours are present in the left panel. **B)** Cell viability measured at 24, 48 and 72 hours by MTS assay (N=4) at two different growth medium conditions (0.5% and 10% DMEM). **C)** *In vivo* CAM assay, where tumour growth capacity was assessed between RKIP KO cells vs Control cells. Representative pictures were taken at 13th day *in ovo* (16X magnification) and *ex ovo* after 17 days (20X magnification) of development using a stereomicroscope (left panel). At the right panel is represented the tumour perimeter, measured at 13th day of development *in ovo* (CTR, N=26; KO, N=35) and percentage of blood vessels around the tumour was measured at 17th day *ex ovo* (CTR, N=10; KO, N=9) using Image J software and represented in the graphs as the mean value.

Moving to the CAM *in vivo* assay (Figure 16C), we observed that they successfully form tumours, being the rate of tumour formation higher when the eggs were inoculated with RKIP KO cells (35tumours/60eggs - 58%) when compared to the control ones (26tumours/54eggs - 48%), as it happened with H292 cells (Figure 15). After tumours perimeter and vessels quantification, no significant

alterations were found between the tumours formed by the RKIP KO vs CTR cells, however, the ones with lower levels of RKIP seems to have grown more (Figure 16C).

Finally, having PC9 cell line demonstrated such promising results, we decided to scale up the work for a more robust *in vivo* assay, the subcutaneous xenograft mouse model, doing a pilot study with a small number of immunocompromised mice (Figure 17). With this in mind, we started by optimizing the technique, namely, the ideal number of cells to inject. Hence, based on the literature, we used the PC9 control cells and started with three conditions: $2x10^6$ cells in a 1:1 ratio with matrigel, $3x10^6$ cells and $5x10^6$ cells per animal. The experiment lasted for 23 days, the time point at which at least a tumour reached the maximum tumour volume established (~1 cm^3) (Figure 17A). Even with a small number of animals, we could observe that when injected with matrigel, the tumours formed tended to be more homogeneous volume wise, even though they took more time to develop. Injecting three million cells enabled the best growth rates, however forming tumours not so homogeneous as the previous group. Finally, with five million cells, all animals at a certain point formed ulcers in the injected spot and had to be sacrificed before the tumour could further develop. Thus, we chose to perform our experiment using $3x10^6$ cells without matrigel.

Analysing Figure 17B, it was evident that animals injected with PC9 RKIP KO cells have a significant higher capacity to form tumours since the early time points, when compared to the control ones, this going in agreement with the tendencies previously observed in the CAM assay. Histologically, we observed, by H&E staining, that tumours formed from PC9 KO cells show a higher cellularity (Figure 17C), with higher number of blood vessels inside the tumours (data not shown). Moreover, by immunohistochemistry it was possible to verify that tumours with almost total absence of RKIP expression have, in fact, increased proliferation rates, with a higher number of cells staining for Ki-67 protein (Figure 17C). Although these results were in line with what we expected, it is important to emphasise that this was a pilot test, performed only once, and so, we would need to repeat this assay in order to be considered relevant.

Thus, bringing all the results together, while H292 cell line was the one where an expected RKIP downregulation-associated phenotype was observed, by the patterns of EMT-related proteins expression upon RKIP KO, a clear impact of RKIP KO in cellular aggressiveness, which, in our case, translated into higher migration and viability rates overtime, was observed with PC9 cell line. However, when inoculated in the CAM, both cell lines tended to grow more when RKIP was downregulated, with a slight decrease of blood vessels in the CAM, indicating that they are being recruited to the tumour (Figure 15D and 16C).

The higher proliferative capacity of PC9 RKIP KO cells was further confirmed using *in vivo* xenograft mouse model.

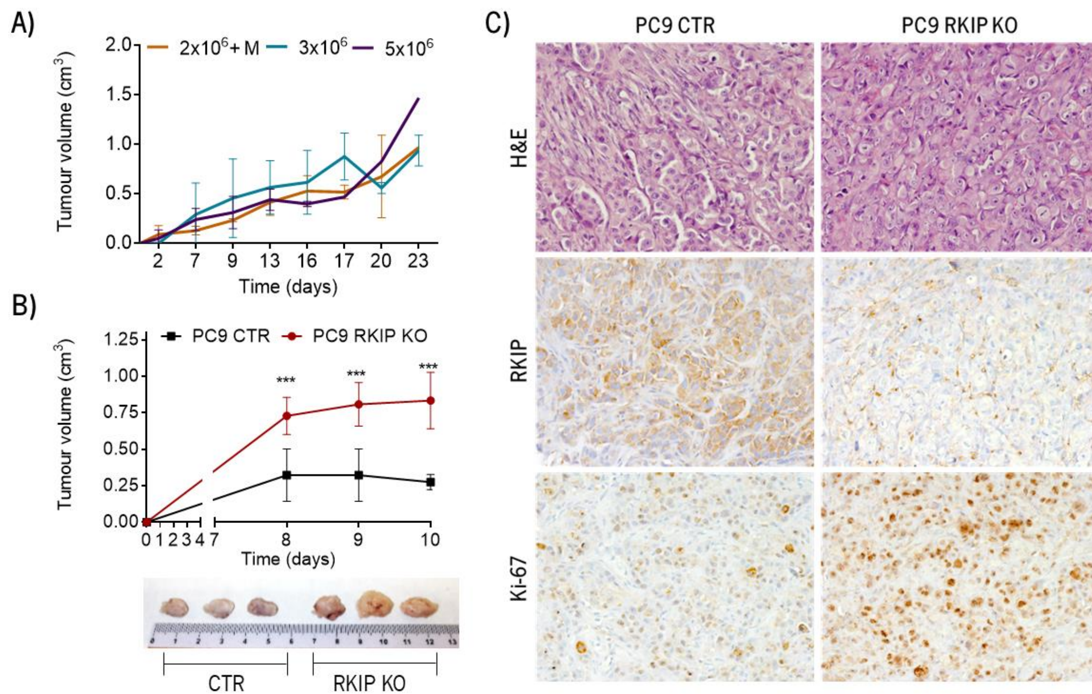

Figure 17- Biological role of RKIP KO *in vivo* using subcutaneous xenograft mouse model. A) A pilot study performed using a subcutaneous xenograft mouse model in which three different suspensions of PC9 CTR cells were injected: $2x10^6$ plus matrigel (M), $3x10^6$ cells and $5x10^6$ cells per animal (N=3 animals per group). The endpoint was considered when at least one tumour reached the maximum tumour volume established (~1 cm^3). **B)** Pilot study follow up, where PC9 RKIP KO and PC9 CTR cells were injected at a concentration of $3x10^6$ cells per animal (N=6 animals per group). In the lower panel, are represented 3 examples of each type of tumour harvested. **C)** Representative pictures (200x magnification) of haematoxylin and eosin (H&E) staining as well as of the immunohistochemical analysis of RKIP and Ki-67 expression in the formalin-fixed and paraffin-embedded isolated tumours.

4.3 Role of RKIP in lung cancer cells response to EGFR targeted therapies

4.3.1 *In vitro* study: Cytotoxic assays

Having characterized our RKIP KO study models we moved forward to our main goal, which was to assess the role of RKIP in lung cancer cell lines response to EGFR inhibitors (EGFRi). To do so, three drugs belonging to the category of small molecule inhibitors, were selected: Erlotinib is a reversible tyrosine kinase inhibitor (TKi), binding exclusively to EGFR. The remaining two drugs, Afatinib and AST1306 are irreversible TKi, having affinity to EGFR and other family receptors. Firstly, in order to determine the range of concentrations to be used in the following assays, we started by screening the

sensitivity of our four cell lines to the different EGFRi (Figure 18). In general, as expected, the EGFR mutant cell lines were responsive to all the drugs and at very low doses. In contrast, although responsive at the used doses, the *KRAS* mutant cell line (A549) was the one for whom the drugs were less effective, even less than for the *WT* one (H292). Additionally, since it is a first generation and reversible drug, proven to be much more useful for *EGFR* mutant cells, Erlotinib showed to be ineffective (at the trial doses used) in *WT* and *KRAS* mutant cell lines, as expected (Figure 18).

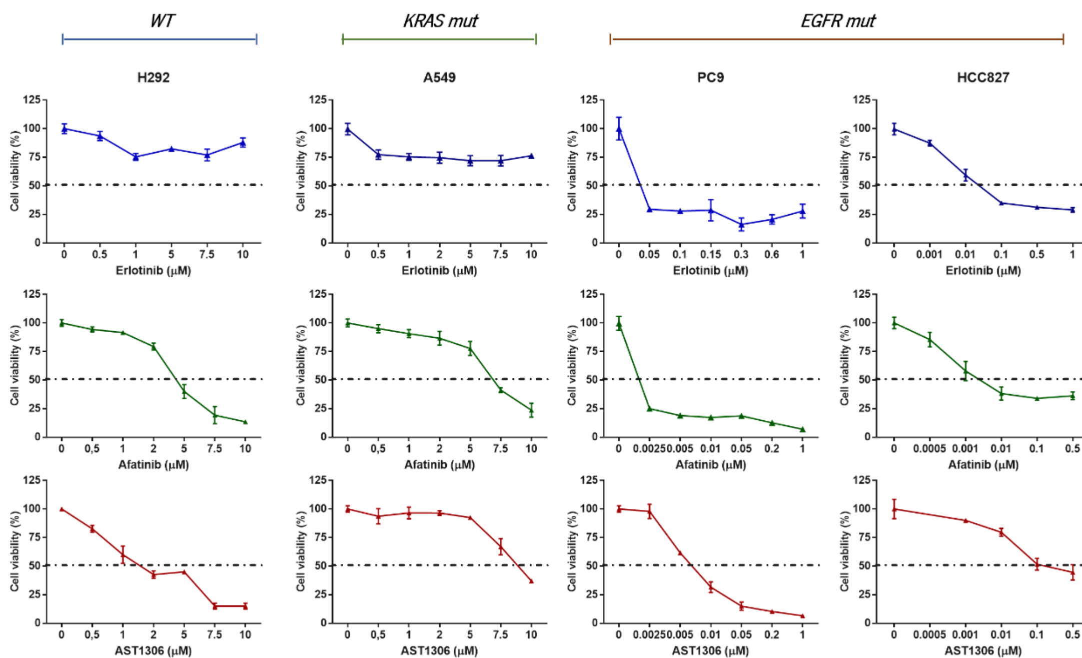

Figure 18- Sensitivity of lung cancer cell lines to EGFR inhibitors. To assess the cytotoxicity of the EGFRi, the cells were incubated with increasing concentrations of all drugs for 72 hours, and the cell viability was assessed by MTS assay. All the experiments were done at least twice in triplicate and the graphs are represented as the mean ± SD, relative to DMSO alone (100% viability).

Next, after this first screening and drug's effect validation, to assess whether RKIP absence influences the cells response to EGFRi, we exposed our RKIP KO cell lines, and the respective controls, to increasing concentrations of all drugs for 72 hours, and determined, by cytotoxic assays, the IC_{50} values for each drug, in each cell line. In Figure 19 and Table 7 are represented the mean IC_{50} values determined. In a first analysis, after doses adjustment, we can observe that the CTR cell lines, even after a transfection process, presented the same pattern of response as the untrasfected (represented in Figure 18) cell lines: second generation drugs (Afatinib and AST1306) were more effective and potent in all cell lines, while Erlotinib was the less potent and effective drug, with the *KRAS* mutant cell line (A549) being completely resistant (IC_{50} > 100 µM) to it (Figure 19 and Table 7).

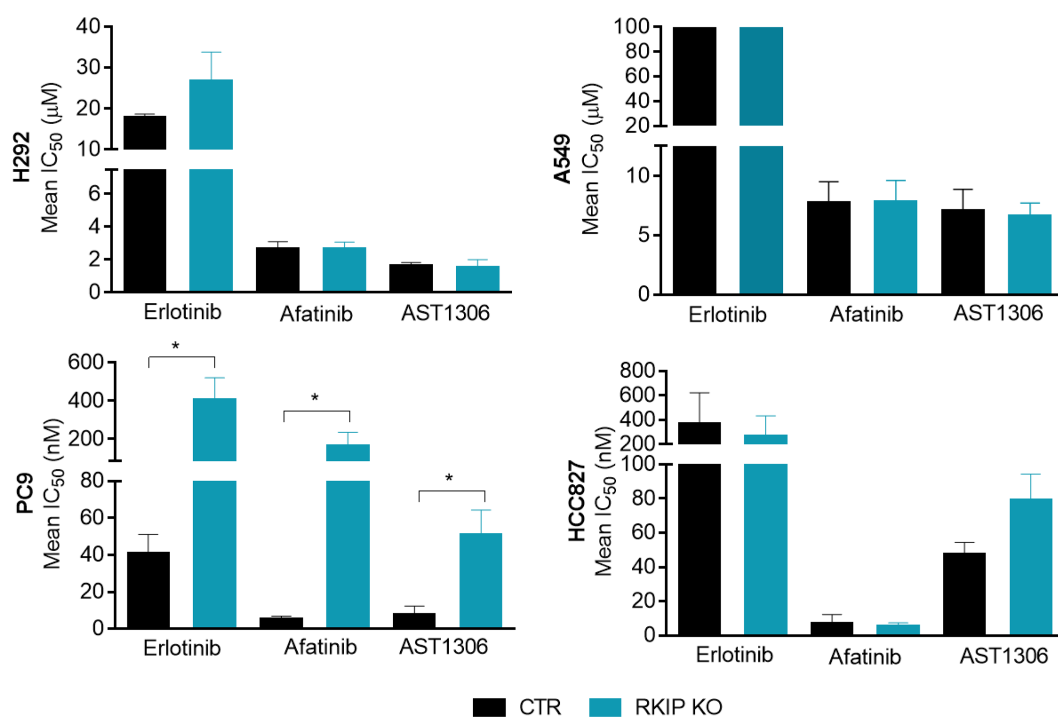

Figure 19- Comparative analysis of EGFR inhibitors IC_{50} values for control and RKIP KO cell lines. IC_{50} values were expressed as the mean of, at least, three independent assays performed in triplicate. Statistically, *p*<0.05 on the student's t teste were considered statistically significant (*).

Table 7- Mean IC_{50} values for Erlotinib, Afatinib and AST1306 in the transfected LC cell lines. For H292 and A549 the IC_{50} values are presented in µM ± SD and for PC9 and HCC827 they are in nM ± SD.

Mean IC_{50}	H292 (µM)			A549 (µM)			PC9 (nM)			HCC827 (nM)		
	N	CTR	RKIP KO	N	CTR	RKIP KO	N	CTR	RKIP KO	N	CTR	RKIP KO
Erlotinib	5	17.62 ± 1.05	26.7 ± 7.10	5	>100	>100	5	40.45 ± 10.70	400.75 ± 97.26	4	338.23 ± 188.2	262.57 ± 171.39
Afatinib	10	2.63 ± 0.46	2.65 ± 0.42	8	7.68 ± 1.81	7.75 ± 1.85	4	4.91 ± 2.04	161.8 ± 72.98	4	6.93 ± 5.33	4.95 ± 2.47
AST1306	8	1.62 ± 0.19	1.52 ± 0.47	8	7.02 ± 1.84	6.55 ± 1.14	6	7.49 ± 4.82	50.49 ± 13.88	4	47.01 ± 7.41	63.04 ± 41.19

Looking with closer detail to each cell line response to the anti-EGFR drugs, we can see that, for H292, the expected tendency for RKIP KO cells to be less sensitive is observed only for Erlotinib, with IC_{50} values being 17.62±1.05 µM for CTR and 26.7±7.10 µM for KO cells, even though not being statistically significant. For Afatinib and AST1306 the obtained IC_{50} values are quite similar among positive and low RKIP expressing cells and also similar between them, being the values around 2.6 µM and 1.55 µM, respectively. Regarding A549 cell line, again, no differences were observed between the CTR and RKIP KO cells when treated with Afatinib and AST1306, even though the values are too in the same range,

around 7 μM. For Erlotinib, as said before was impossible to determine the IC_{50} (>100 μM) (Figure 19 and Table 7).

Analysing PC9 cell line, it was observed a huge difference between RKIP positive and negative cells (Figure 19) response to the three anti-EGFR drugs used: For Erlotinib the IC_{50} values are 10 times higher in KO cells (400.7±97.26 nM) when compared with the control ones (40.45±10.70 nM) (p=0.009); For AST1306, the observed tendency is maintained, with a six times difference among the IC_{50} values (p=0.006) (7.49±4.82 nM vs 50.49±13.88 nM). The most impressive difference was observed for Afatinib, with PC9 KO cells being around 30 times less sensitive than the control ones (p=0.020), having 161.80±72.98 nM and 4.91±2.04 nM mean IC_{50}, respectively (Figure 19 and Table 7).

Finally, regarding HCC827 cell line, for Erlotinib and Afatinib no differences were spotted between RKIP KO and control cells, however, for AST1306, it was observed a not significant tendency for KO cells to be less responsive, with an IC_{50} value of 63.04±41.19 nM comparatively to 47.01±7.41 nM for control cells. It is interesting also to note that HCC827 cell line, independently of RKIP expression, is the most sensitive cell line among all the tested for all the drugs (Table 7).

Thus, this *in vitro* analysis of RKIP role in response to EGFR inhibitors enabled to determine that RKIP downregulation could be associated with lower response rates to anti-EGFR therapies in LC, a result that was evident in PC9 *EGFR* mutant cell line.

4.3.2 *In vivo* study: CAM assay

Faced with the previous results, we aimed to validate them in an *in vivo* model. For that we started with the CAM assay and chose Afatinib to be used in the experiment, since it was the one for which higher differences in the IC_{50} values were found between control and KO PC9 cells.

For this experiment, the formed tumours at 13th day of development were treated with Afatinib or vehicle during 4 days. Thus, the experiment ended at day 17, when tumour perimeter was re-measured *in ovo* and blood vessels counted *ex ovo* (Figure 20A). In that case, the results of tumour perimeter were expressed as the difference between the perimeter at day 17 and 13, meaning that if the value is positive, the tumour grew and if the value is negative the tumour regressed or shrunk. As it can be observed in Figure 20, in tumours formed by PC9 CTR and RKIP KO cells, it is possible to see that some of the tumours shrunk without any treatment, but on average, the tumours formed by KO cells tended to grow more than ones formed by the CTR cells, as it was already observed before in Figure 16 assay, at 13 day of development. In agreement, a decrease on the percentage of blood vessels around the tumour was again observed in the tumours formed by KO cells inoculation and independently of tumours treatment

with Afatinib, meaning that these tumours are probably recruiting the vessels to them and that Afatinib seems do not have anti-angiogenic activity (Figure 20B).

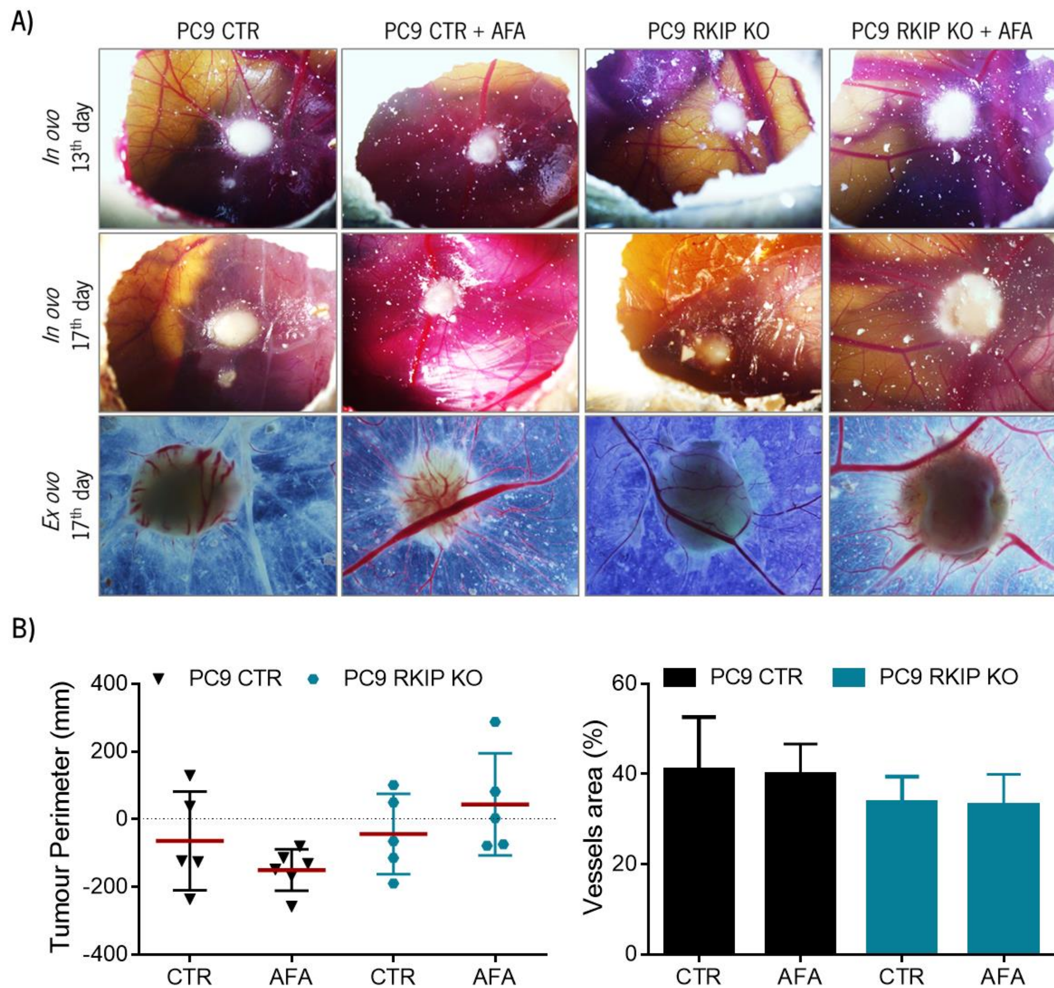

Figure 20- ***In vivo*** **role of RKIP in PC9 tumours growth after treatment with Afatinib. A)** Representative pictures were taken *in ovo* at 13th day (16X magnification) of development and then tumours were treated with 10nM of Afatinib (AFA) for four days. In total, in the group of tumours formed by CTR cells, 6 were treated with AFA and other 6 with the vehicle. In the group of tumours formed by PC9 KO cells, 5 were treated with AFA and other 5 with the vehicle. At day 17 the tumours were again photographed *in ovo* (16X magnification) and also *ex ovo* after 17 days (20X magnification). All pictures were taken using a stereomicroscope**. B)** Tumour perimeter is expressed as the difference between the perimeter of each tumour at day 17 and 13. In total, in the group of tumours formed by CTR cells, remained 6 treated and 5 AFA untreated tumours, while in the KO ones all the embryos remained alive (5 tumours per group). The red bold lines represent the mean perimeter of each experimental group. At the right panel is represented percentage of blood vessels around the tumour, measured at 17th day *ex ovo* (CTR, N=5; CTR+AFA, N=6; KO, N=5; KO+AFA, N=5) using Image J software.

Importantly, even though no statistically significant differences between the groups were found, because not all of them grew or regressed, it is interesting to note that Afatinib was, in fact, able to

decrease the mean tumour perimeter in the tumours formed by the PC9 CTR cells. The opposite was observed in the tumours formed by the KO cells, where the majority of tumours grew, being the mean tumours perimeter higher in the treated group than in the KO untreated tumours (Figure 20A and B).

The results herein obtained, even with a small group of tumours, are in agreement with what was observed *in vitro*, with the PC9 RKIP KO tumours being less responsive to Afatinib than the ones that express RKIP.

4.3.3 RKIP impact in signalling pathways modulation before and after EGFR inhibition

Given the observed association between RKIP expression loss and the difference in the responses to anti-EGFR drugs, particularly in PC9 cell line, it was intended to understand through which pathway RKIP could be modulating therapy response. For that, we assessed by western blot the activation levels of the EGFR receptor and important intermediates of its downstream signalling pathways such as MAPK and AKT, before and after drugs treatment.

Firstly, and analysing Figure 21, it is possible to state the influence of RKIP KO in EGFR-related signalling pathways. Looking at the control conditions, it is visible that PC9 RKIP KO cells have higher activation levels of EGFR, even at basal conditions without EGF stimulation. However, this overactivation does not directly influences the activation of AKT and MAPK, as PC9 KO cells have lower activation levels of AKT, ERK1/2 and MEK1/2, when compared to the PC9 CTR cells (Figure 21A and b). Additionally, considering only the PC9 CTR cells, it was curious to observe that RKIP and mainly p-RKIP tend to be downregulated upon EGFR stimulation, an observation already made in Figure 13, but upregulated upon EGFR inhibition (Figure 21A and C), suggesting that EGFR and RKIP can be regulating each other by a negative feedback mechanism.

Scoping the effect of the anti-EGFR in the signalling pathways, it is very clear that the most effective drug, inhibiting more extensively the target EGFR and its downstream signalling, was Afatinib, followed by AST1306, being Erlotinib the less effective, as expected (Figure 21A and B).

Regarding the effectiveness of EGFR inhibition in RKIP KO cells, as the PC9 CTR and PC9 RKIP KO cells presented different levels of EGFR signalling activation upon EGF stimulation (Figure 21B), and EGFR inhibition was done in EGF-stimulated conditions, in order to accurately interpret the results, the western blots quantification, upon drugs treatment, were presented in relation to the EGF-stimulated CTR, that was set as 1. By doing this, it is possible to observe in Figure 21B that all the drugs have the same capacity to inhibit EGFR and MEK activation, in both CTR and PC9 KO cells, but, in contrast, presented a lower capacity to inactivate ERK and mainly AKT, when RKIP was absent (Figure 21B).

As a conclusion from this part, we observed that RKIP and EGFR can regulate each other, at least in PC9 cells, and that RKIP mediated modulation of AKT singling pathway could be the underlying mechanism of the apparent resistant phenotype observed in PC9 RKIP KO cells.

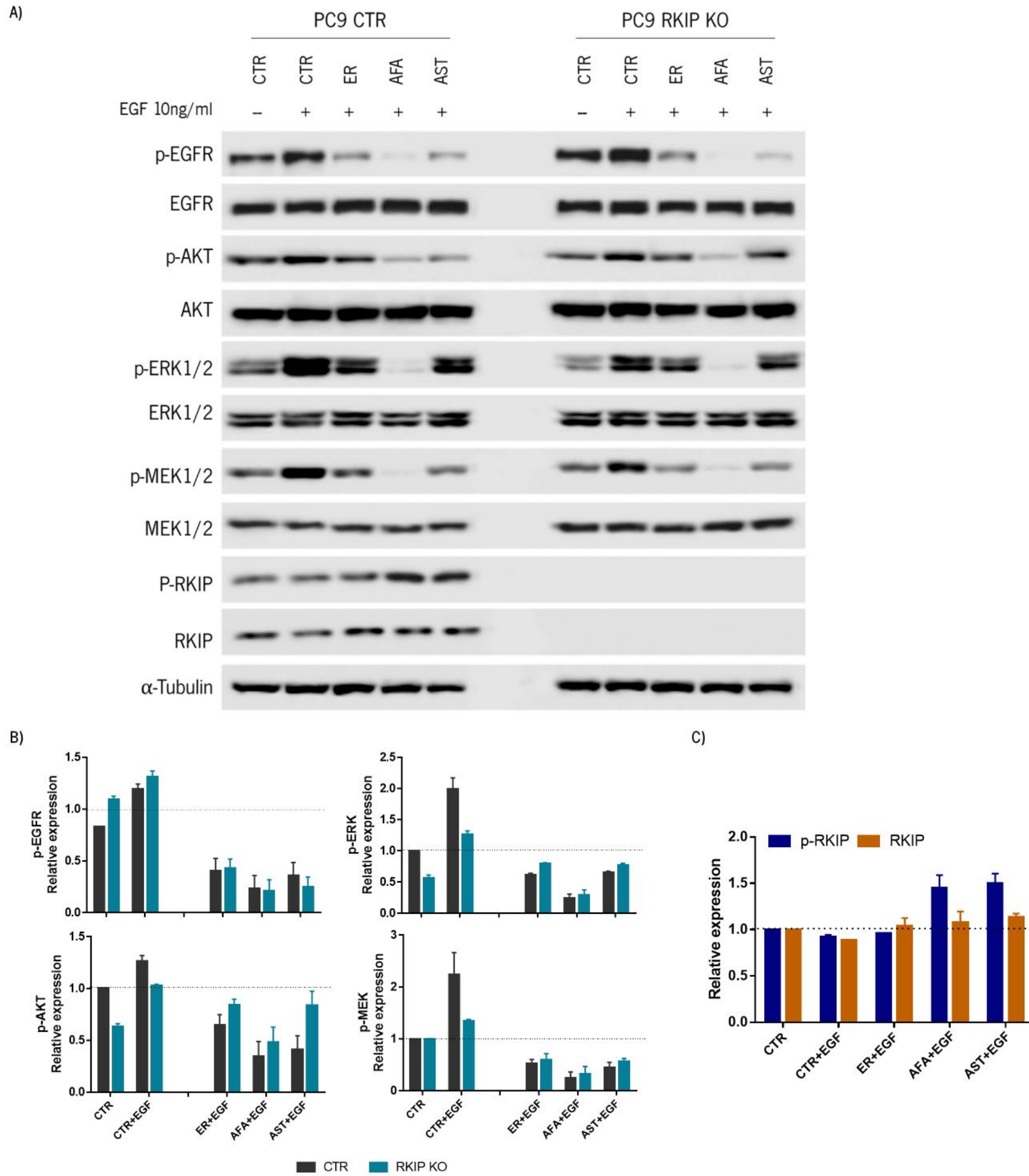

Figure 21- Western Blot analysis for EGFR receptor and downstream signalling pathways, AKT and MAPK, in PC9 transfected cell line, upon treatment with EGFR inhibitors. A) Cells were serum starved for 2 hours and treated with 10nM of Erlotinib (ER), Afatinib (AFA) and AST1306 (AST) for 2 hours. At the end, the cells were stimulated with 10 ng/ml of EGF by 15 minutes. RKIP expression was also assessed and α-Tubulin was used as loading control. This a representative assay of three independent experiments. **B)** Quantification of WB results was performed using band densitometry analysis with Image J software. Relative protein expression results for p-EGFR, p-MEK, p-ERK and p-AKT are shown as the ratio between the respective total proteins, EGFR, MEK ERK and AKT. Concerning the bars correspondent to the drugs plus EGF stimulation,

they were quantified as before, as ratio between the phosphorylated and total protein, but presented in relation to the EGF-stimulated CTR that was set as 1 (pointed line). **C)** Quantification of WB results regarding RKIP and p-RKIP in PC9 CTR cells, that are shown as the ratio between the proteins and α-Tubulin. All the WB quantification results are shown as the mean value of the 3 independent assays.

4.3.4 RKIP molecular signature in lung cancer: *in silico* analysis

The advantage of analysing TCGA data is the possibility to stablish correlations between one protein of interest and millions of other genes, both at mRNA and protein level. Hence, we tried to design a RKIP molecular signature in lung cancer to help us better understand the main results achieved in this thesis, using for such, the TCGA provisional available data at cBioPortal database (www.cbioportal.org).

First, by using mRNA data (RNA Seq V2) we determined which genes are co-expressed, both positively and negatively, with RKIP. Through this, thousands of genes came out, which we restricted to the Top25 more related genes. As expected, among the Top25 genes positively related with RKIP were genes that are located in the same cytoband as RKIP (12q), meaning copy number variations (data not shown). As RKIP is considered a tumour suppressor in cancer, we explored specifically for the Top25 genes negatively co-expressed with RKIP. When comparing between adenocarcinomas (AC) and squamous cell carcinomas (SCC), only 3 genes were found in common: ADGRF4 (Adhesion G Protein-Coupled Receptor), ERO1A (endoplasmic reticulum oxidoreductase 1 alpha), and LAMC2 (Laminin Subunit Gamma 2) (Supplementary Table S1 – Appendix I). Functionally, when we plot the 25 genes in a functional protein association network called STRING (https://string-db.org/), we can observe that, although different, the genes negatively associated with RKIP in both histological types are significantly related with cellular differentiation, focal adhesion, adherent junctions, cell metabolism (HIF1α signalling) and are intervenient of NF-κB, PI3K, JAK/STAT and MAPK pathways (Supplementary Figure 1 and 2 – Appendix I).

Interestingly, the TCGA provisional data has available information for protein expression (www.cbioportal.org). Although RKIP protein expression is not available, we were able to do an enrichment analysis, which consists in taking the samples with RKIP mRNA overexpression as a set and determine whether other genes are concomitantly over or underexpressed in the same set of samples, at the protein level. As above, considering only the proteins that are underexpressed when RKIP is upregulated, we found 118 proteins for AC and 92 for SCC, being around 35% post-transcriptional modified proteins by phosphorylation, which fits well with the already described role of RKIP as a modulator of signalling molecules [191]. At supplementary Table S2 (Appendix I) are discriminated the Top50 enriched proteins in the set of patients with RKIP upregulation.

Curiously, we found that EGFR is among the Top50 proteins that are downregulated when RKIP protein is overexpressed, only for adenocarcinomas (Supplementary Table S2 (Appendix I). By crossing the data concerning EGFR and RKIP expression levels in adenocarcinoma patients, we found that at mRNA level they are in fact statistically significantly inversely correlated (Figure 22A). Moreover, both EGFR protein and its activated form (p-EGFR) are commonly downexpressed in the set of patients that presents RKIP mRNA uperugulation (Figure 22B).

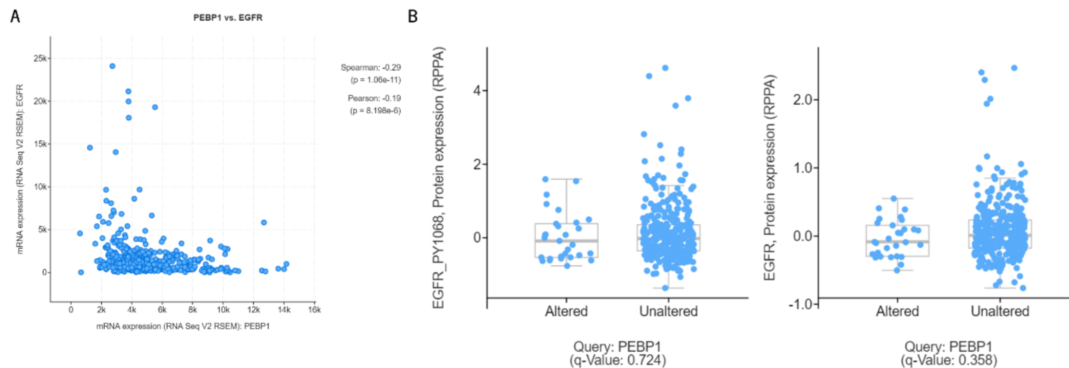

Figure 22- ***In silico*** **correlation between RKIP and EGFR in adenocarcinomas patients with lung cancer. A)** RNA Seq V2 data for AC patients (566 patients), showing the RKIP (PEBP1) and EGFR mRNA expression is inversely correlated. **B)** Data from enrichment analysis in the same AC patients, showing that both total EGFR and phosphorylated EGFR (Tyr1068) are downexpressed in the set of samples with RKIP mRNA upregulation (set as altered in the analysis). All data belongs to the TCGA provisional database for lung AC and is available at www.cbioportal.org.

In Table 8 are shown the 60 proteins that co-occurred in AC and SCC patients with RKIP upregulation, and thus constitute our so called "RKIP-signature" in lung cancer (Table 8). Analysing it deeply, it is very enthusiastic to verify that RKIP could be a central modulator of lung carcinogenesis, due to its involvement with important oncogenic driver proteins in these tumours. Using functional annotation tools such as STRING (https://string-db.org/) and DAVID Bioinformatic Resources (https://david.ncifcrf.gov/), we categorized the proteins by function and observed that the great majority of them are signalling molecules either being receptors, adapters, mediators or transcription factors involved cancer pathways, such as RTK mediated signalling, PI3K/AKT, MAPK, mTOR, NF-κB and hippo signalling (Table 8 and Figure 23). Additionally, RKIP is also inversely related with proteins involved in transcription regulation, cell cycle and cellular stress response (p53 as autophagy) as well as with apoptosis, autophagy, tight junction and adhesion processes (Table 8).

Concluding, we were able with this *in silico* analysis to validate some of the results obtained through the thesis as well as determine an RKIP-associated signature in lung cancer that will be very useful in the near future to deepen our work.

Table 8- Proteins underexpressed in the subset of LC patients depicting RKIP mRNA upregulation (common between AC and SCC). TCGA provisional data (www.cbioportal.org)

Protein	Cytoband	Function / Signaling pathway	
IRS1	2q36.3	Insulin receptor binding	RTKs signaling
CAV1	7q31.2	Protein kinase binding	RTKs signaling
VEGFR2	4q12	Tyrosine-protein kinase	RTKs signaling
MET	7q31.2	Tyrosine-protein kinase	RTKs signaling
PRKCA	17q24.2	Protein Kinase	RTKs signaling
PRKCA_PS657	17q22-q23.2	Protein Kinase	RTKs signaling
PRKCD_PS664	3p21.31	Protein Kinase	RTKs signaling
SERPINE1	7q22.1	Serine protease inhibitor	RTKs signaling
SRC_PY416	20q12-q13	Tyrosine-protein kinase	RTKs signaling
SRC_PY527	20q12-q13	Tyrosine-protein kinase	RTKs signaling
ESR1	6q25.1-q25.2	Estrogen receptor	RTKs signaling
GATA3	10p14	Transcriptional activator	RTKs signaling
ARAF_PS299	Xp11.4-p11.2	Serine/threonine-protein kinase	MAPK signaling
MEK1	15q22.31	Mitogen-Activated Protein Kinase	MAPK signaling
MEK1_PS217_S221	15q22.1-q22.33	Mitogen-Activated Protein Kinase	MAPK signaling
ERK2_PT202_Y204	22q11.21	Mitogen-Activated Protein Kinase	MAPK signaling
P38_PT180_Y182	6p21.3-p21.2	Mitogen-Activated Protein Kinase	MAPK signaling
ERK1_PT202_Y204	16p11.2	Mitogen-Activated Protein Kinase	MAPK signaling
RAF1_PS338	3p25	Serine/threonine-protein kinase	MAPK signaling
RPS6_PS240_S244	9p21	Serine/threonine-protein kinase	MAPK signaling
RPS6KA1_PT359_S363	1p	Serine/threonine-protein kinase	MAPK signaling
AKT1_PS473	14q32.32	Serine/threonine-protein kinase	PI3K/AKT signaling
AKT1_PT308	14q32.32	Serine/threonine-protein kinase	PI3K/AKT signaling
AKT2_PS473	19q13.1-q13.2	Serine/threonine-protein kinase	PI3K/AKT signaling
AKT2_PT308	19q13.1-q13.2	Serine/threonine-protein kinase	PI3K/AKT signaling
AKT3_PS473	1q44	Serine/threonine-protein kinase	PI3K/AKT signaling
AKT3_PT308	1q44	Serine/threonine-protein kinase	PI3K/AKT signaling
PIK3R1	5q13.1	Phosphatidylinositol 3-Kinase	PI3K/AKT signaling
PIK3R2	19p13.11	Phosphatidylinositol 3-Kinase	PI3K/AKT signaling
TGM2	20q11.23	Transglutaminases	PI3K/AKT signaling
LKB1	19p13.3	Serine/threonine-protein kinase	mTOR/AMPK signaling
mTOR_PS2448	1p36.2	Serine/threonine-protein kinase	mTOR signaling
RICTOR	5p13.1	Rapamycin-insensitive companion	mTOR signaling
RICTOR_PT1135	5p13.1	Rapamycin-insensitive companion	mTOR signaling
SYK	9q22.2	Tyrosine-protein kinase	NF-kappa B signaling
CD20	11q12.2	B-lymphocyte antigen	NF-kappa B signaling
ANXA1	9q21.13	Ca(2+)-regulated phospholipid-binding	NF-kappa B signaling
LCK	1p35.2	Tyrosine-protein kinase	T cell receptor signaling
TAZ	3q25.1	Transcriptional coactivator	Hippo signaling pathway
YAP1_PS127	11q13	Transcriptional coactivator	Hippo signaling pathway
BCL2L1	20q11.21	Bcl-2-like protein 1	Apoptosis
CASP7	10q25.3	Caspase-7	Apoptosis
CASP8	2q33.1	Caspase-8	Apoptosis
p62	5q35.3	Autophagy receptor	Autophagy
BECN1	17q21.31	Beclin-1	Autophagy
CDKN1B_PT198	12p13.1-p12	Cyclin-dependent kinase inhibitor	Cell Cycle/ p53 signaling
CHEK1	11q24.2	Serine/threonine-protein kinase	Cell Cycle/ p53 signaling
TP53	17p13.1	Cellular tumor antigen	Cell cycle/ p53 signaling
ATM	11q22.3	Serine-protein kinase	DNA Repair/ p53 signaling
NDRG1_PT346	8q24.3	Stress-responsive protein	DNA Repair/ p53 signaling
RAD51	15q15.1	DNA repair protein	DNA Repair/ p53 signaling
HSPA1A	6p21.33	Heat shock protein	Cellular response to stress
SETD2	3p21.31	Histone-lysine N-methyltransferase	Transcription regulators
XBP1	22q12.1|22q12	Transcription factor	Transcription regulators
YBX1	1p34.2	pre-mRNA alternative splicing	Transcription regulators
YBX1_PS102	1p34	pre-mRNA alternative splicing	Transcription regulators
FN1	2q35	Fibronectin type III domain containing	Cell adhesion
ITGA2	5q11.2	Integrin alpha-2	Cell adhesion
MYH11	16p13.11	Cellular myosin	Tight junction
MYH9_PS1943	22q13.1	Cellular myosin	Tight junction

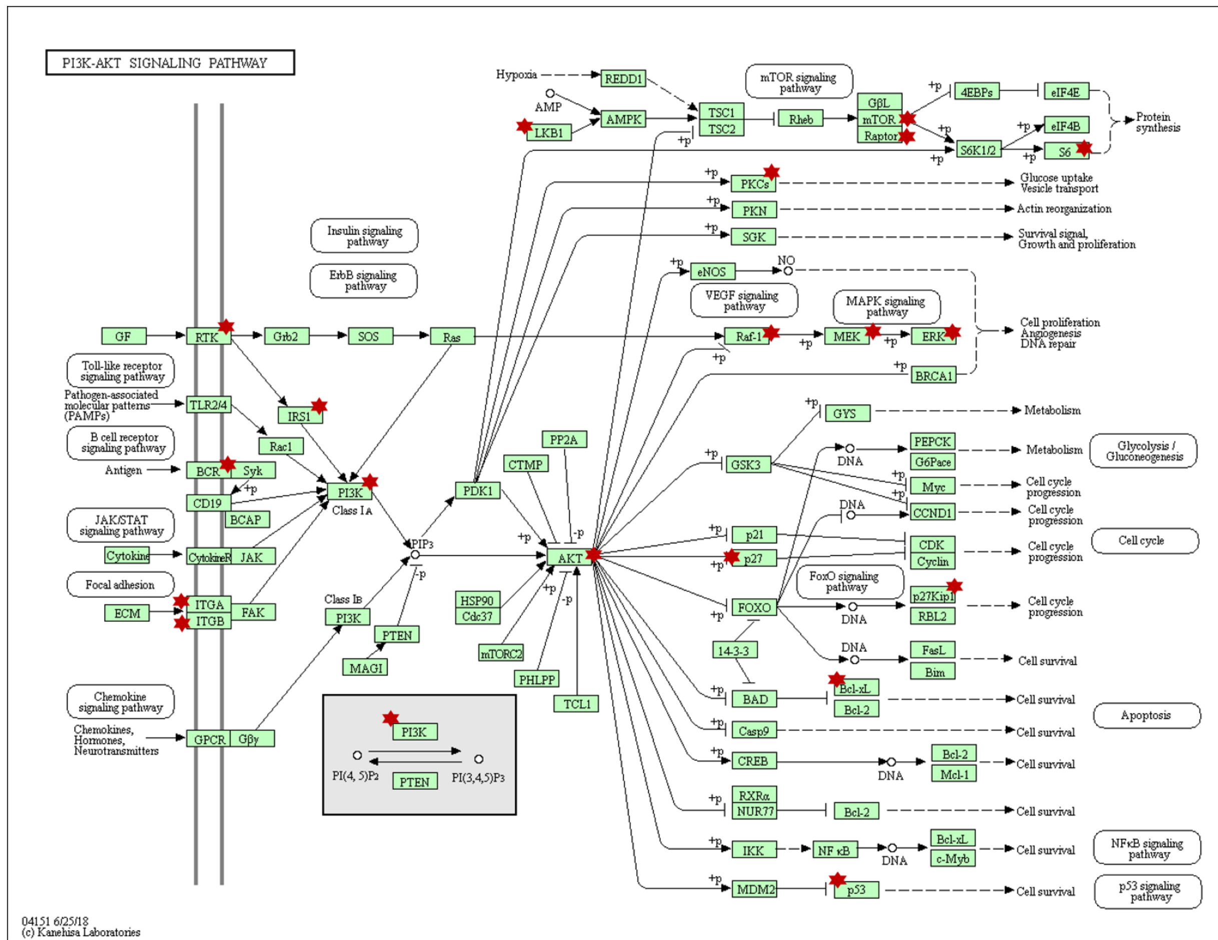

Figure 23- RKIP related pathways in lung cancer. Using DAVID functional annotation tools (https://david.ncifcrf.gov/), with the list of 60 common proteins to AC and SCC that were underexpressed in the set of tumours depicting RKIP downregulation (Table 3), we determined in which singling pathways those proteins are players. DAVID Functional annotation chart tool, indicated us that PI3K/AKT pathway is as one of the more enriched with the list of 60 proteins. The scheme of the pathway is provided by KEGG database (https://www.genome.jp/kegg/).

CHAPTER 5:

General Discussion

5. General discussion

Lung cancer is the most fatal type of cancer among men and women, and upon diagnosis, patients are usually not expected to live more than five years[12,202]. Associated with this poor prognosis is the late detection and the ineffective treatments in advanced stages[203]. Besides conventional therapies, there has been a great effort in the last decades to develop novel molecular targeted therapies, with these considering specific mutations in tumour's patients, this way applying a more personalized treatment, avoiding unnecessary toxicities[81]. Fortunately, these kind of therapies are already integrated in the clinic for the treatment of NSCLC, being the inhibitors of the EGFR one of the most important. Coupled to them, strategies to determine the responsiveness of the patients to the treatment are applied, which are based on predictive biomarkers[55]. Two of the most important are mutations in *KRAS* or *EGFR* genes, which are mutually exclusive. Patient's tumours with mutations in the first mentioned gene will not be responsive to anti-EGFR drugs, otherwise, mutations in EGFR are predictors of good response[57,76]. However, it is important to refer that only 30% and 32% of the patient's exhibit mutations in *KRAS* and *EGFR*, respectively, being the remaining percentage unknown regarding the response to these particular therapies. Unfortunately, patients tend to become resistant to the treatment, through the acquisition of secondary mutations by the tumours[204,205].

In this sense, for NSCLC, it is still important to discover and understand new biomarkers capable of giving a trustworthy prognostic for the patient; new biomarkers of therapy response in order to fill the existing gaps among patients' profiles; and also new ways to tackle associated mechanisms of resistance, preventing them from happening. In this sense, Raf kinase inhibitory protein arise to us as a potential prognostic and predictive biomarker of therapy response in lung cancer.

RKIP is a multifunctional protein that has been described as being critical in several physiological process, due to its interaction with several intracellular signalling pathways[99]. The first and most described role is its ability to modulate MAPK pathway, inhibiting it[94]. The quest to unravel more about the influence of RKIP came upon discovery that, in cancer, this protein was downregulated, particularly in metastatic cancer cells[114]. It is now known that, in various human cancers, RKIP acts as an endogenous onco-supressing protein affecting negatively tumorigenic processes, being the most well-reported role as a metastasis suppressor[206]. In fact, RKIP expression has been described in several types of cancer, with its downregulation ranging from a minimum of around 20% in bladder cancer and acute myeloid leukaemia to a maximum of more than 80% in hepatocellular carcinoma. Such studies, demonstrated RKIP as an independent prognostic factor, being its downregulation predictor of worse overall survival (OS) and/or lower disease free survival (DFS)[117].

Taking a closer look into what is known about RKIP, namely its expression levels, in lung cancer, the information was quite dispersed[207]. However, we were able to collect from the literature all what is known until now, and came up with some interesting facts. On one hand, downregulation of RKIP was observed in 36-62% of patients, with no differences detected between different types of NSCLC, namely, AD and SCC[207]. This ample difference might be explained by the very different approach adopted, such as PCR, IHC and WB, and even between the same technique, the fact that different antibodies were used to detect RKIP might make a difference preventing a good comparison between studies. Across these same studies, a clinical correlation was always found between lower levels of RKIP and lymph node metastasis occurrence and even higher TMN stage[207]. However, only two of these studies attempted to understand whether RKIP could have a prognostic value, and those reached quite different conclusions as well. Huerta Yepez did not find a predictive value in the total levels of RKIP, finding however for pRKIP and, on the other hand, Shi-Yang and colleagues determined that lower RKIP levels were predictor of worse OS, this being closely related with resistance to radiotherapy[164,172]. With this, it was possible to comprehend that RKIP expression has great potential to be considered a prognostic value, however more understanding is needed in order to state, with certainty, its role in the matter.

With this review, information regarding RKIP's role in signalling and implications in LC treatment were also put together in a clearer and concise way, something also never done previously[207]. RKIP is then implicated, in LC cells, not only in MAPK but too in EMT-controlling pathways in a so called NFKB/SNAIL/YY1/RKIP loop. Also, it has been implicated in the resistance to conventional therapy, such as adryamicin, cisplatin, both chemotherapeutic agents, and radiotherapy. Altogether, understanding how RKIP is behind the conventional therapies resistance is relevant as it might potentially open doors for it to be considered a predictor of therapy response and even be helpful in bypassing mechanisms of resistance.

Importantly, an *in silico* analysis of RKIP alterations was performed, focussing in lung cancer, which were divided into two main topics: understanding the molecular alterations behind RKIP downregulation and unravel the molecular signature of RKIP, in lung cancer. Only more recently studies were published trying to understand what is responsible for RKIP's expression variations, so this topic is still elusive. As demonstrated by Yesilkanal and Rosner, we too observed that gene alterations in NSCLC are rare (1% and <0.5%)[191,207]. However, we found that genomic alterations in *RKIP* were significantly associated with poor OS, which, even though rare, is still interesting to take in consideration for future studies. Besides this, the possibility of RKIP dysregulation occurring through transcriptional or post-transcriptional mechanisms has been slightly more explored. In the *in silico* analysis performed, it was established a

good negative correlation between low mRNA expression levels and hypermetilation of *RKIP*[207]. This goes according to various studies which have established a strong correlation between higher methylation status of the RKIP gene promoter and lower expression levels of the protein, such as in breast[208], coloretal[127,209], gastric[210] and esophageal[129] cancers, being this correlated with worse prognosis in the last two.

For the next part of the work we moved on to a more practical approach, starting by characterizing the NSCLC cell line models to be used. The four cell lines were chosen according to their histological and genetic characteristics and were firstly characterized, on one hand, for their RKIP levels of expression and, on the other hand, for their levels of expression of EGFR, as this is the major target for therapies in LC.

Based on the literature, only the A549 cell line has been characterized for RKIP expression, with it being positive[171]. From the first analysis, several interesting things were spotted. All cell lines expressed RKIP, but in quite different extensions. We believe this might be due to their different origins and molecular characteristics. PC9 demonstrated to have relatively normal levels of RKIP, compared to the other cell lines. Regarding H292's RKIP expression levels these are relatively lower. The reason behind this might be based on the metastatic origin of this cell line, as RKIP tends to be downregulated in a cancer metastasis context, justifying these lower levels[113,114,211]. However, this characteristic does not help to explain why HCC827 has such relatively lower RKIP levels, as there is no record in the literature of this cell line having a metastatic origin. It is known that there is a correlation between lower RKIP levels and tumour progression and so, having this in mind, these lower levels observed in HCC827 might be due to this cell line coming from a more advanced primary tumour, where RKIP would then be suffering a downregulation. Additionally, the fact that this cell line is the one with the considerably highest expression/activation levels of EGFR, which in turn could be responsible for the lower levels of RKIP, as demonstrated by us in the present work. Under this circumstances, EGFR-related downstream pathways will be always activated, impairing the activity of its inhibitor RKIP.

Still regarding RKIP expression levels, it is possible to see that, A549 cell line has similar RKIP levels as the H292 cell line, even though being originated from a primary tumour. Interestingly, it was recently described in pancreatic cancer that KRAS activation promotes the repression of RKIP expression, *in vitro* and *in vivo*, via MAPK-ERK pathway, leading to metastization and resistance to therapy[165]. This negative correlation might be the cause for A549 cells having relatively lower levels of RKIP, as this cell line is mutated for *KRAS,* thus having KRAS protein always activated, repressing RKIP. Additionally, it was possible to see that only in A549, upon EGF stimulation, pRKIP levels increased. When RKIP is

phosphorylated, its inhibitory function is reversed [106], exacerbating the activation of pathways such as MAPK and AKT. However, it is interesting to note that in the remaining cell lines, mainly in PC9, RKIP's expression levels decrease upon EGF stimulation and is significantly upregulated when EGFR is inhibited. Quite interestingly, Giovannetii *et al.* have reported in LC, that upon treatment with Erlotinib, RKIP expression increased, and that this could be explained by a reduction in NF-κB activation caused by EGFR inhibition[190]. Further we validated, by *in silico* analysis, that RKIP and EGFR are also inversely correlated in LC patients. These results pointed RKIP's loss as possible oncogenic modulator in LC, through a negative feedback mechanism regulation of the activation of one of the major players in this tumours, the EGFR.

Focussing now on EGFR expression levels, both H292 and A549, do not have relatively high levels of this receptor, however, upon stimulation, the same receptor and downstream signalling pathways activate extensively, revealing their 'oncogene addiction' to EGFR. Nonetheless, it is important to emphasise that the MAPK pathway in A549 is too activated without upstream stimulation, demonstrating the previously stated about it being *KRAS* mutant. As for the cell lines PC9 and HCC827, the observed high levels of pEGFR, even at basal levels, can also be explained by their genetic background, as these two are *EGFR* mutant being always activated regardless of stimulation, obviously influencing further activation of downstream signalling. Xu and colleagues too have demonstrated the differences in EGFR expression among both cell lines[212].

Further, to unravel the biological role of RKIP loss in lung cancer, the gene was knocked out through CRISPR/Cas9 technology. This process was effective for all cell lines, however, in H292, RKIP expression was only substantially reduced instead of completely eradicated, only being considered a knockdown. From this genetic alteration, it was expected to see changes in the expression levels of key proteins, known to modulate and be modulated by RKIP, such as EMT process. According to the literature, the loop NF-κB/Snail/YY1/RKIP was described in metastatic prostate cancer and melanoma, which states that NF-κB and Snail can repress RKIP, potentiating EMT processes, but at normal RKIP levels this mechanism is controlled[119,122,124,126]. Thus, it was expected that, upon RKIP KO, proteins such as Snail, YY1 and NF-κB would have higher levels of expression, and also that expression levels of epithelial markers, such as E-cadherin, would decrease and mesenchymal markers, like N-cadherin and Vimentin, would increase[213,214]. However, quite different results were obtained among the cell lines used.

Snail expression did not increase as much as expected, and in fact, it decreased in A549, PC9 and HCC827 cell lines. Regarding H292, there was a slight increase in Snail and a considerable increase of YY1. As referred before, the NF-κB/Snail/YY1/RKIP loop is only relatively well explained in metastatic

prostate cancer, so, the fact that all the cell lines, except H292, are from primary lung tumours might have an influence in these apparently non-expected results.

It was also expected with the RKIP KO to see changes in the EMT markers as direct influence of the loop referred before. Even though it was expected to observe a downregulation of E-cadherin in KO cells, which was in fact observed for PC9 and HCC827, this ends up to not be coherent with the decrease Snail expression, as it is described in the literature, that Snail directly downregulated E-cadherin by directly binding with *CDH1* promoter (which encodes E-cadherin)[215,216]. Having this into consideration, A549 has more consistent results, with an increase of E-cadherin. Still regarding PC9 cell line, it was possible to see that N-cadherin was expressed, and reduced upon KO (too consistent with lower levels of Snail). This might be explained not with the same cell expressing both markers, but rather by the initial cell culture, used to extract protein, having some cells in a more mesenchymal phenotype and others exhibiting a more epithelial phenotype. Interestingly, for H292 cell line, there is a clear expression of the mesenchymal markers N-cadherin and Vimentin. This is consistent with the fact that H292 is a metastatic cell line, and so exhibit a mesenchymal phenotype, a consequence of the EMT process.

Moreover, pSTAT3 levels were analysed, and, as it was already described in several types of cancer, including in NSCLC, RKIP is able to block STAT3 activation via phosphorylation, inhibiting metastasis formation[155,166,171]. So having this in mind, in our results, it was expected to see that upon RKIP KO, pSTAT3 would have higher expression levels. However, such difference among clones was not observed indicating that, at least at basal levels, RKIP KO does not directly influence the levels of pSTAT3 in our experimental conditions.

Finally, pGSK3β levels were analysed as this suppresses tumour progression[110]. Thus, it was expected that a RKIP loss would lead to an inactivation of GSK3β, driving the promotion of EMT. However, we did not observe consistent results. In A549 cell line, GSK3β in fact decreased upon RKIP KO, but this does not add up to the observed decrease of Snail and pSTAT3. On the other hand, in HCC827, GSK3β increases expression upon RKIP KO, and although not expected, it makes more sense as snail, YY1 and pSTAT3 levels decreased.

As we are faced with apparently unexpected results, it is important to emphasise that this characterization was performed with no stimulation conditions, and so, this is a representation of the cell lines at basal levels. Additionally, our results were compared with studies in which RKIP was downregulated and not completely absent through KO processes, us being the first ones to perform such studies. Thus, the disparities with the literature might, at some level, be explained by this fact. Also, we cannot exclude the hypothesis of these cells having miss targets from the CRISPR/Cas9 process.

To circumvent this, their phenotype was assessed and compared with what is described upon RKIP loss, giving strength to our models, and, at the same time, exploring the influence of RKIP in the biological behaviour of LC cells. Being RKIP a well-established metastasis suppressor, upon its downregulation, tumorigenic processes such as proliferation, migration, invasion and matastization capacity tend to be exacerbated. This relationship between loss of RKIP and increase tumour aggressiveness has been described *in vitro* in several cancer types such as breast[114,217], cervical[115], colorectal[142], gliomas[149], prostate[113], among others. Concerning NSCLC, a clear exploration of RKIP's role in such processes was not yet done, being only demonstrated that NSCLC metastasis are inversely correlated with RKIP expression levels[171].

Thus, for the *in vitro* and *in vivo* evaluation, the two different histological types available were chosen, H292 and PC9, which are mucoepidermoid carcinoma and adenocarcinoma, respectively. For the *in vitro* assays cells were put under two different conditions, 0.5% and 10% DMEM, enabling the comparison of the behaviour of the tumour cells when put under challenging conditions of low nutrients media. The obtained results, both *in vivo* and *in vitro*, were different between the two cell lines.

Starting with H292, no significant differences in cellular viability and migration rates were observed between cells with and without RKIP. In respect to the *in vivo* CAM assay, only a slight tendency for KD clones to form bigger tumours was observed, although not statistically significant, and no changes were found in the vessel area formed. These results are intriguing as changes were seen in the expression of EMT proteins, which did not translate into the migration and proliferation assays as expected. However, because in H292 clones it was not observed a shift from expression of epithelial to mesenchymal markers, but rather a slight increase in the levels of expression of mesenchymal markers, might be the reason for no greater changes have been detected. Also, the fact that RKIP was only knockdown in H292, might have impaired the observation of major differences, as the cell line is already adapted to have low levels of this protein.

Regarding PC9 KO cells, it was interesting to see differences in migration and viability rates between cells with and without RKIP, where the last ones presented an advantage over time, as it was hypothesized. Differences in the migration rate are only observed in 0.5% media as at this conditions, cells are put under stress and so only migration occurs, and not uncontrolled proliferation, as it happens at 10% media, where no differences were found. Regarding the proliferative behaviour, the tendency for RKIP KO cell to proliferate more (higher viability over time) is well visible. It is interesting that at 10% media differences statistically significant were observed but at 0.5% such was not. When dealing with these cells it was clear for us that these tend to enter in a quiescent state when are seeded at low density. For this assay, this low density factor was mandatory and so this might have impaired cells to grow,

impeding statistical differences to be observed. We believe that, if differences can be spotted when these cells grow in rich media, the loss of RKIP has a true effect on raising the proliferation rate.

As for the *in vivo* evaluation of PC9 clones, because the previous results were promising, adding to the CAM assay evaluation, a study in mice was also performed. Regarding the CAM assay, there was a tendency for RKIP KO cells to form bigger tumours, though not statistically significant. Also, similar to H292, no differences were spotted in vessel area. Although the technique is well-established in the group, we were faced with challenges, which might have compromised the observation of differences between clones: embryos harbouring well-formed tumours from PC9 KO cells tended to die before the predicted time; additionally, from the 13th to the 17th day of development, some tumours (both clones) tended not to grow. Unfortunately, the time limitation prevented a further analysis of these problems, nonetheless, and based on the cells behaviour *in vitro*, we hypothesize that PC9 KO cells invade tissues beyond the CAM, possibly compromising the viability of the embryo, leading to its death. The fact that it was possible to observe the formation of smaller secondary tumours, indicative of metastization, leads us to believe the previously stated. However, this is only a conjecture, and further analysis is needed.

Concerning the pilot study using immunocompromised mice, after optimising the ideal number of cells to be injected, tumour growth, using the PC9 KO and control cells, was assessed. Statistically significant differences were found between the tumour volumes formed in the mice, with cells without RKIP forming considerably bigger tumours. Although in the *in vivo* CAM assay the tendency observed was not significant, with the *in vivo* mice assay we were able to provide more support towards our stating that RKIP loss contributes to an increase in aggressiveness. Taking all into consideration, both *in vitro* and *in vivo* assays, we can say with some certainty that our new model for studying RKIP loss, the knockout by CRISPR/Cas9, could be reliable, as, upon RKIP loss, PC9 cells demonstrated to behave in a way concordant with the described in the literature for other tumour types, which is, the loss of this protein leads to a more aggressive phenotype.

Besides the role of RKIP in cancer progression, in NSCLC, RKIP downregulation was also related with increased radioresistance[164]. Interestingly, this has also been shown in other types of tumours with loss of RKIP being implicated in resistance to conventional treatments, frequently through the inhibition of apoptosis[153,157,186-188]. However, until now, the role of RKIP in response to molecular targeted therapies has not been explored. As referred before, one of the main limitations of these kind of drugs is that patients tend to develop acquired resistance. So, exploring the role of RKIP in response to EGFR targeted therapies in NSCLC, might provide a new insight regarding the response of cells, hence exploring a new potential predictive biomarker and also look into possible resistance mechanism, in order to circumvent them.

Thus, to assess the sensitivity of our NSCLC cell line panel to EGFR inhibitors we performed citotoxicity assays using three different drugs: Erlotinib, which is a reversible inhibitor, belonging to the first generation of this kind of inhibitors; Afatinib and AST1306 which are second generation EGFR inhibitors that bind irreversibly to EGFR, providing a more potent response.

It was anticipated that, due to being *KRAS* mutant, A549 cell line, would be resistant to anti-EGFR drugs[218], and that the cell lines PC9 and HCC827, which are *EGFR* mutant, would be highly sensitive to the same drugs[219]. For H292 we were expecting the drugs to be effective but at higher concentrations, due to being wild type for EGFR. Adding to this, our group has done the largest *in vitro* screen to assess AST1306 cytotoxicity performance, in which our four-panel of NSCLC were too analysed[68]. So in this study, and confirming our premises, H292, HCC827 and PC9 were considered highly sensitive to this drug and, appositively, A549 was classified as being resistant to AST1306[68].

Thus, a first screen to assess the efficacy of this drugs in our NSCLC cell lines was performed. Erlotinib exhibited the higher IC_{50} levels across all the cell lines, except for A549, which IC_{50} levels could not even be calculated due to the extreme lack of efficiency. This low effectiveness across our cell line panel can be justified because this drug binds to the receptor in a reversible way, thus, higher doses are needed to impact the cells viability. The remaining EGFR inhibitors, Afatinib and AST1306, demonstrated to be much more efficient, and this is explained because they bind irreversible not only to the target EGFR, but to other receptors of the same family such as HER2, enabling better results at lower concentrations[220]. Emphasizing that, in cell lines where *EGFR* is not mutated (H292) the IC_{50} values were higher, as these drugs are designed to specifically target the mutant isoforms of *EGFR*.

As RKIP is an endogenous inhibitor of EGFR-activated pathways, our hypothesis was that, upon RKIP loss, these same pathways would no longer be tightly controlled, this way becoming more activated, leading to a worse response of the cell to EGFR inhibitors. By testing our hypothesis very different results came up depending on the cell line.

For H292 cell line, there was a tendency for cells without RKIP to be less responsive to Erlotinib. The response observed for this drugs might not have been statistically significant due to RKIP expression not have been totally inhibited but only downregulated. It appears that cells without EGFR mutations and with loss of RKIP might be resistant to 1st generation TKI, so it would be interesting to further explore this matter. As for the irreversible EGFR inhibitors AST1306 and Afatinib, the expected tendency was not observed, being IC_{50} values quite similar, which again might be due to their associated higher potency.

Regarding A549 cell line, no differences were observed regarding the two clones. The reason behind this might be laid on the fact that this cell line is *KRAS* mutant, and so, altering RKIP expression, might not have much impact, as the downstream pathways are already constitutively active.

Nonetheless, it was impressive to see that, in PC9 cell line, our initial hypothesis was observed, in a remarkable extension. Significant differences were observed with Erlotinib, Afatinib and AST1306, with cells without RKIP being less responsive than its control. Additionally, from the *in vivo* CAM assay, in which tumours were treated with Afatinib, it was possible to see that tumours formed by PC9 KO cells do not have a reduction on tumour perimeter after treatment, opposed to what happens with the control group. This tendency was consistent with the observed in the *in vitro* assay.

Because PC9 is *EGFR* mutant, it was quickly questioned whether this response could somehow be associated with the *EGFR* mutations status. Hence, having the cell line HCC827, which has the same mutation in EGFR (exon del19), we tried to attest our theory. Unfortunately, the results were not at all similar to the PC9 ones, with HCC827 showing only tendency to be less responsive to AST1306 upon RKIP loss. However, HCC827 has very low levels of RKIP expression, and so, eradicating RKIP expression through knockout will probably not make much difference, response wise. Adding to this, the fact that this cell line is the most sensitive to all the drugs, the influence of RKIP KO can be insignificant. Thus, to further validate our hypothesis, it would be interesting to test other cell lines known to have the same or different *EGFR* mutations (e.g. HCC4006 and H1975), as well as overexpress RKIP instead of knocked out it in HCC827 cell line, to better understand if in fact there is a relation between RKIP, *EGFR* mutational status and anti-EGFR drugs response.

Having demonstrated that the loss of RKIP lead to a more resistant phenotype in PC9 cell line, our final aim was to shed light on which pathways were being modulated upon treatment with EGFR inhibitors and RKIP KO. Regarding the cells response to EGFRi, as it was graphically demonstrated, all the drugs were able to bind to its target inhibiting it, impairing the activation of proteins like MEK in both clones. However, there was a tendency for PC9 KO cells to have less inhibition of the activation of ERK and mainly AKT. This inability to inactivate completely AKT and MAPK might be the reason behind the apparent resistant phenotype observed in the *in vitro* assays. Although no report was found in the literature relating RKIP with AKT mediated resistance, this is not surprising, as the reactivation of the phosphorylation of AKT, activating PI3K/AKT pathway, has been well described as a mechanism of lung cancer cells resistance to EGFR targeting [221-223].

Finally, an *in silico* analysis was performed, using the TCGA database, to understand which proteins were underexpressed upon RKIP overexpression, this way revealing RKIP's control length in LC patients.

It was very interesting to verify the involvement of RKIP in oncogenic proteins, in both AC and SCC tumours, particularly with players of the PI3K/AKT signalling pathway, as it was possible to see in our results. This analysis also showed an involvement of RKIP in pathways such as NF-κB, Hippo and mTOR signalling pathways, as well as with important cellular processes such as apoptosis, which makes the continuous study of this protein in lung cancer extremely relevant.

CHAPTER 6:

Conclusions and Future perspectives

6. Conclusion and future perspectives

In this work, the RKIP's role in the biological behaviour, in signalling modulation and response to EGFR targeted therapies was evaluated in lung cancer. Also, we comprised all known information about RKIP and lung cancer, and evaluated its potential as a prognostic biomarker as well as its implication in cancer therapy. Through an *in silico* analysis we demonstrated that lower gene copy numbers and higher methylation status of RKIP promoter are significantly associated with RKIP downregulation in lung cancer, that mutations in *RKIP,* although rare, are significantly associated with poor overall survival of NSCLC patients and importantly we corroborated that RKIP can be a central modulator of lung carcinogenesis. With this analysis, it was possible to realize that, in future studies, it would be interesting to include genetic analysis of RKIP, to understand if those could be considered as possible prognostic markers or even therapeutic targets.

Also, we were able to demonstrate that the loss of RKIP is implicated in processes known in tumorigenesis such as migration and proliferation and importantly, we tried to validate this *in vivo*, demonstrating that cells without RKIP have a higher capacity to form tumours. On the other hand, we found that PC9 cell line, *EGFR* mutant, upon RKIP KO, becomes less responsive to EGFR inhibitors, and also that this apparently resistance might be probably through the RKIP modulation of AKT signalling pathway. Having in consideration the molecular signature of RKIP that we determined by *in silico* analysis, it would be of upmost interest in the future to further explore other pathways identified that can be behind the observed gain of resistance to EGFR inhibitors.

Finally, and in regard to future perspectives, we intend to deeply explore whether the resistance to EGFR inhibitors in NSCLC cells is dependent on the mutation status of *EGFR*. So, performing a similar analysis in other *EGFR* mutant cell lines or even genetically alter *EGFR* in WT cell lines by expressing the mutations of interest. Importantly, it will be mandatory to validate the results in more sophisticated *in vivo* model, such as orthotropic mouse models for example, particularly because RKIP role might be dependent on the tumour's microenvironment and also, proceed to validate RKIP as a predictive biomarker to EGFR targeted therapies in LC by analysing a series of patient's cases with clinical information for EGFRi response, which we already have available.

Concluding, we hypothesized in the beginning that RKIP would be a modulator of EGFRi in lung cancer. Actually, we gained a huge amount of new and interesting data that put us in the good way to prove our hypothesis as gained many others of new hypothesis to test in the future.

CHAPTER 7:

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7. References

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APPENDIX I

Supplementary data

Supplementary Tables

Table S1- Top25 genes negatively co-expressed (at mRNA level) with RKIP mRNA expression in lung cancer. TCGA provisional data (www.cbioportal.org).

SCC				AC			
Gene	Cytoband	Spearman's Correlation	p-Value	Gene	Cytoband	Spearman's Correlation	p-Value
ERO1A *	14q22.1	-0.416	2.05e-22	**AVL9**	7p14.3	-0.510	1.67e-35
TEP1	14q11.2	-0.404	4.48e-21	**GREB1L**	18q11.1-q11.2	-0.499	7.79e-34
HRH1	3p25.3	-0.401	9.10e-21	**GNA12**	7p22.3-p22.2	-0.493	4.56e-33
TNFRSF12A	16p13.3	-0.365	3.17e-17	**LAMC2 ***	1q25.3	-0.477	9.95e-31
TGFBI	5q31.1	-0.363	4.69e-17	**RASAL2**	1q25.2	-0.468	1.81e-29
SOCS4	14q22.3	-0.360	9.37e-17	**ITGAV**	2q32.1	-0.460	1.82e-28
AHNAK2	14q32.33	-0.357	1.58e-16	**CD109**	6q13	-0.456	6.00e-28
KLF10	8q22.3	-0.356	2.13e-16	**BICD1**	12p11.21	-0.456	6.42e-28
HIF1A	14q23.2	-0.354	2.87e-16	**SLC16A3**	17q25.3	-0.452	2.17e-27
DAAM1	14q23.1	-0.353	3.76e-16	**BCL10**	1p22.3	-0.446	1.33e-26
NUMB	14q24.2-q24.3	-0.347	1.37e-15	**COLGALT1**	19p13.11	-0.444	2.29e-26
SERPINE1	7q22.1	-0.346	1.67e-15	**SPATS2L**	2q33.1	-0.441	4.64e-26
ACTN1	14q24.1|14q22-q24	-0.345	1.98e-15	**ANLN**	7p14.2	-0.436	2.28e-25
XDH	2p23.1	-0.344	2.54e-15	**PPP1R18**	6p21.33	-0.433	5.10e-25
LAMC2 *	1q25.3	-0.342	3.40e-15	**OSBPL3**	7p15.3	-0.432	6.52e-25
OSMR	5p13.1	-0.341	4.20e-15	**GPRIN1**	5q35.2	-0.432	7.28e-25
S100A2	1q21.3	-0.341	4.30e-15	**MMD**	17q22	-0.430	1.11e-24
KLF7	2q33.3	-0.340	4.68e-15	**CPD**	17q11.2	-0.429	1.43e-24
MMP14	14q11.2	-0.339	6.04e-15	**ADGRF4 ***	6p12.3	-0.427	2.70e-24
IL4R	16p12.1	-0.339	6.46e-15	**ELK3**	12q23.1	-0.427	2.73e-24
IL1RAP	3q28	-0.337	9.65e-15	**UBA6**	4q13.2	-0.426	2.98e-24
EHD2	19q13.33	-0.333	2.12e-14	**ERO1A ***	14q22.1	-0.426	3.16e-24
DUSP7	3p21.2	-0.332	2.45e-14	**SLC2A1**	1p34.2	-0.424	5.57e-24
TRIP11	14q32.12	-0.332	2.55e-14	**C1GALT1**	7p22.1-p21.3	-0.424	5.83e-24
ADGRF4 *	6p12.3	-0.329	4.03e-14	**TPBG**	6q14.1	-0.424	5.96e-24

* Common genes to Adenocarcinoma (AC) and Squamous cell carcinoma (SCC).

Table S2- Top50 Proteins underexpressed in the subset of AC and SCC patients depicting RKIP mRNA upregulation. TCGA provisional data (www.cbioportal.org)

AC				SCC			
Protein	**Cytoband**	**p-Value**	**q-Value**	**Gene**	**Cytoband**	**p-Value**	**q-Value**
INPP4B	4q31.21	-0.35	1.047e-3	**CDKN1A**	6p21.2	6.126e-4	0.127
ERRFI1	1p36.23	-0.13	3.712e-3	**CCND1**	11q13.3	6.357e-3	0.401
MAPK1_PT202_Y204	22q11.21	-0.33	3.766e-3	**NDRG1_PT346**	8q24.3	0.0131	0.401
MAPK3_PT202_Y204	16p11.2	-0.33	3.766e-3	**BAX**	19q13.33	0.0355	0.401
RB1_PS807_S811	13q14.2	-0.23	7.177e-3	**BECN1**	17q21.31	0.0400	0.401
FN1	2q35	-0.47	0.0127	**CAV1**	7q31.2	0.0423	0.401
AKT1_PS473	14q32.32	-0.27	0.0130	**MYH11**	16p13.11	0.0446	0.401
AKT2_PS473	19q13.1-q13.2	-0.27	0.0130	**MAPK14**	6p21.31	0.0600	0.457
AKT3_PS473	1q44	-0.27	0.0130	**HSPA1A**	6p21.33	0.0625	0.457
ESR1	6q25.1-q25.2	-0.14	0.0160	**YAP1_PS127**	11q13	0.0709	0.473
STAT5A	17q21.2	-0.40	0.0218	**SERPINE1**	7q22.1	0.0737	0.477
XBP1	22q12.1|22q12	-0.14	0.0229	**RPS6_PS235_S236**	9p21	0.103	0.551
AKT1_PT308	14q32.32	-0.13	0.0232	**SRC_PY416**	20q12-q13	0.107	0.551
AKT2_PT308	19q13.1-q13.2	-0.13	0.0232	**MAPK14_PT180_Y182**	6p21.3-p21.2	0.109	0.551
AKT3_PT308	1q44	-0.13	0.0232	**FN1**	2q35	0.126	0.621
TSC2_PT1462	16p13.3	-0.13	0.0245	**EIF4EBP1_PT70**	8p12	0.134	0.645
PXN	12q24.23	-0.38	0.0293	**PEA15**	1q23.2	0.158	0.677
NFKB1_PS536	4q24	-0.30	0.0367	**YBX1**	1p34.2	0.164	0.677
RPS6KA1_PT359_S363	1p	-0.09	0.0394	**MTOR_PS2448**	1p36.2	0.165	0.677
ANXA1	9q21.13	-0.28	0.0489	**YAP1**	11q22.1	0.173	0.677
CCNE1	19q12	-0.13	0.0493	**SYK**	9q22.2	0.179	0.677
MYH11	16p13.11	-0.74	0.0538	**MS4A1**	11q12.2	0.180	0.677
DVL3	3q27.1	-0.08	0.0550	**ARAF_PS299**	Xp11.4-p11.2	0.198	0.694
SRC_PY527	20q12-q13	-0.23	0.0551	**PRKCA_PS657**	17q22-q23.2	0.200	0.694
RICTOR	5p13.1	-0.35	0.0605	**STK11**	19p13.3	0.205	0.694
ATM	11q22.3	-0.24	0.0748	**PECAM1**	17q23.3	0.210	0.698
SYK	9q22.2	-0.15	0.0897	**PARK7**	1p36.23	0.214	0.698
YBX1	1p34.2	-0.23	0.0909	**FOXO3_PS318_S321**	6q21	0.216	0.698
RAF1	3p25.2	-0.05	0.104	**EIF4E**	4q23	0.224	0.698
SERPINE1	7q22.1	-0.36	0.110	**LCK**	1p35.2	0.227	0.698
ERBB2	17q12	-0.24	0.116	**YWHAB**	20q13.12	0.230	0.698
MTOR_PS2448	1p36.2	-0.11	0.127	**RPS6_PS240_S244**	9p21	0.236	0.698
EGFR	7p11.2	-0.09	0.127	**ITGA2**	5q11.2	0.239	0.698
CAV1	7q31.2	-0.31	0.131	**SRC_PY527**	20q12-q13	0.243	0.698
RPS6KB1	17q23.1	-0.12	0.147	**PDK1**	2q31.1	0.270	0.719
MS4A1	11q12.2	-0.05	0.155	**PIK3R1**	5q13.1	0.279	0.719
AKT1S1_PT246	19q13.33	-0.05	0.155	**PIK3R2**	19p13.11	0.279	0.719
PRKAA1_PT172	5p12	-0.18	0.160	**YWHAZ**	8q22.3	0.296	0.722
MAP2K1	15q22.31	-0.13	0.176	**PRDX1**	1p34.1	0.326	0.722
RPTOR	17q25.3	-0.10	0.183	**MAP2K1_PS217_S221**	15q22.1-q22.33	0.327	0.722
TSC2	16p13.3	-0.18	0.192	**MAP2K1**	15q22.31	0.346	0.722
SETD2	3p21.31	-0.13	0.197	**ESR1**	6q25.1-q25.2	0.348	0.722
COL6A1	21q22.3	-0.13	0.199	**MAPK8_PT183**	10q11.22	0.356	0.722
PRKCA_PS657	17q22-q23.2	-0.15	0.201	**SETD2**	3p21.31	0.359	0.722
YBX1_PS102	1p34	-0.06	0.202	**IRS1**	2q36.3	0.363	0.723
GATA3	10p14	-0.05	0.224	**CASP8**	2q33.1	0.378	0.746
RICTOR_PT1135	5p13.1	-0.06	0.228	**MYH9_PS1943**	22q13.1	0.386	0.746
IRS1	2q36.3	-0.04	0.229	**BCL2L1**	20q11.21	0.404	0.761
TP53BP1	15q15.3	-0.15	0.232	**KDR**	4q12	0.433	0.793
ITGA2	5q11.2	-0.11	0.243	**MAPK1_PT202_Y204**	22q11.21	0.449	0.803

AC - Adenocarcinoma; SCC - Squamous cell carcinoma.

Supplementary Figures

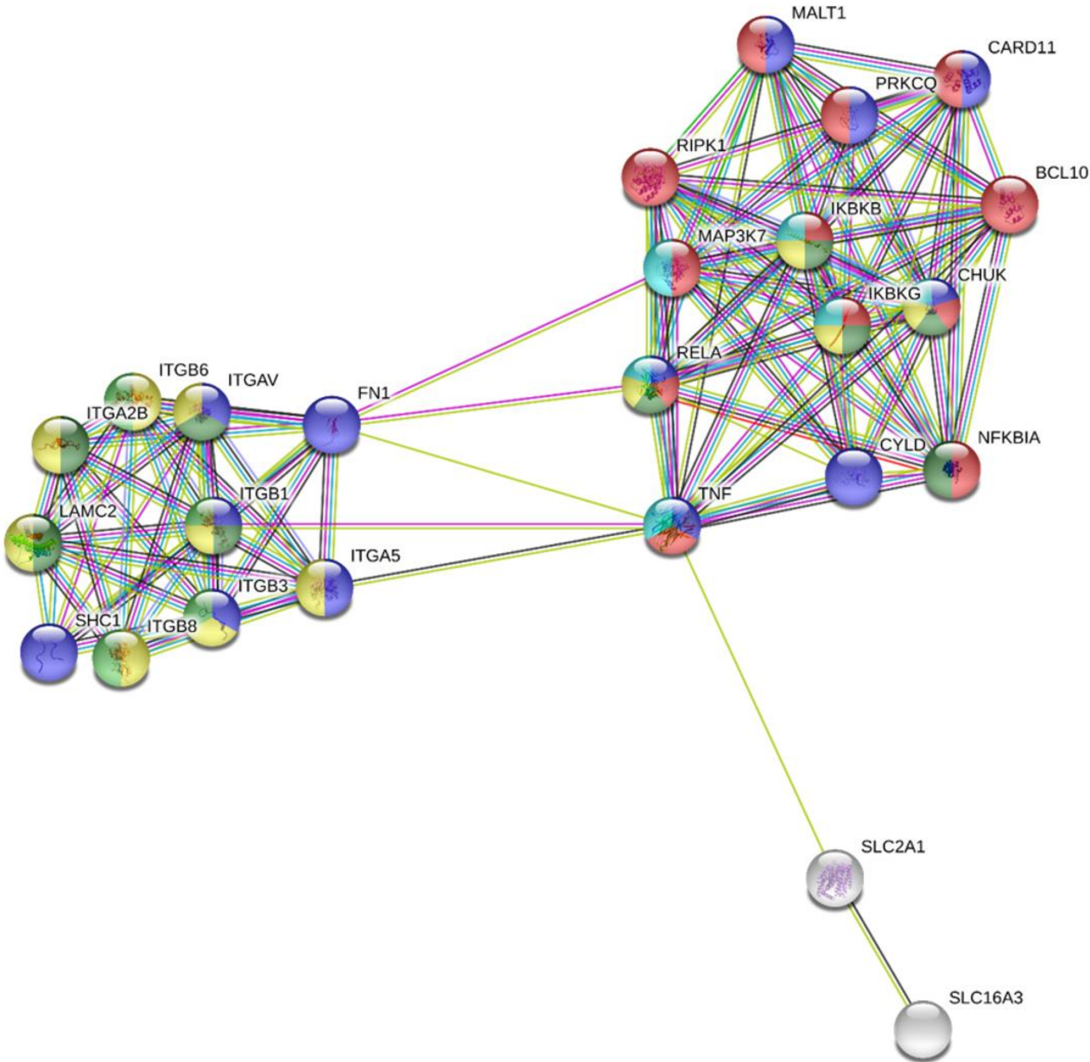

Figure S1: Functional protein association network done in STRING (https://string-db.org/) with the Top25 genes (Supplementary Table S1) negatively co-expressed with RKIP at mRNA level in lung AC. Blue: cell differentiation genes; Red: NF-KappaB associated genes; Yellow: PI3K/AKT associated genes; Light Blue: MAPK associated genes; Green: EGFlike domain associated genes; White: Glucose Metabolism associated genes.

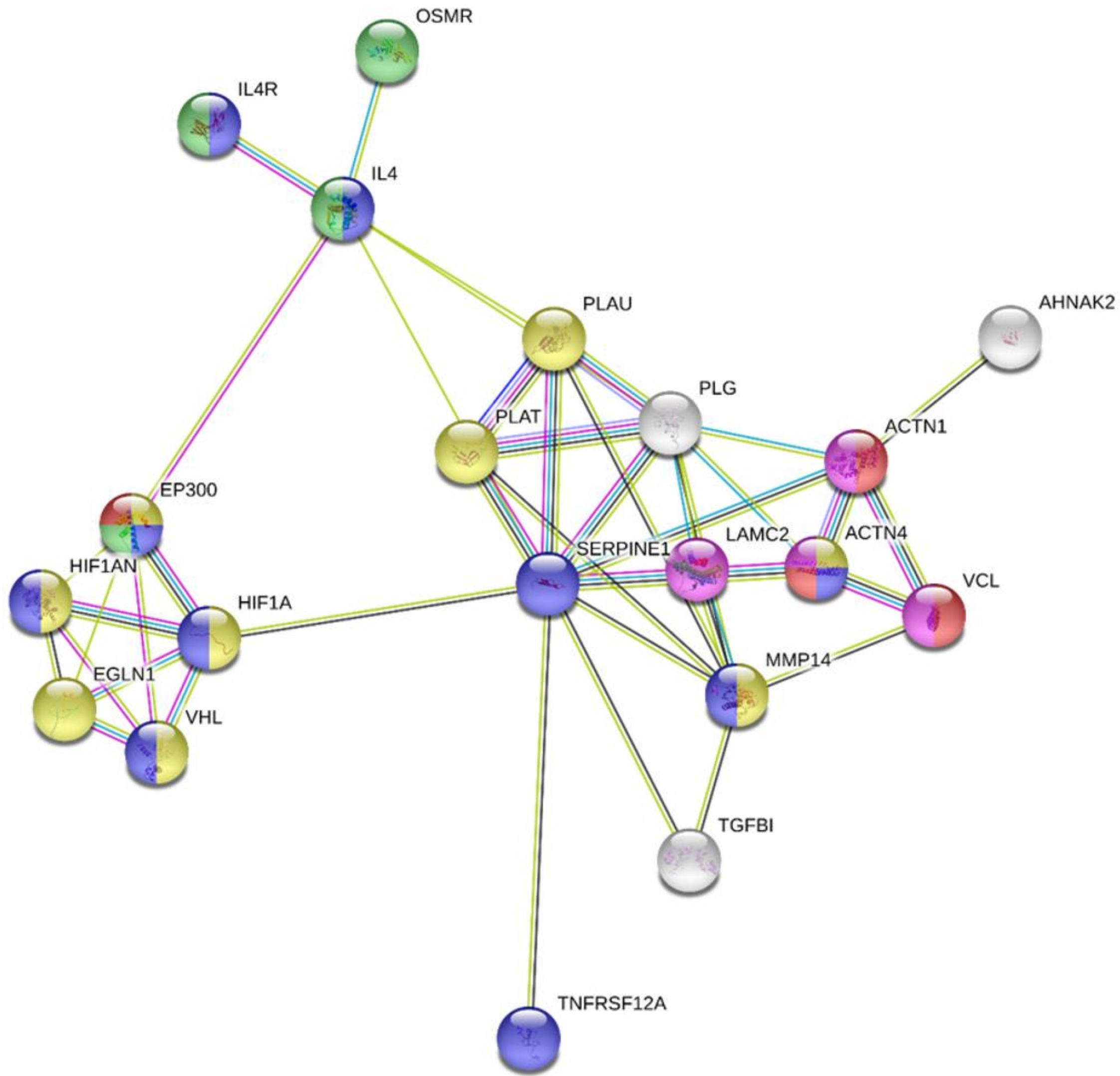

Figure S2: Functional protein association network done in STRING (https://string-db.org/) with the Top25 genes (Supplementary Table S1) negatively co-expressed with RKIP at mRNA level in lung SCC. Blue: cell differentiation genes; Red: adherent junctions associated genes; Yellow: response to hypoxia associated genes; Pink: focal adhesion associated genes; Green: JAK/STAT associated genes.