Genetic modeling of B- and C-Raf kinase inhibition for the treatment of K-RasG12V driven lung adenocarcinoma

DOCTORAL THESIS

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Genetic modeling of B- and C-Raf kinase inhibition for the treatment of K-RasG12V driven lung adenocarcinoma

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i. SUMMARY/RESUMEN
SUMMARY

Even though Raf proteins share a high percentage of homology they have been proven to be non-redundant in specific tissues and physiological contexts. In K-RasG12V driven lung adenocarcinoma, genetic elimination of C-Raf blocked tumor initiation and maintenance, whereas the absence of A- or B-Raf did not impair tumorigenesis. It was therefore necessary, first, to validate C-Raf kinase activity as a therapeutic target for K-Ras driven lung cancer patients. However, B-Raf kinase inhibition has been reported to paradoxically hyperactivate the MAPK pathway in Ras mutated cells. Importantly, Raf inhibitors are ATP analogues that target all Raf kinases. Therefore, it was necessary to deciphered if inhibition of B-Raf catalytic activity could be counterproductive for these patients.

We have observed that the elimination of C-Raf kinase activity impairs tumor initiation and growth of p53 wild type tumors, and moreover, the FDG-Glucose uptake of the most aggressive lesions. However, preliminary data suggest the lack of C-Raf catalytic activity limited the therapeutic benefit in p53 deficient tumors.

Conversely, in the case of B-Raf, the inhibition of its kinase activity concomitantly with the expression of K-RasG12V in the mouse lung dramatically decreases lifespan. We have determined that the cause of death of these mice is due to increased tumor burden, which was dependent on C-Raf kinase activity. Surprisingly, we also found that inhibition of B-Raf catalytic activity induces tumor formation in the absence of the K-RasG12V oncogene, as it has been also observed in human lung adenocarcinomas.

Together these data suggest that there are important differences between B- and C-Raf inhibition that should be taken into account when designing inhibitors for the treatment of K-RasG12V driven lung cancer.
RESUMEN

Aunque las proteínas Raf comparten un alto grado de homología, se ha visto que en determinados tejidos y contextos fisiológicos no son redundantes. En tumores de pulmón inducidos por el oncogén K-RasG12V, mientras que la eliminación de la proteína C-Raf bloquea la iniciación y el desarrollo tumoral, la ausencia de B-Raf no impide la tumorigénesis. Por tanto era necesario, validar la actividad quinasa de C-Raf como diana terapéutica para pacientes con cáncer de pulmón inducido por la proteína K-RasG12V. Sin embargo, se ha descrito que la inhibición de la actividad catalítica de B-Raf en células que tienen Ras mutado produce la hiperactivación de vía MAPK. Por tanto, era fundamental, describir si la inhibición de la actividad quinasa de B-Raf era contraproducente para estos pacientes.

En el caso de C-Raf, se observó que la eliminación de la actividad quinasa impide la iniciación y el crecimiento de tumores en los que la proteína p53 está en su forma salvaje, e incluso disminuye la toma de FDG de los tumores más agresivos. Sin embargo, datos preliminares sugieren que la falta de la actividad quinasa de C-Raf en tumores en los que p53 está mutado, no recapitula el efecto terapéutico anterior.

Por otro lado, en el caso de B-Raf, la inhibición de su actividad catalítica de forma concomitante a la expresión de K-RasG12V en el pulmón causa la disminución de la esperanza de vida en ratones. Asimismo, se determinó que la causa de muerte de estos ratones es la carga tumoral en el pulmón, y que el desarrollo de ésta depende de un proceso en el que media la actividad quinasa de C-Raf. Sorprendentemente, se observó que la inhibición de la actividad quinasa de B-Raf también puede inducir la formación de tumores en ausencia de K-RasG12V.

De forma conjunta, estos datos sugieren que hay importantes diferencias entre la inhibición de la actividad quinasa de B- y C-Raf que deberían tenerse en cuenta a la hora de diseñar inhibidores de Raf para el tratamiento de cáncer de pulmón inducido por K-RasG12V.
1 INDEX
# INDEX

1 INDEX ................................................................................................................................. 1

2 ABBREVIATIONS .................................................................................................................. 5

3 INTRODUCTION ...................................................................................................................... 4
  3.1 LUN CANCER .................................................................................................................... 6
    3.1.1 Epidemiology ............................................................................................................. 6
    3.1.2 Histopathologic lung cancer subtypes ....................................................................... 7
    3.1.3 Frequent mutations in lung adenocarcinomas ........................................................... 8
  3.2 Ras proteins ....................................................................................................................... 8
    3.2.1 Ras family .................................................................................................................. 8
    3.2.2 Ras structure ............................................................................................................. 9
    3.2.3 Ras activation .......................................................................................................... 9
    3.2.4 Ras effectors ........................................................................................................... 11
  3.3 The Raf-Mek-Erk pathway ............................................................................................... 12
  3.4 The Raf proteins .............................................................................................................. 13
    3.4.1 A-Raf protein ........................................................................................................... 15
    3.4.2 C-Raf protein ........................................................................................................... 15
    3.4.3 B-Raf protein ........................................................................................................... 17
    3.4.4 B-Raf/C-Raf heterodimers ....................................................................................... 19
  3.5 The Mek proteins ............................................................................................................ 20
  3.6 The Erk proteins ............................................................................................................ 20
  3.7 The Mapk inhibition as a therapeutic strategy ............................................................... 21

4 OBJECTIVES ........................................................................................................................ 24

5 MATERIALS AND METHODS ................................................................................................ 28
  5.1 Maintenance of the mouse lines .................................................................................... 30
    5.1.1 Generation of C-Raf LmLD468A mice ................................................................. 30
    5.1.2 Proceeding of the mouse lines used in this work .................................................. 30
    5.1.3 Maintenance of mice ............................................................................................... 31
    5.1.4 Standard necropsy ................................................................................................ 31
    5.1.5 MEFs extraction ...................................................................................................... 31
    5.1.6 Genotyping ............................................................................................................. 32
  5.2 In vivo proceedings ......................................................................................................... 34
    5.2.1 Tamoxifen injection ................................................................................................ 34
    5.2.2 Tamoxifen diet ........................................................................................................ 34
    5.2.3 Adenoviral intratracheal infection .......................................................................... 34
    5.2.4 Micro X-ray computed tomography (micro-CT) .................................................... 35
  5.3 Processing of mouse tissues ............................................................................................ 35
    5.3.1 X-Gal whole mount staining ................................................................................. 35
    5.3.6 Histopathology and immunohistochemistry ......................................................... 36
  5.4 Quantification of tumors .................................................................................................. 36
    5.4.1 Quantification of the relative areas occupied by the different types of lung epithelia... 36
  5.5 In vitro procedures ......................................................................................................... 36
    5.5.1 Culture conditions ................................................................................................. 36
  5.6 RNA extraction and cDNA sequencing ............................................................................ 37
    5.6.1 RNA extraction ....................................................................................................... 37
5.6.2 cDNA synthesis ...........................................................................................................37
5.6.3 Primers for cDNA amplification ..................................................................................37
5.7 Western Blot ..................................................................................................................38
5.7.1 Protein extraction .........................................................................................................38
5.7.2 Gel run .........................................................................................................................38
5.7.3 Transfering the denatured proteins to a membrane .....................................................39
5.7.4 Blocking and antibodies .............................................................................................39
5.8 Southern Blot ..................................................................................................................39
5.9 Statystics .......................................................................................................................40
6 RESULTS ..........................................................................................................................42
6.1 Study of C-Raf kinase inhibition in K-RasG12V driven lung cancer ..............................44
  6.1.1 Generation of an inducible C-Raf kinase dead allele: C-RafD468A .........................44
  6.1.2 C-RafD468A protein has impaired kinase activity ......................................................45
  6.1.3 C-Raf kinase activity is not necessary for mouse embryo development ....................46
  6.1.3 C-RafD468A protein impairs tumor formation ............................................................50
  6.1.4 C-Raf kinase activity stops tumor growth in K-RasG12V, p53 proficient lung tumors ...51
  6.1.5 C-Raf kinase activity ablation does not stop tumor growth in K-RasG12V, p53 deficient lungs ..........................................................54
6.2 Study of B-Raf kinase inhibition in K-RasG12V driven lung cancer ..............................56
  6.2.1 B-RafD594A cooperates with K-RasG12V to increase tumor burden .................56
  6.2.2 Coexpression of K-RasG12V and B-RafD594A in the lung shortens mice lifespan .......58
  6.2.3 Ubiquitous co-activation of K-RasG12V and B-RafD594A leads to hyperplastic growth of certain epithelial tissues .................................................60
  6.2.4 C-Raf kinase activity is essential for the paradoxical tumor growth acceleration ..........61
  6.2.5 Loss of B-Raf wild type allele in K-RasG12V and B-RafD594A co-expressing lung cells increases Clara Cell transformation and diminishes adenoma/adenocarcinoma growth ..........63
  6.2.6 Loss of B-Raf kinase activity can promote lung tumorigenesis .................................67
7 DISCUSSION .......................................................................................................................68
  7.1 Validation of C-Raf kinase activity inhibition to treat K-RasG12V driven lung adenocarcinomas ..............................................................71
  7.2 Study of the effect of B-Raf kinase inhibition in K-RasG12V driven lung adenocarcinomas.78
8 CONCLUSIONS/ .................................................................................................................85
CONCLUSIONES .....................................................................................................................85
10 APPENDIX/ ......................................................................................................................103
PUBLICATIONS .....................................................................................................................103
2 ABBREVIATIONS
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ad-Cre</td>
<td>Adenovirus codifying Cre recombinase.</td>
</tr>
<tr>
<td>Ad-FIP</td>
<td>Adenovirus codifying Flp recombinase.</td>
</tr>
<tr>
<td>ASK-1</td>
<td>Apoptosis Signal-regulating Kinase 1.</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 Antagonist of cell Death.</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidasa.</td>
</tr>
<tr>
<td>β-geo</td>
<td>β-galactosidasa / neomicina.</td>
</tr>
<tr>
<td>CC10</td>
<td>Clara Cell 10. Proteína de las células clara.</td>
</tr>
<tr>
<td>CR</td>
<td>Conserved Region.</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-Rich Domain.</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography.</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium.</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinases.</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum.</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-Activating Protein.</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate.</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange-Factor.</td>
</tr>
<tr>
<td>GGTLasal</td>
<td>Gerani Gerani Transferasa I.</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate.</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxilin &amp; Eosine.</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site.</td>
</tr>
<tr>
<td>Mapk</td>
<td>Mitogen-activated protein kinases.</td>
</tr>
<tr>
<td>Mek</td>
<td>Map Erk Kinase.</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse Embryonic Fibroblasts.</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis 1.</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-kappaB.</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small-Cell Lung Cancer.</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-hidroxitamoxifeno.</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphatidylinositol-Dependent Kinase-1.</td>
</tr>
</tbody>
</table>
PFU: Plaque-forming unit.
PI3K: Phosphoinositol-3-Kinase. Fosfoinositol-3-Quinasa.
PKA: Protein Kinase A.
PKB: Protein Kinase B.
PKC: Protein Kinase C.
PLCe: Phospholipase Ce.
PP1: Proteinphosphatase 1.
PP2A: Proteinphosphatase 2A.
PTP: Protein Tyrosine Phosphatase.
p90RSK: p90 Ribosomal S6 Kinase.
RalGDS: Ral Guanine nucleotide-Dissociation Stimulator.
RB: Retinoblastoma.
RBD: Ras Binding Domain.
RT: Room Temperature.
RTK: Receptor Tyrosine Kinase.
SCLC: Small-Cell Lung Cancer.
SPC: Surfactant Protein C.
TF: Transcription Factor.
3 INTRODUCTION
3.1 LUN CANCER

3.1.1 Epidemiology

Lung cancer is the leading cause of cancer related diagnosis and deaths in Spain, Europe and worldwide (Figure 1) (Ferlay et al., 2015). In 2012, it was the fourth cause of death in the world after ischaemic heart disease, stroke and chronic obstructive pulmonary disease and lower respiratory infections (2). This disease has a poor prognosis being the five years survival less than 15% (Davidson et al., 2013).

![Figure 1. Mortality rates in Spain in 2012 of the most frequent type of cancers. Adapted from (Ferlay et al., 2015)](image)

Among the risk factors to lung cancer, tobacco is responsible for 75% of the cases (Parkin et al. 20015). Nicotine-derived nitrosaminoketone (NKK) causes DNA adducts that if persist can cause mutations in key driver lung cancer oncogenes. However, the risk(s) factor(s) for the 25% remaining cases has not been identified yet. There are many environmental, genetic and hormonal factors that have been related to lung cancer in never-smokers, however, any of them seem to have a predominant role (Sun et al., 2007).

Lung cancer in smokers and in never-smokers should be considered two separate diseases. The distinct carcinogens are probably the cause of the predominant histopathological subtype and the specific landscape of mutations on each smokers and never-smokers. Therefore, the difference in gender, region and response to inhibitors could also be attributed to them (Sun et al., 2007).
3.1.2 Histopathologic lung cancer subtypes

Primary lung carcinomas are classified as:

- **SCLC (Small Cell Lung Cancer):** it accounts for 18-20% of the cases and it is a highly malignant type consisting of small cells expressing neuroendocrine markers. It is characterized by a highly metastatic spread. The majority of the cases are connected with tobacco exposure (Sun et al., 2007) and it rarely benefits from chemo- and radiotherapy.

- **NSCLC (Non Small Cell Lung Cancer):** it represents the 80% of the cases (Zugazagoitia et al., 2014) and it is subdivided in:
  
  **SCC (Squamous Cell Carcinoma):** morphologically, they are characterized by the presence of intercellular bridges, individual cell keratinisation and squamous pearl formation (Zugazagoitia et al., 2014). They arise from the bronchiolar epithelium of the central airways even though a significant percentage is identified in the periphery. They present loss of heterozygosity in different locus, p53 mutations and deregulation of cell proliferation (cyclin D1 and E) and apoptosis (Bcl-2) markers (Davidson et al., 2013). The most accepted and widely used molecular marker for SCC is p63. In addition, CK5/6 are other markers of pulmonary squamous tumors (Zugazagoitia et al., 2014).

  **Lung adenocarcinoma:** it is the most frequent type of all lung cancers and particularly the most common in non-smokers (Davidson et al., 2013). Histologically they present glandular differentiation and/or mucin production, even though the majority of adenocarcinomas have a mixed pattern. They develop in peripheral areas and are thought to arise from the alveolar or bronchiolar epithelium (pneumocytes or Clara cells). Regarding the molecular alterations of these lesions, EGFR mutations appear more frequently in non-smokers whereas in smokers K-Ras is the most common altered gene. TTF1 is the most widely used molecular marker for identifying lung adenocarcinoma. It is a lineage-specific transcription factor involved in maintaining proper alveolar maturation together with GATA6 (Zhang et al., 2007). In addition to TTF1, Napsin A could also be used (Zugazagoitia et al., 2014). Some samples can present a mixed immunohistochemistry pattern of both SCC and adenocarcinoma. Curiously, p63 can be positive in up to 1/3 of adenocarcinomas. Generally, tumors co-expressing TTF1 and p63 are commonly classified as adenocarcinomas (Zugazagoitia et al., 2014).

  **Large Cell Carcinoma:** they might represent poorly or undifferentiated forms of the other types of cancers (Sun et al., 2007). This subtype of lung cancer is a diagnosis of exclusion, when the tumor does not have features diagnostic of adenocarcinoma, SCC or SCLC. These
tumors are normally large, partially necrotic and composed of sheets and nest of large polygonal cells with vesicular nuclei and prominent nucleoli (Davidson et al., 2013).

### 3.1.3 Frequent mutations in lung adenocarcinomas

There are different genes that are commonly altered in lung adenocarcinomas. The most frequent driver events are K-RAS mutations (25%), EGFR mutations and amplifications (23%), EML4-ALK transversions (6%) and B-RAF (3%) and PI3K (3%) mutations. Interestingly, K-RAS mutations appear in a mutually exclusive manner with EGFR alterations or B-RAFV600E activating mutation (Cheng et al., 2012). Conversely, mutations in p53 tumor suppressor gene appear in 40-60% of NSCLC, independently of the EGFR and K-RAS mutation status. However, they are also associated with tobacco cigarette smoking (Lee et al., 2011). The histologicpathologic lung cancer subtype differs depending on the tobacco smoking history and the driver mutation. Likewise, whereas EGFR mutations correlate to woman, Asian race and never smoking status, mutant K-RAS cases are more frequently in smokers. As expected, EGFR mutant cancer cases respond to RTKIs (Receptor Tyrosine Kinase inhibitors) whilst K-RAS driven tumors are resistant to this therapy and mutations in this protein is prognostic of poor survival (Sun et al., 2007).

As K-RAS mutations are linked to lung adenocarcinomas its inhibition was thought to be an effective therapy to treat K-RAS driven lung cancer patients. However, Ras inhibitors have not succed and currently approaches try to inhibit downstream targets. In the present thesis we used two conditional mouse models that recapitulate human adenomas and adenocarcinomas (Guerra et al., 2003)(Drosten et al. unpublished).

### 3.2 Ras proteins

#### 3.2.1 Ras family

RAS (Rat Sarcoma) is a subfamily of small Guanosine TriPhosphatases (GTPases) that changes between a GDP-bound inactive state and a GTP-bound active form (Campbell et al., 1998). In mammals, there are three genes that codify the four RAS isoforms whose names come either from the researchers who reported two retroviruses causing sarcoma in rats: H-RAS (from Harvey) and K-RAS4A and K-RAS4B (from Kirsten); or from the tumor type where it was first isolated N-RAS (from neuroblastoma). Particularly, K-RAS4A and 4B are splice variants of exons 4A and 4B of the K-RAS gene, however, K-RAS4B is the isoform predominantly
Introduction

expressed in human cells (Cox and Der, 2010a) and very likely in established tumors (Stephen et al., 2014). Thus, K-Ras normally refers to K-Ras4B.

3.2.2 Ras structure

RAS proteins share a common structure: a conserved N-terminal domain, named G-domain, responsible for GDP/GTP binding and hydrolysis; followed by the HVD (HyperVariable Domain), where residues involved in activation and membrane interaction are located. HPV domain contains a -CAAX box where C is a cysteine, A an aliphatic aminoacid acid and X is a serine in H-Ras and a methionine in N- and K-Ras. The farnesylation of this cysteine residue by Farnesyl Transferase I (FTI) was thought to be essential for subsequent -CAAX modifications to activate the protein (Cox and Der, 2010a). This is true in the case of H-Ras, however, in the absence of FTI, K-Ras and N-Ras can be modified by GeranylGeranyl Transferase I (GGTI), allowing them to interact with endoplasmic reticulum membrane. It is in this organelle where all Ras proteins suffer the proteolysis of the –AAX tripeptide by the Ras and a-factor converting enzyme (Rce1) to be then methylated by the Isoprenylcystein carboxyl methyltransferase (lcm). Afterwards, the six polysyn residues in K-Ras are responsible for its subsequent localization to the plasma membrane, while H- and N-Ras need to be further palmitoylated (Figure 2A). This divergence in post-translational modifications is responsible for different localization in plasma membrane regions. While H-Ras is mainly in lipid rafts (more rigid areas), K-Ras is in microdomains in non-lipid rafts (more fluid areas that allow more movility) (Hancock, 2003).

3.2.3 Ras activation

Ras activation is regulated by Guanine-nucleotide Exchange Factors (GEFs) that facilitate the load of GTP to Ras. The recruitment of GEFs to the membrane is mediated by the adaptor protein Growth Factor Receptor-Bound 2 (Grb2), which contains SH2 domains that bind to the phosphorylated and therefore activated Receptor Tyrosine Kinases (RTKs). GEFs proteins, for instance SOS (Son of Senseless), thus bind to Grb2 and catalyse the binding of GTP to Ras. The inactivation of Ras proteins is mediated by GTPase Activating Proteins (GAPs), for example Nf1 (Neurofibromatosis), which accelerate the RAS-GTPase activity, controlling therefore the duration of the signal upon activation. Ras members regulate crucial cellular processes such as proliferation, apoptosis, autophagy/metabolism, vesicular trafficking,
morphological changes and gene expression among others (Cox and Der, 2010a) (Figure 2B and 3).

Albeit Ras proteins are highly similar at the structural level, there are important evidences that suggest selective physiological functions of each isoform. First of all, K-Ras protein is essential for mouse embryo development (Johnson et al., 1997; Koera et al., 1997) whereas the absence of H-Ras and N-Ras either individually or in combination is perfectly tolerated (Esteban et al., 2001). Secondly, the variations in Ras post-translational modifications and thus in localization could involve differences in function (Stephen et al., 2014). Moreover, it has been described that K-Ras is poorly transcribed in comparison to H-Ras due to the use of rare codons (Lampson et al., 2013). And finally, mutations in each of these proteins trigger transformation of specific and different tissues.

**Figure 2.** RAS proteins primary structure and activation mechanism. A. (Adapted from Hancock, Nat Rev Mol Cell Biol, 2003) The 4 RAS proteins share a 90% to 100% sequence identity in the G domain, which contains the sequences required for GTP binding and hydrolysis. On the other hand, they diverge in the HVD domain (8% identity). Cys (in blue) in the CAAX box is farnesylated to anchor the membrane. Additional Cys (in orange) are palmitoilated in H- (in 2 Cys) and N-Ras (in 1 Cys). Lysines (in orange) in K-Ras constitute the polybasic domain. B. (Adapted from Ahearn, Nat Rev Mol Cell Biol, 2011) Growth factors activate RTKs promoting their phosphorylation. GRB2 adaptor proteins bind to phosphorylated residues of RTKs by the SH2 domains. SOS-GEFs interact with Grb2 and Ras proteins to facilitate GTP loading. Ras-GTP binds to the Ras Binding Domain (RBD) of its downstream effectors and in order to switch Ras off, GAPs accelerate GTPase-Ras intrinsic activity up to $10^{5}$-fold.

Described as oncogenes codified by retroviruses in the 60’s decade, later in the 80’s RAS genes were the first oncogenes identified in human cancer. RAS mutations appear in 30% of the human cancers. Even though the three isoforms are all expressed widely in adult tissues and tumors, it is still unknown the rationale for the prevalence of mutations of one isoform over the rest depending on the tissue. Intriguingly, H- and N-Ras although being transforming oncogenes in model systems are mutated in a lower frequency than K-Ras in human cancers (Stephen et al., 2014). Whereas, K-RAS mutations are frequent in colorectal (50%), pancreas (70-90%) and lung cancer (25-50%), N- and H-RAS mutations are more frequent in skin (Johnson et al., 2001). As demonstrated in in vitro and in vivo models, Ras oncoproteins are
Introduction

key cancer drivers and moreover, they have been reported to be necessary for tumor maintenance. There are residues that account for more than 95% of the mutations of these genes: G12, G13 and Q61 (Cox and Der, 2010b). Substitutions of glycine in the residues G12 and G13 creates steric hindrance impairing Ras GAPs stimulation. On the other hand, mutations at Q61 disrupt the coordination of water molecule necessary for GTP hydrolysis. As a result, mutant Ras proteins accumulate with elevated GTP-bound proportion (Stephen et al., 2014).

3.2.4 Ras effectors

Since RAS proteins were first described in the 80's decade, these isoforms have been described to regulate eleven different effectors (Vigil et al., 2010). Therefore, RAS proteins control a high variety of cellular processes. All the effectors share a common Ras Binding Domain that promotes the interaction with GTP-bound Ras. Six of them have been reported to be involved in Ras driven oncogenesis: Rapidly Accelerated Fibrosarcoma (Raf) serine/threonine kinases, class I phosphatidylinositol-4,5-biphosphate 3 kinases (PI3K), GEFs for the Rak (RalGEFs) and Rac1 (Tiam 1), small GTPases, phospholipase C epsilon and RASSF1A. Of them, Raf and PI3K are frequently altered in RAS driven tumors. Moreover, in vitro and in vivo models confirmed that inhibition of these proteins could have a therapeutic effect in RAS human cancers. In addition, both have catalytic activities potentially targeted by small molecule inhibitors. Therefore, Raf and PI3K are the most promising targets for RAS driven tumors (Figure 3).
Figure 3. Ras-GEFs and –GAPs and downstream effectors involved in Ras tumor initiation, progression and/or maintenance (Adapted Berndt et al., Nat Rev Mol Cell Biol, 2011; and Ahearn et al., Nat Rev Mol Cell Biol, 2012). GEFS activate Ras protein and GAPs accelerate the Ras-GTPase intrinsic activity in order to deactivate it. Downstream effectors involved in Ras transformation include the p110 catalytic subunits of class I PI3Ks, GEFS for the small GTPases (RalGEFs; RalGDS; Rgl; Rgl2 and Rgl3), the Tiam Rac small GTPase GEFS (RacGEF) and PLCε whose function are necessary for tumor growth. Conversely, RASSF1A family members are negative regulators because their expression is lost in cancer.

3.3 The Raf-Mek-Erk pathway

The first of the four Mitogen-Activated Protein Kinase (MAPK) pathways operating in vertebrates to be discovered was the Raf-Mek-Erk pathway. This three-tiered protein cascade transmits mitogenic stimuli from membrane receptors to downstream effectors in the nucleus and in the cytoplasm, controlling therefore a huge variety of responses (Wimmer and Baccarini, 2010). The first tier of the pathway involves the three Raf isoforms: A-Raf, B-Raf and C-Raf. Briefly, upon Ras dependent activation these three isoforms will phosphorylate their only widely accepted substrates Mitogen/Extracellular signal-regulated Kinases 1 and 2 (Mek1 and Mek2) (Matallanas et al., 2011), which will phosphorylate and activate Extracelullar signal Regulated Kinases 1 and 2 (Erk1 and Erk2) straightaway. Therefore, the Erk phosphorylation status has been used as a common readout of the Ras-Raf signalling activation. These last
Introduction

Serine/Threonine (Ser/Thr) kinases can phosphorylate more than 150 substrates in the cytosol as well as in the nucleus (Yoon and Seger, 2006), where they promote the transcription of a wide variety of factors (Figure 4A). A higher regulation level is the interaction of these Raf-Mek-Erk isoforms with scaffold proteins, which modulate interactions and phosphorylations in this signalling pathway (Matallanas et al., 2011).

Whereas in yeast there are no Raf proteins (Matallanas et al., 2011), in mammals there are three. Moreover, every isoform have different splicing variants that can have important roles (Barnier et al., 1995; Hmitou et al., 2007). Additionally, there are two MEK and two ERK proteins with no redundant roles. And finally, Raf, Mek and Erk proteins can act as mono-, homo- or heterodimers. These facts together with the appearance of feedback loops, scaffold proteins and crosstalk with other pathways finely tune the signalling trough Raf-Mek-Erk, and therefore twist the linearity and fake simplicity of the pathway.

3.4 The Raf proteins

Raf isoforms are Ser/Thr kinases that share a common structure. They have three Conserved Regions (CR): CR1, CR2 and CR3 (Heidecker et al., 1992) and other divergent areas which may be responsible for the differences in regulation and function (Rushworth et al., 2006).

On the first hand, CR1 contains the RBD and the Cystein Rich Domain (CRD) necessary for RAS and membrane phospholipids interaction respectively. Secondly, CR2 incorporates Ser and Thr phosphorylation sites that negatively regulate RAS binding and RAF activation. Finally, CR3 involves the Activation-loop (A-loop), the Phosphate-binding loop (P-loop) and the Ser/Thr kinase domain (Figure 4B). Appart from the CRD, there are other regions that accomplish important functions, such as the Negative charge regulatory region (N-region), which minimally consists on four residues that need to be phosphorylated for activation in A- and C-Raf proteins (Roskoski, 2010).
Figure 4. MAPK pathway activation and B- and C-Raf protein structure. A. (Adapted from Ahearn, Nat Rev Mol Cell Biol, 2011) Mapk effectors transmit the signal from activated RTKs to downstream targets in the cytosol or in the nucleus. B. (Adapted from Wellbrock, Kasarides and Marais Nat Rev Mol Cell Biol, 2004) B- and C-Raf residues and domains implicated in protein activation are depicted. Conserved domains (CR1, yellow), (CR2, orange), (CR3, red). Residues interacting with 14-3-3 protein (green), residues of the N-region (pink) and in the A-loop (blue) that must be phosphorylated for activation.

Generally, Ras proteins activate B- and C-Raf similarly, whereas A-Raf is only weakly activated by Ras. In the case of C-Raf, the cytoplasmic inactive conformation is stabilised by the interaction of 14-3-3 to pS259 and pS621 phosphorylated residues. The dephosphorylation of S259 by PP1 and PP2A leads the subsequent unbinding of 14-3-3, and the recruitment of C-Raf to the membrane. A conformational change renders the N-terminal autoinhibitory domain and exposes CRD and RBD allowing the interaction with GTP-Ras and yielding the protein in an open inactive conformation (Roskoski, 2010). In order to be completely active and interact with Mek, the residues S338 and Y341 in the N-region should be phosphorylated by known (PAK, CK2, Raf, Src6 and Jak) and unknown kinases (Cox and Der, 2010a). Two additional residues T491 and S494 must be phosphorylated in order to be active. Finally, phosphorylations mediated by Erk and AKT or PKA and PP2A dephosphorylations render Raf proteins on its inactive conformation (Wellbrock et al., 2004).

Even though activation mechanisms in Raf proteins are very similar, there are important differences. In B-Raf protein, the residue corresponding to S338 in C-Raf is constitutively phosphorylated (S446 in B-Raf) and the N-terminal region is already negatively charged because the residues corresponding to YY340/341 in C-Raf are aspartic acids in B-Raf. Therefore, the inhibitory role of the N-terminal domain towards the catalytic region is neutralized and the 3-dimensional catalytic domain is stabilized (Matallanas et al., 2011). Due to this fact, B-Raf is considered to be prone to activation.

Raf knockout mouse models have demonstrated that Raf isoforms are not functionally redundant during embryo development. Likewise, C-Raf" mice die at a wide range from E11.5
**Introduction**

to birth depending on the genetic background due to placental defects and apoptosis in liver (Mikula et al., 2001; Wojnowski et al., 1998). B-Raf−/− mice die also before birth due to defects in placental vascularization (Galabova-Kovacs et al., 2006; Wojnowski et al., 1997). On the contrary, A-Raf−/− mice die soon after birth due to gastrointestinal and neurological disorders (Pritchard et al., 1996).

**3.4.1 A-Raf protein**

Expression of the protein kinase domain of the 3 Raf proteins revealed that A-Raf is 10 times less efficient than C-Raf kinase domain in terms of Mek phosphorylation and 500 times less than B-Raf kinase domain (Pritchard et al., 1995). Unique residues in the A-Raf regulatory domain and in the N-region are predicted to stabilize the ‘closed’ conformation, to weaken the binding to H-Ras and adversely affect activation, accounting for the low kinase activity of A-Raf. Moreover, phosphorylation of amioacids between 248 and 267 stimulated both activation and disassociation from the plasma membrane, suggesting that A-Raf activity is driven in other cellular compartments. Accordingly, A-Raf has been detected in the mitochondria, endosomes and Golgi apparatus (Matallanas et al., 2011).

A-Raf mutations in cancer are rare. A small percentage of lung adenocarcinoma patients carry A-Raf mutations, however it has not been determined if they are oncogenic drivers. Cell cultures studies, confirmed that A-RafS124C had transforming potential and it is sensitive to RAF inhibition (Imielinski et al., 2014).

**3.4.2 C-Raf protein**

C-Raf isoform was the first Raf protein to be described: C-Raf or Raf-1. This protein was reported to be the cellular homologue of the transduced protein of 3611-MSV oncogenic retrovirus that caused Rapidly Accelerated Fibrosarcomas and the first Ser/Thr oncogene to be described (Matallanas et al., 2011), thus far the general thought was that only tyrosine kinases could act as oncogenes (Moelling et al., 1984).

Apart from the well-known Mek substrate, C-Raf can phosphorylate other targets. On the first hand, C-Raf has been reported to translocate to the nucleus, where it can complex to and phosphorylate Rb. Even though this phosphorylation does not release Rb from the E2F1 promoters it has been suggested to be essential as the initial steps required for Cdk-Cyclin complex activity. Moreover, when C-Raf binds to E2F1-regulated promoters, Brg1, a chromatine modelling protein, is released. All these events favour the transcription of proteins
dependent on E2F regulated promoters and the progression through G1/S phase. This C-Raf function has been recently reported to be essential for Neural Stem Cells proliferation and to depend on K-Ras activation (Bender et al., 2015). Likewise, C-Raf:Rb complex increases in human NSCLC in comparison to surrounding tissue. Inhibition of C-Raf:Rb complexes with small molecules has been described to impair the growth of several cell lines introduced in immunocompromised mice. Additionally, BAG1 has been proposed to bind to Bcl2, a survival factor, and to C-Raf, and mediate the interaction with the proapoptotic protein BAD in order to phosphorylate it or mediate its phosphorylation, promoting its inactivation and stimulating survival (Wellbrock et al., 2004). Finally, C-Raf activates NF-κB transcription factor, which is implicated in protection from apoptosis. C-Raf kinase activity has been reported to necessary for MEKK1 activation very MEK independent functions, as a dominant negative form of Mek did not block C-RAF-mediated NF-κB activation (Baumann et al., 2000) (Figure 5).

Yes-associated Protein (Yap) promotes the expression of antiapoptotic and pro-proliferative genes and is frequently upregulated in many cancer types. Hippo pathway is the canonical regulator of Yap and it is a kinase cascade that consists on MST1/2 proteins that phosphorylate LAST1/2, which subsequently phosphorylate Yap, impeding its nuclear translocation and favouring its degradation. C-Raf protein can regulate Yap by kinase dependent and independent functions. Firstly, oncogenic K-Ras signals through the MAPK pathway to modulate the transcriptional activity of Yap in pancreatic ductal cells (Zhang et al., 2014). Secondly, C-Raf has been described to impair Mts2 homodimerization and to recruit Mts2 inactivating phosphatases, therefore avoiding Yap inactivation.

C-Raf has been also implicated in other cellular processes based on protein/protein interactions. First of all, it inhibits Ask1, a Ser/Thr kinase that triggers apoptosis in response to genotoxic stress and reactive oxygen species through Jnk and p38. On the second hand, C-Raf also inhibits Rockα, which is a RhoGTPase target that is implicated in cytoskeleton rearrangements. Remarkably, C-Raf ablation in a Squamous Cell Carcinoma (SCC) model impeded tumor initiation; and moreover, established tumors regressed (Ehrenreiter et al., 2009). In this situation Rockα inhibition abrogated STAT phosphorylation (and its nuclear translocation), and Myc transcription (which is implicated in the keratinocyte progenitors proliferation). Thus, C-Raf-dependent Rockα inactivation promotes proliferation. In this SCC model, C-Raf elimination induced Rockα hyperactivation, increased differentiation and therefore restrained tumor growth. Finally, C-Raf has also been reported to form complex with Cdc25, which activates the cyclin E-Ckd2 and promotes G1-S transition. Likewise, C-Raf
downregulation by siRNA or inhibition with Sorafenib has been shown to downregulate cyclin E and induce G1 arrest (Takezawa et al., 2009).

Increased C-Raf expression has been identified in human cancers such as osteosarcomas (Ikeda et al., 1989), bladder (Simon et al., 2001) and lung (U.Rapp, 1988) among others. However, C-Raf mutations are rare events in oncogenic processes even though mutations in S259 have been identified in lung, colon or ovarian cancer (Holderfield et al., 2014). C-Raf fusions with epithelial splicing regulatory protein 1 (ESRP1) have also been reported in prostate cancer (Palanisamy et al., 2010). Importantly, experiments with Genetically Engineered Mouse Models (GEMMs), revealed that C-Raf protein could be a potential therapeutic target for NSCLC patients as its ablation impaired lung (Blasco et al., 2011a) and skin tumor initiation (Ehrenreiter et al., 2009) as already mentioned.

Figure 5. Kinase-independent functions of Raf kinases. (A) General structure of MAPK pathways. (B) Raf-1 controls cell migration and differentiation by inhibiting the Rho effector kinase ROK-α. (C) Raf-1 controls TNF- and Fas-mediated apoptosis by inhibiting apoptosis signal-regulating kinase-1 (ASK). (D) Raf-1 and A-Raf bind and inhibit the pro-apoptotic mammalian sterile 20-like kinase (MST2) thereby interfering with its dimerization, autophosphorylation, and activation.

3.4.3 B-Raf protein

B-Raf is the phylogenetic oldest Raf isoform which appears in vertebrates (Matallanas et al., 2011) and it has the highest basal kinase activity compared to A- and C-Raf which is attributed to the constitutive negative charge in the N-region (Roskoski, 2010). The absence of B-Raf in proliferating MEFs significantly diminishes the activation of the MAPK pathway.
B-Raf gene mutations in human cancers were first described in 2002. (Davies et al., 2002). This protein is mutated in around 8% of all human cancer: the most common mutated driver in melanomas, 60% of thyroid cancers, 10% of colorectal carcinomas and a 6% of lung cancers among others (Holderfield et al., 2014). In melanoma, which is the B-Raf mutated more frequent cancer, a half of the cases bear B-RafT1799A transversion, which encodes the constitutively active B-RafV600E protein (Holderfield et al., 2014). Most of the B-Raf mutations affect the A- and P-loops, which interact one to each other to regulate the inactive and active state of the protein (Wan et al., 2004). These mutations mimic the T599 and S602 phosphorylation so that the A- and P-loop cannot interact rendering the protein in a constitutive active state. Even though the tumor-promoting capacity of the majority of the B-Raf mutations have not been demonstrated yet in vivo, the well-known B-RafV600E was reported to drive tumorigenesis in lung, melanocytes, thyocytes, colorectal tract, pancreatic ductal cells and prostate cancer among others when expressed in these tissues in Genetically Engineered Mouse Models (GEMMs). Specifically in the lung, B-RafV600E mutation caused lung adenomas; and further progression to adenocarcinoma was observed when p53 was homozygously deleted (Dankort et al., 2007). Likewise, when expressed in melanocytes, cooperation with loss of Pten or p16 tumor suppressors yield melanoma with metastatic potential (Dankort et al., 2009; Dhomen et al., 2009).

Even though V600E substitution is the most common alteration (90% of the cases) (Davies et al., 2002), other activated mutants (G469) and catalytically inactive isoforms (D594, G466) appear in human cancers. In contrast to B-RafV600E mutations, that do not occur in Ras mutant cells, four of the kinase inactive B-Raf mutations appeared coincident, which suggest interaction between both (Heidorn et al., 2010). Moreover, co-expression of the oncogene K-RasG12D and kinase inactive B-RafD594A protein in a melanoma mouse model accelerated tumor progression (Heidorn et al., 2010). This effect referred as MAPK paradoxical activation has been described to rely in B-Raf inactive/C-Raf active heterodimers formation, which is dependent on Ras activations and the subsequent hyperactivation of C-Raf (Heidorn et al., 2010; Rushworth et al., 2006). Moreover, it has been reported that P-loop also contains an autophosphorylation site that markedly reduces the activity of the wild type enzyme (Holderfield et al., 2013), what would explain why some kinase dead mutants are constitutively activated.

Similarly as in C-Raf protein, B-Raf protein fusions have also been reported. In all of them, the N-terminal autoinhibitory domain was missing in the fusion protein. Together, these facts support the therapeutic potential of targeting B-Raf oncoprotein (Holderfield et al., 2014).


Introduction

3.4.4 B-Raf/C-Raf heterodimers

Raf Ras-dependent activation induces the homo- or heterodimerization of the Raf kinase domain (Garnett and Marais, 2004; Rajakulendran et al., 2009; Rushworth et al., 2006; Weber et al., 2001). Surprisingly, wild type heterodimers have more kinase activity than monomers or homodimers (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001) and are probably the Mek activating unit (Wimmer and Baccarini, 2010). At the same time, Raf activation in D. melanogaster was reported to be dependent on dimerization with Kinase Suppressor of Ras (KSR) (Douzieh et al., 2006), a scaffold protein that contains a pseudokinase domain, similar to that of C-Raf and that connects Raf and MEK (Wimmer and Baccarini, 2010). In fact, KSR can also bind Raf proteins resulting in protomer transactivation in mouse (Nguyen et al., 2002). There are discrepancies whether Raf homo ir heterodimerization is crucial for its activation. On the one hand, it has been reported that the active Raf kinase consists on a side-to-side dimer in which C-Raf conform a productive allosteric conformation (Rajakulendran et al., 2009). However, it is still not clear that Raf and Erk signalling always correlate with Raf dimerization (Freeman et al., 2013; Poulidakos et al., 2011; Röring et al., 2012).

The importance of Raf heterodimerization for MAPK signalling in physiological and tumorigenic context has been recently emphasized. In normal cells B-/C-Raf heterodimerization is positively regulated by extracellular signals (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001; Yasuda et al., 2009). Despite the fact that these heterodimers are unstable (Rushworth et al., 2006), they seem to be stabilized in Ras activated cells (Weber et al., 2001)(Rushworth et al., 2006). The physiological relevance of heterodimerization was highlighted when catalytically inactive B-Raf mutants found in human tumors were shown to enhance ERK signaling by their increased capacity to associate with C-Raf and hyperactivate it. Dimers in which one of the protomers is inhibited are in fact active, and surprisingly, the inactive isoform act as an activator independently of its intrinsic kinase activity. However, it remains unclear whether both B- and C-Raf are equally active in this respect (Garnett et al., 2005; Rushworth et al., 2006).

Due to the fact that B-Raf dimer interface has a low affinity in vitro, it seems probable that there are other factors that promote dimer formation. In that sense, Ras-dependent dimer formation is not yet completely understood. It has been proposed that Ras dimerization and nanocluster formation in the plasma membrane could favour to increase Raf concentration in specific regions, therefore facilitating coupling two Raf molecules. Surprisingly, pharmacologic inhibition-induced dimer formation also seems to require Ras activation and reciprocally, these dimers prompt Ras colocalization at plasma microdomains.
Intriguingly, this suggests that these events cooperate reciprocally (Lavoie and Therrien, 2015). Finally, 14-3-3 has also been described to promote dimer formation. In this case, it is though the positive regulation comes form the 14-3-3 complexes interaction with both S621 in C-Raf and S279 in B-Raf, therefore stabilizing Raf heterodimers (Rajakulendran et al., 2009).

### 3.4 The Mek proteins

There are two Mek proteins in mammals Mek 1 and 2 that share an 85% of peptide homology. Mek1b is an splicing variant from Mek1 from which kinase activity has not been reported yet. Raf proteins phosphorylate Mek isoforms in the activation loop (S218/S222 in Mek1 and S222/226 in Mek2 both in human). PP2A and other kinases dephosphorylate these residues rendering Mek proteins inactive. There are other phosphorylations implicated in Mek regulation, such as Pak1-mediated S298 phosphorylation in Mek1, which is thought to favor C-Raf interaction. Once activated, Mek1 and 2 can phosphorylate Ser/Thr and Tyr residues (dual kinases) (Shaul and Seger, 2007).

Mek1 and 2 knockout mouse models reveal that these proteins have tissue specific functions that cannot be compensated reciprocally. Mek1−/− mice die at E10.5 due to placental vascularization defects similarly to B-Raf−/− mice (Bissonauth et al., 2006; Giroux et al., 1999) whereas Mek2−/− mice reach adulthood (Bélanger et al., 2003). Some scaffold proteins have been identified to interact specifically with Mek1 or Mek2 (Brunet et al., 1994; Roy et al., 2005; Schaeffer et al., 1998; Yin et al., 2004), which could determine Mek 1 and 2 tissue specific roles even though their targets are the same: Erk 1 and 2. (Brunet et al., 1994; Roy et al., 2005; Schaeffer et al., 1998; Yin et al., 2004),

### 3.5 The Erk proteins

There are two Erk proteins Erk1 (p44) y Erk2 (p42). As Raf and Mek proteins, Erk proteins are not redundant either. Mek kinases phosphorylate Thr202 and Tyr204 of human ERK1, and Thr185 and Tyr187 of human ERK2 (Lavoie and Therrien, 2015). Once activated Erk can phosphorylate targets in the cytoplasm and in the nucleus. As in the case of Raf and Mek, albeit sequence homology, Erk proteins are not redundant during embryonic development either. In contrast to Erk1−/− mice which are born and fertile (Pagès et al., 1999), Erk2−/− mice die during embryo development at different stages depending on the genetic background. This was due to defects in placental vascularization as in the cases of Mek1−/− and B-Raf−/− mice (Hatano et al., 2003; Yao et al., 2003). Scaffold proteins that differentially interact with Erk
Introduction

isoforms could explain the different phenotype. Likewise, MP1 that is located in late endosomes only interacts with Mek1 and Erk1 (Schaeffer et al., 1998).

### 3.6 The Raf-Mek-Erk feedback loops

Feedback loops in the MAPK pathway are not yet completely understood although the majority seems to come from the Erk activity at all different levels: GEFS, Raf and Mek proteins.

On the one hand, Erk can phosphorylate T292 residue in Mek1. Even though this residue is missing in Mek2 protein it is essential for Mek heterodimer formation. Therefore the intensity and duration of the signalling is mediated by Erk-dependent Mek heterodimer destabilization (Wimmer and Baccarini, 2010).

Secondly, ERK-induced phosphorylation has been also described at the Raf level. On the one hand, B-Raf residues Ser151, Thr401, Ser750, and Thr753 are associated with feedback inhibition (Ritt et al., 2010). In particular, Erk-mediated phosphorylation on T753 promoted the disassembly of Raf heterodimers (Rushworth et al., 2006). In the case of C-Raf, Erk phosphorylation has been related either with activation (Balan et al., 2006) and inactivation (Dougherty et al., 2005). While Dougherty and collaborators identified 6 feedback sites, Balan identified 3 out of these 6 (Ser9, 43, 289, 296, 301, and 642). The identification of 2 of these 3 activating residues in A-Raf suggested that phosphorylation of a subset of ERK feedback sites has a positive effect, whereas in order to be inhibited, C-Raf must be phosphorylated in all these 6 residues (Roskoski, 2010).

Finally, active Erk phosphorylates Sos in the C-terminal region that contains the proline rich domains that bind to Grb2. It therefore promotes its dissociation and inhibits the signalling through Ras (Corbalan-Garcia et al., 1996).

These Erk-mediated negative feedback loops are futile in B-RafV600E cells due to the fact that they do not depend on Ras activation of Raf heterodimerization on orer to proliferate (Lavoie and Therrien, 2015).

### 3.7 The Mapk inhibition as a therapeutic strategy

The design of K-Ras inhibitors nowadays is still challenging. As an alternative, and due to the frequent Raf-Mek-Erk signaling alterations found in human cancers this pathway is currently at the forefront of drug discovery. The first-generation Raf inhibitors started with Sorafenib, which was developed as a C-Raf inhibitor to treat RAS mutant cancers (Lyons et al.,
Introduction

2001). Even though the FDA approved it for the treatment of a variety of cancers the efficacy of the treatment remains still unknown due its multikinase inhibitory effect. Even though in renal cell carcinoma the benefit is probably due to VEGFR2 inhibition, in hepatocellular carcinomas response correlates with ERK phosphorylation (Abou-Alfa et al., 2006) although this is not related to RAS mutational status (Holderfield et al., 2014). Although B-RafV600E melanoma patients were predicted to benefit from this therapy any response was observed (Eisen et al., 2006). Therefore more specific inhibitors designed to target B-RafV600E were developed.

Vemurafenib (PLX-4032) and Dabrafenib are small molecules that showed to inhibit more potently B-RafV600E than the B-Raf wild type form (Gibney and Zager, 2013; Tsai et al., 2008). Even though tumor regression and partial responses were observed in metastatic melanoma patients, toxicities and resistance were reported to appear few months after the treatment (Flaherty et al., 2010). In fact, around 20% of patients were intrinsic resistant to B-Raf inhibitors. Mechanisms of resistance activate the Mek/Erk pathway in the majority of the cases: RTKs amplification or upregulation, secondary mutations in N-Ras, amplification of B-Raf or alternative splicing of mutant B-Raf mRNA, upregulation of COT or Mek mutations (Villanueva, 2015). In addition to resistance, all ATP-competitive RAF inhibitors trigger RAS-dependent MAPK pathway paradoxical activation in B-RAF wild type cells (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulakis et al., 2010). In most melanomas, B-RafV600E exists as a monomer that is effectively inhibited the drug (Poulakis et al., 2011), however, in B-Raf wild type cells in which Ras is altered, Erk is paradoxically activated due to the transactivation of wild-type Raf dimers when one protomer of the dimer is bound to the drug (Poulakis et al., 2010). Therefore, cutaneous squamous cell carcinomas, keratoacanthomas and other skin lesions have reported in B-Raf inhibitors treated patients (Anforth et al., 2013; Chapman et al., 2011). Even though skin lesions are easily removable and monitored, N-RAS-mutant chronic myelomonocytic leukaemia (Abdel-Wahab et al., 2014) and tumor relapsed from K-RasG12D colorectal cancer (Andrews et al., 2013) have been also reported under the vemurafenib treatment. It seems that inhibition of Erk signalling in B-Raf mutant tumors impairs Erk-mediated negative feedback loops that control the duration of the signal (Lito et al., 2012).

With the objective to avoid MAPK paradoxical activation and enhance the efficacy of B-Raf inhibition, Mek allostERIC inhibitors were implemented. Surprisingly, even though this combination have improved clinical outcome, resistance still emerge due to B-Raf ultra-amplifications or N-RasQ61K amplification (Girotti et al., 2015). Fortunately, the second-generation B-Raf inhibitors, called paradox breakers, are pan-Raf inhibitors and have been
Introduction

reported to inhibit B-RafV600E melanoma cell lines without inducing MAPK pathway paradoxical activation. Moreover, they potently block Mek/Erk signalling in resistant cells that express different B-Rafv600E splice variants or mutant N-Ras (Basilie et al., 2014; Girotti et al., 2015). Even though it has been recently reported that intermittent inhibition of Mapk signalling could delay resistance mechanism, paradox breakers seem to be an effective alternative as a first- and a second-line treatment (Moriceau et al., 2015).
4 OBJECTIVES
There is still no functional targeted therapy for K-RasG12V driven lung adenocarcinoma. In a mouse model that recapitulates this disease, C-Raf elimination has been shown to impair tumor initiation and maintenance. As current Raf inhibitors target the kinase activity of these proteins and C-Raf protein has kinase-independent functions, it was necessary to elucidate whether ATP-competitive inhibitors could be a suitable therapy for these tumors. Raf inhibitors are not 100% selective against Raf isoforms and B-Raf kinase inhibition hyperactivates tumor growth in Ras mutant cells, therefore the use of Raf inhibitors in this context could be detrimental for patients.

Hence, the objectives of this thesis were the following:

1. To validate the inhibition C-Raf kinase activity as a therapeutic target for K-RasG12V driven lung adenocarcinomas in p53 proficient and deficient contexts. In addition, to model the possible side effects derived from C-Raf systemic inhibition.

2. To study the effect of inhibition of B-Raf kinase activity in K-RasG12V driven lung cancer to uncover if the MAPK pathway’s paradoxical effect is also present in lung tissue.

3. To study whether MAPK paradoxical activation in the lung is C-Raf dependent.

4. To model by genetic means the consequences of systemic inhibition of B-Raf kinase activity.
5 MATERIALS AND METHODS
5.1 Maintenance of the mouse lines.

5.1.1 Generation of C-Raf LmLD468A mice

For the generation of the murine C-Raf\textsubscript{LmLD468A} allele, the genomic region encoding the murine C-Raf gene was cloned from the BAC RP23-37K24. The D468A point mutation and the loxP-cDNA-STOP-neo-loxP cassette were inserted in the C-Raf genomic locus by Red/ET (“triple recombination”) and PCR analysis confirmed correct modification of the locus. The modified C-Raf genomic locus from the BAC was subcloned into a high copy plasmid backbone incorporating NotI and Sall restriction recognition sites for convenient linearization of the targeting construct prior to introduction into ES cells. Finally, restriction analysis and sequencing confirmed basepair-precise subcloning and modification of the modified C-Raf genomic locus and integrity of the subcloning junctions and all functional elements. These steps were carried out by Gene Bridges (P307.26.11.10).

The linearized vector was electroporated into E14.1 C57BL/6 embryonic stem (ES) cells by the Transgenic Mice Unit of the CNIO. Homologous recombination events were identified by Southern Blot as described below. ES cell clones having undergone proper homozygous recombination with both arms of the targeting vector were identified by Southern Blot as described below. Single cell suspensions of two independent and positive ES cell clones (ESSD17.11 and ESSD17.14) were microinjected into FVB donor blastocysts, which were then implanted into pseudopregnant C57Bl/6J females. Two male founder chimera pups (with 100% penetrance of coat color) were utilized to generate germline transmission and were backcrossed to C57Bl/6J mice to establish pure genetic background.

5.1.2 Proceeding of the mouse lines used in this work

The K-Ras\textsuperscript{LSLG12Vgeo} (Guerra et al., 2003), the K-Ras\textsuperscript{FSG12V} (Drosten, unpublished) and the C-Raf\textsubscript{LmLD468A} lines were generated in Mariano Barbacid’s laboratory (Spanish National Cancer Centre, Madrid, Spain). The B-Raf\textsubscript{LmLD594A} was generated in Richard Marais’ laboratory (Paterson Institute for Cancer Research, Manchester, UK) designated as B-Raf\textsubscript{LSL-D594A} (Heidorn et al., 2010) in the original publication. The B-Raf\textsubscript{Lox} was generated in Acino Silva’s labratoty (Brain Research Institute, UCLA. Los Ángeles, CA, USA) designated as B-Raf\textsuperscript{F} (Chen et al., 2006) in the original publication. The C-Raf\textsubscript{Lox} was generated in Manuela Baccarini’s laboratory (Max F Perrutx Labratories, Center for Molecular Biology, Viena University, Austria) and designated as c-Raf-1\textsubscript{Lox} (Jesenberger et al., 2001) in the original publication. The p53\textsuperscript{frt} (Lee et al., 2012) was generated in David Kirsch’s laboratory (Duke University Medical Center, Durham, NC, USA) and designated by p53\textsuperscript{frt} . The Tg.hUBC-CreERT2\textsuperscript{T} (Ruzankina et al., 2007) was generated in
Materials & Methods

Eric J. Brown’s laboratory (University of Pennsylvania School of Medicine, Philadelphia, PA, USA).

5.1.3 Maintenance of mice

All mice used in this project were housed in the animal facility of the CNIO (Spanish National Cancer Research Centre, Madrid, Spain) in accordance with the FELASA’s (Federation of European Laboratory Animal Science Association) recommendations and following the European Union legislation. All experiments described in this thesis were previously approved by the Bioethics and Animal Welfare Committee of the Institute for Health Care Carlos III (CBA PA 17_2012-v2). Mice were submitted to a light/darkness cycle of 12 hours each. The daytime cycle came from fluorescent lamps that emit a white light (TLD 36W/840 and TLD 58W/840). Mice were fed ad libidum the Harlan diet except in cases where the Tamoxifen inducible Cre recombinase was induced, then animals were fed a high-fat transition diet for 10 days (Harlan) followed by a Tamoxifen-containing diet (Harlan).

5.1.4 Standard necropsy

Mice were sacrificed by cervical dislocation. Tissues of interest were collected in tissue sample blocks and fixed in formalin. Samples were processed in paraffin, cut and stained as needed by the Comparative Pathology Unit at CNIO. Some organs were frozen in OCT (Sakura) and stored at -80 °C for later cryostat sectioning. In addiction, partial tissue samples were also collected in eppendorf tubes and flash frozen in dry ice for biochemical analysis. Besides, some organs were also stained through X-Gal whole mount staining protocol (see section 5.3.1). In this case they were fixed in the adequate solution.

5.1.5 MEFs extraction

MEFs were isolated from 13.5 embryos. Uterus was removed and put into PBS at 37°C. Embryo’s head and liver were removed and the head was used for genotyping. The embryo was minced with a razorblade for 3 min. 3 ml of 0.5% trypsin were added (10X diluted in PBS; Invitrogen) and the plate was incubated for 10 min at 37°C, dispersed with a Pasteur pipette and incubated for another 10 min. Cells were transferred to a 15 cm-diameter dish and grown with 20 ml of the DMEM + 10% FBS + Pen/strp.
5.1.6 Genotyping

Mice were routinely genotyped by extracting DNA from tail samples.

1. 490 µl of lysis buffer (20mM Tris/HCl pH8.0, 100mM NaCl, 0.5% SDS, 10mM EDTA pH8.0 and milli-q H$_2$O) and 10 µl of proteinase K were added to each tail, and left overnight (o.n.) at 55°C.
2. 300 µl of saturated NaCl were added.
3. The solution was mixed and incubated for 20 min at 4°C.
4. The samples were centrifuged at 13000 rpm for 20 min.
5. The supernatant was transferred to a clean eppendorf.
6. 800 µl of isopropanol were added, then the solution was mixed vigorously.
7. The samples were centrifuged at 13000 rpm for 30 min.
8. The supernatant was discarded.
9. 200 µl of 70% ethanol (EtOH) were added.
10. The samples were centrifuged at 13000 rpm for 10 min.
11. The supernatant was discarded.
12. The pellets were left to dry.
13. The pellets were resuspended in 300 µl of milli-q H$_2$O.

Once the DNA was isolated we performed the genotyping of the mice using Polymerase Chain Reaction (PCR) technology. Adding for each reaction:

- 1 µl MgCl$_2$ 25mM.
- 2ul Taq Polymerase Buffer 10X.
- 0.25 µl dNTPs 10mM.
- 0.2 µl BSA 10 mg/ml.
- 0.1 µl Taq Polymerase (5 u/µl EcoTaq, Ecogen).
- 0.75 µl of each of the primers (10µM, Sigma).
- 1 µl of DNA.
- Fill up to 20 µl with milli-q H$_2$O.

The oligonucleotides used for genotyping were:

**K-Ras$^{LSL\text{G12Vgeo}}$**

K-Ras I0: 5’ CGTCCAGCGTGCCTAGACTTTA 3’
K-Ras3’Ex: 5’ CTCAGTCATTTTTCAGCAGGC 3’
STOP: 5’ TAGTGCCTTGACTAGAGATCA 3’

**K-Ras$^{LSS\text{G12Vgeo}}$** allele: 590bp (STOP + K-Ras3’Ex)
K-Ras$^{+}$ allele: 402bp (K-RasI0 + K-Ras3’Ex)
Materials & Methods

*K-Ras*\(^{G12Vgeo}\) allele: 669bp (K-Ras\(^{I0}\) + K-Ras\(^{3'Ex}\))

*K-Ras*\(^{F56G12V}\)

K-Ras\(^{3'Ex}\) allele: 5’ CTCAAGTCATTTCAGCAAGC 3’
STOP: 5’ TACTGCCTTGACTAGATCA 3’

K-Ras\(^{2F_8B2}\) allele: 5’ CCACAGGTTAGCGTACTATGCAG 3’

K-Ras\(^{*}\) allele: (STOP- K-Ras\(^{3'Ex}\)) 507bp
K-Ras\(^{G12Vgeo}\) allele: 358bp (K-Ras\(^{3'Ex}\) + K-Ras\(^{2F_8B2}\))
K-Ras\(^{G12Vgeo}\) allele: 441bp (K-Ras\(^{3'Ex}\) + K-Ras\(^{2F_8B2}\))

*C-Raf*\(^{lmlD468A}\)

C-RafFw: 5’ GGTGGTGCTATCTTTATCCC 3’
C-RafRv: 5’ GTGGATGATGTTCTTG 3’

*C-Raf*\(^{lmlD468A}\) allele: 560bp
*C-Raf*\(^{D468A}\) allele: 593bp
C-Raf\(^{*}\) allele: 501bp

*B-Raf*\(^{lmlD594A}\)

Primer A: 5’ GCCCAAGCTTTTATGAGAA 3’
Primer B: 5’ GCTTGGCTGGGTGAAACTC 3’
Primer C: 5’ AGTCAATCATCCACAGAGACCT 3’

B-Raf\(^{lmlD594A}\) allele: 140bp (Primer A + Primer B)
B-Raf\(^{D594A}\) allele: 518bp (Primer A + Primer C)
B-Raf\(^{*}\) allele: 467bp (Primer A + Primer C)

*p53*\(^{FRT}\)

p53 Fw: 5’ CAAGAGAAGCTGCTAAGAG 3’
p53 FRT Rv: 5’ CCTTCTAACAGAGGCAAG 3’
p53 Δ Rv: 5’ ACTCGGAAACAGAAGGCAGA 3’
p53* FRT allele: 292 bp (p53 Fw + p53 FRT Rv)
p53* Δ allele: 352 bp (p53 Fw + p53 Δ Rv)
p53* allele: 258bp (p53 Fw + p53 FRT Rv)
The PCR program used was:

94°C 1 min
94°C 30 sec
60°C 30 sec \times 35 cycles
72°C 1 min
72°C 10 min

The length of the amplified fragments was assessed by 1-3% agarose gel electrophoresis.

Alternatively, the mice were genotyped by Transnetyx (Córdova, TN, USA).

5.2 In vivo proceedings

5.2.1 Tamoxifen injection

The 4-OHT has a steroid type chemical structure that makes it only soluble in organic solvents, which cannot be used as a vehicle for intraperitoneal administration due to significant toxic effects. For administration of 4-OHT, the steroid was dissolved in corn oil.

1. 50 mg of 4-OHT (Sigma) were dissolved in 10 ml of corn oil (Sigma).
2. The mixture was sonicated until the 4-OHT was completely dissolved (2 min at 42% amplitude).
3. 100 μl of the dissolved 4-OHT were administered once through intraperitoneal injection to each mouse.

5.2.2 Tamoxifen diet

Mice were fed a tamoxifen containing diet ad libitum (Teklan CRD Tam^400/CreER) for treatments that required complete excision of the gene.

5.2.3 Adenoviral intratracheal infection

All the adenoviral preparations used in this work were purchased from the Iowa University (Iowa City, Iowa, USA).

At 8 weeks of age, mice were inoculated 30 μl of the adenoviral solution. In the case of Ad-Cre virus, 6x10^8 Plaque Forming Units per mouse (pfu/mouse) was used in the cases of high titter infections; normally with the aim to sacrifice the mice at a short time after the infection. 6x10^6 pfu/mouse was used to performe survival curves. In the case of Ad-FIpO, mice were infected with 6x10^5 pfu/mouse. Mice were previously anesthetized via intraperitoneal injection of a ketamine (75 mg/kg, Imalgene®) xilacine (12 mg/kg, Rompum®) solution. The virus was introduced directly in the trachea through a cannula (Simpson et al., 2001).
Materials & Methods

5.2.4 Micro X-ray computed tomography (micro-CT)
The CT acquisition was performed by the Molecular Imaging Unit at the CNIO, with an eXplore Vista PET CT (GE Healthcare) using an amperage of 200 µA and a voltage of 35 kV. Four-month old mice were anesthetized with a continuous flow of 1% to 3% isoflurane/oxygen mixture. 3D images of the skeleton were generated and analyzed with MMWS/Vista Software (GE Healthcare).

5.3 Processing of mouse tissues

5.3.1 X-Gal whole mount staining
1. Lungs and other organs were obtained during the necropsy of the mice.
2. The tissues were fixed at RT for 60-90 min in:
   - 0.2% glutaraldehyde (Sigma)
   - 1.5% formalin solution (Sigma)
   - 2mM MgCl₂
   - 5mM EGTA
   - 100mM sodium phosphate pH7.3
   - milli-q H₂O
3. The tissues were washed 3 times (20 min each at RT) in washing solution:
   - 0.2% NP-40
   - 0.1% sodium deoxycolate (Sigma)
   - 2mM MgCl₂
   - 100mM sodium phosphate pH7.3
   - milli-q H₂O
4. Following the washes, the tissues were stained for 48 hours at 37°C in the staining solution:
   - 0.2% NP-40
   - 0.1% sodium deoxycolate (Sigma)
   - 2mM MgCl₂
   - 100mM sodium phosphate pH7.3
   - 5mM K₃Fe(CN)₆ (ProLab)
   - 5mM K₃Fe(CN)₆ (ProLab)
   - 1mg/ml X-Gal (dissolved in dimethylformamide)
   - milli-q H₂O
5. The tissues were washed 3 times and for 10 min with the above mentioned washing solution.
6. The samples were postfixed o.n. in phosphate buffered 10% formaldehyde.
7. The fixed and stained tissues were washed twice with 1X PBS for 5 min.
8. The tissues were washed twice with 50% EtOH and twice with 70% EtOH (1 hour each).
9. At this point tissues were processed by the Comparative Pathology Unit at CNIO, which produced the standard 4 μm sections on a 3-aminopropyltrethoxylan coated slides and counter-stained with Nuclear Fast Red (NFR).

5.3.6 Histopathology and immunohistochemistry
Tissues including pancreas, spleen, thymus, liver, kidney, lung, heart, skeletal muscle, intestine, stomach, colon, skin, ovary, uterus, prostate, testis, mammary gland, white adipose tissue, brown adipose tissue, brain, eye, femur were dissected, fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. 4 μm tick sections were cut and stained with hematoxylin and eosin (H&E). Antibodies used for immunohistochemistry included rabbit polyclonal anti-SP-C (Millipore), goat polyclonal anti-CC10 (Santa Cruz), mouse monoclonal anti-p63 (Thermo Scientific), rabbit monoclonal anti-TTF1 (EPITOMICS). For detection of β-Galactosidase activity in adult and embryonic tissues, samples were included in OCT compound (Sakura) and frozen. X-Gal staining of 10 μm tick criosections was performed as previously described (section 5.3.3).

5.4 Quantification of tumors

5.4.1 Quantification of the relative areas occupied by the different types of lung epithelia
In order to estimate the relative areas occupied in a normal lung by the different types of lesions, we scanned whole mount X-Gal stained 2D sections from paraffin-embedded wild type lungs (n=3 mice, three sections per mouse) and we calculated the areas based on the K-Ras reporter marker β-Galactosidase activity using the DOTSLIDE (3DHISTECH).

5.5 In vitro procedures

5.5.1 Culture conditions
Cells were maintained in an incubator at 37°C with 16% O₂ and 5% CO₂. Mouse embryonic fibroblasts (MEFs) and HEK293T cells (ATCC® Number CRL-11268™) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco) and 1% antibiotic/antimycotic (Gibco).
5.6 RNA extractin and cDNA sequencing

5.6.1 RNA extraction
Total RNA was extracted from MEFs (see section 5.1.4) using RNeasy Micro Kit (Quiagen). Briefly, the cells were lysed by suspending them into BLT buffer supplied with β-mercaptoethanol. The RNA was then purified using the appropriate columns following the manufacturer’s instructions. On-column DNAase digestion was performed to eliminate residual genomic DNA. RNA was eluted in 20 µl of elution buffer. Finally, RNA was quantified using Nano-drop spectrophotometer. Also, to assess the quality of RNA for subsequent gene expression microarray experiments.

5.6.2 cDNA synthesis
cDNA synthesis was performed using SuperScript II RT kit from Invitrogen.

1. For each sample the following mix was prepared:
   - 200 ng random primers
   - 40-100 ng total RNA
   - 1 µl dNTP mix (10mM each)
   - Sterile, distilled H₂O

2. The mixture was incubated at 65°C for 5 min.
3. The samples were chilled on ice and spunned.
4. For each sample the following mix was added:
   - 4 µl 5X First Strand Buffer
   - 2 µl 0.1M DTT
   - 1 µl RNaseOUT (40 units/µl)

5. The content of the tubes was gently mixed. Samples were incubated at 25°C for 2 min.
6. 1 µl of SuperScript II RT enzyme was added and samples were mixed by pipetting.
7. Samples were incubated at 25°C for 10 min.
8. Samples were incubated at 42°C for 50 min.
9. The reaction was inactivated by heating at 70°C for 10 min

5.6.3 Primers for cDNA amplification

C-Raf
C-Raf forward: 5’ GATCCTAAAGGTGGTTGACCC 3’
C-Raf reverse: 5’ GTGGGCTTTCAACCTG 3’
5.7 Western Blot

5.7.1 Protein extraction

1. Either tissues or cells were resuspended in lysis buffer: 50mM Tris-HCl (pH7.4) solution, containing 150mM NaCl, NP-40 0.5% and phosphatase inhibitors (100mM vanadate sodium, Roche and 1mM NaF, Sigma) and either PMSF protease inhibitor (PMSF 100mM, Roche) or a cocktail of proteases inhibitors (Complete Mini, Roche).
2. For tissue protein extraction we used an homogenizer to mechanically breakdown the tissue samples and maximize the efficiency of lysis. Scirconium beads were added to a 1.5 ml screw capped tube together with the sample and 4 times of lysis buffer.
3. The samples were incubated on ice for 15 min.
4. The extracts were centrifuged for 15 min at 13000 rpm to remove the undigested membranes.
5. The supernatant was transferred to a new tube.
6. In order to quantify the amount of protein obtained, we used the Bradford method (1 μl of the protein extract was added to 1 ml of Bradford reagent and the absorbance was read on a spectrophotometer).

5.7.2 Gel run

1. The quantity of tissue/cell extracts needed to load the same total amount of protein was estimated (30-50 μg).
2. 4X loading buffer and 20X reducing agent (Biorad) were added.
3. The samples were boiled for 5 min. at 95°C.
4. The samples were placed on ice for 5 min.
5. Criterion XT precast gels (Biorad) were prepared into the cassette with running buffer (MES 1X, Biorad).
6. The samples were spinned and loaded into the gel.
7. 10 μl of the molecular weight color marker were loaded as well.
8. The gel was run with a constant voltage of 160V for usually 1.5 hours.
Materials & Methods

5.7.3 Transferring the denatured proteins to a membrane

1. A piece of nitrocellulose transfer membrane was cut as well as six pieces of Whatman paper.
2. The membrane and the papers were dipped on transfer buffer (1x Tris-Glycine, 20% methanol).
3. The gel was also dipped in transfer buffer.
4. The transfer “sandwich” was assembled in the transfer machine.
   - 4 Whatman papers- membrane- gel- 4 Whatman papers.
5. The gel was transferred for 50 min at constant 0.45 A.
6. When the transfer was finished, the amount of protein transferred was checked by staining with Ponceau S solution (Sigma).

5.7.4 Blocking and antibodies

1. The membrane was incubated in the blocking solution for 1 hour at RT: 5% BSA in 1x TBS-T for phosphorylated proteins and 5% non-fat milk in 1x TBS-T (1x Tris-Buffered Saline (TBS) solution with a 0.1% of Tween-20) for the rest of the proteins.
2. The membrane was incubated with the primary antibody (C-Raf, B-Raf, A-Raf, pErk, Erk1/2, Gapdh 1:20000; Sigma-Aldrich-Aldrich) diluted in the blocking solution o.n. at 4°C.
3. The membrane was washed twice with 1X TBS-T.
4. The membrane was incubated with the appropriate HRP-conjugated secondary antibody (1:2000, Dako).
5. The membrane was washed twice with 1X TBS-T.
6. Protein visualization was carried out with ECL detection reagent (Amersham) using different exposure times depending on the antibody.

5.8 Southern Blot

The nucleic acid probe is a DNA fragment with a sequence complementary to a specific region of the locus. Therefore it is necessary that both, the probe and the genomic DNA fragments were denatured, in a single-stranded DNA to the complementary binding. For this reason, the DNA gel was placed into an alkaline solution (1.5M NaCl and 0.5N NaOH) for 20 minutes at room temperature (RT) to denature the double-stranded DNA. For DNA fragments larger than 15 Kbp, prior to denaturalization, the gel must be treated with an acid solution to depurinate the DNA fragments, breaking the DNA into smaller pieces that are easily transferred from the gel to the membrane. To this aim agarose gel was treated with an acid solution.
(0.25N HCl) for 15 minutes at RT. Finally, since the transfer needs a pH lower than 9, the gel was incubated in a neutralizing solution (0.5M Tris HCl pH 7.4 and 1.5M NaCl) during 30 minutes at RT to equilibrate the pH. All these steps were performed on a rotary platform.

After the gel treatment, the DNA fragments were transferred to nylon membrane (Hybond-N+, Amershand) by capillary action using 10X Saline Sodium Citrate (SSC) as a buffer transfer from 12 to 24 hours. Ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and the positive charge of the membrane. Then the membrane was exposed to 1.2x105 µJ of ultraviolet radiation using a UV Stratalinker 1800 (Strategene) to permanently attach the DNA to the membrane by covalent interactions.

After that, the DNA within the membrane was exposed to hybridization with the specific probe. Before that, the membrane was blocked during 4 hours with the hybridization solution that contains salmon sperm DNA, formamide and detergents such as SDS to reduce non-specific binding of the probe and the probe was radioactively labeled. 20 µg of purified probe was denatured by heat (5 minutes boiling) and labeled with 50 µCi of α32P-CTP according to the manufacture’s instructions (Amersham Rediprime II Random primer). The membrane was incubated with labeled probe in the hybridization buffer (5mM Polyvinylpyrrolidone, 2mM Ficoll, 0.1M Dextran Sulfate, 25mM Na2HPO4, 20mM NaH2PO4, 0.9M NaCl, 35mM SDS, 2.5M EDTA pH 8, 10 mg/ml of salmon sperm DNA, 13M formamide and water) for 24 hours at 42oC.

After hybridization, excess probe was washed from the membrane using first 2X SSC/0.1% SDS, second 1X SSC/0.1% SDS and third 0.1X SSC/0.1% SDS incubations for 20 minutes at 42oC.

Finally, the pattern of hybridization was visualized by exposing the membrane to the Phosphoimagener screen for 24 hours and scanning the screen afterward (Molecular Dynamics).

### 5.9 Statistics

The statistics calculations reported in this work were performed using the paired Student’s t-test. Equality or difference in the variance was determined by the Fischer’s F test. All the calculations were performed with the Excel software (Microsoft).
6 RESULTS
6.1 Study of C-Raf kinase inhibition in K-RasG12V driven lung cancer

C-Raf ablation has been reported to impair initiation (Blasco et al., 2011a; Karreth et al., 2011) and progression (unpublished data) of lung adenocarcinoma driven by oncogenic mutations in K-RasG12 residue. However, so far, elimination of the protein is not possible in the clinic and current Raf inhibitors are small molecules that target Raf kinase activities. Additionally, and contrary to A- and B-Raf, C-Raf has antiapoptotic and antidifferentiation kinase independent functions (Niault and Baccarini, 2010) some of which are involved in skin tumor initiation and development in Ras mutant cells (Ehrenreiter et al., 2009). As the relevance of the distinct C-Raf protein functions can be tissue-specific, it was mandatory to investigate whether the therapeutic benefit observed in lung adenocarcinoma relied on C-Raf catalytic activity or on kinase independent functions.

6.1.1 Generation of an inducible C-Raf kinase dead allele: C-RafD468A

We therefore designed an inducible C-Raf kinase dead knock-in allele that expressed a catalytic inactive C-Raf protein under its own regulatory elements upon Cre-mediated excision. With this purpose, the mouse endogenous exon 13 of C-Raf gene was targeted by homologous recombination. In order to ensure the proper expression of both C-Raf wild type and catalytic inactive proteins, we designed a targeting vector that contained the following elements in the specified order:
- A minigene, which is the fragment of mouse C-Raf cDNA encoding exons 13 to 17.
- A stop transcriptional sequence.
- The PGK-Neomycin resistance gene.
- LoxP sites flanking the aforementioned elements to allow their excision upon Cre recombinase activity.
- The exon 13 containing the A1403C point mutation, resulting in the substitution of the aspartic acid D468 for an alanine in the HRD domain of C-Raf protein. This mutation eliminates the proton acceptor residue and was therefore thought to truncate the catalytic activity of the protein (Figure 6A).

This allele will be referred to as C-Raf\textsuperscript{LmL\textsubscript{D468A}} where LmL accounts for Lox-minigene-Lox. The generation of C-Raf\textsuperscript{LmL\textsubscript{D468A}/LmL\textsubscript{D468A}} mice was done by standard methods (see Materials and methods). Briefly, the targeting vector was introduced in embryonic stem cells, and homologous recombination events were identified (Figure 6B). Mice were generated from
positive clones (11 and 14) and a breeding colony was established in a mixed 129/C57BL6 background.

![Diagram of C-Raf alleles](image)

**Figure 6. Schematic representation of C-Raf †, C-Raf^LmLD468A and C-Raf^D468A alleles.** A. The mouse genomic C-Raf wild type locus (C-Raf†), the inducible (C-Raf^LmLD468A) and the catalytic inactive (C-Raf^D468A) knock-in alleles are depicted. External 5′ Southern Probe (5′SP) location and the size of EcoRV restriction enzyme fragments are indicated. Exons (squares), LoxP sites (white triangles), Minigene (cDNA from exon 13 to 17), Neomycin resistance gene and STOP transcriptional cassette depicted. The modified exon 13 encoding D468A is indicated (*). B. Southern blot analysis performed with DNA isolated from microinjected ESC. DNA was digested with EcoRV. Diagnostic DNA band for each allele is indicated.

### 6.1.2 C-Raf^D468A protein has impaired kinase activity

Raf knockout mouse models confirmed that the absence of a single Raf protein does not interfere with the physiological MAPK signalling in the majority of the tissues (Hüser et al., 2001; Mikula et al., 2001; Wojnowski et al., 1998). Likewise, proliferation is not arrested in MEFs and multipotent hematopoietic precursors lacking C-Raf protein (Blasco et al., 2011a; Mikula et al., 2001). This suggests that the remaining Raf isoform(s) can compensate this absence. Therefore, in order to analyze C-Raf^D468A catalytic activity in terms of Erk phosphorylation in a cellular context we had to ablate both A- and B-Raf so that they did not compensate the probably reduced or absent C-Raf^D468A catalytic activity. We took advantage of A-Raf^LmLD594A, B-Raf^LmLD594A, C-Raf^LmLD594A, RERT2^ert/ert MEFs that enabled us to ablate all
endogenous Raf proteins upon Cre-mediated recombination. We thought this approach has a more significant value than kinase cascade assays (Marais et al., 1997) due to the fact that all cellular scaffold proteins and interactors needed for Raf activity are present, and moreover, in physiological conditions. C-RafD468A resulted to have an impaired kinase activity compared to the C-Raf wild type protein (Figure 7). Fortunately, this implied that C-RafD468A could be used to decipher whether the therapeutic benefit of C-Raf protein elimination in K-RasG12V lung tumor initiation and development relied on C-Raf kinase activity.

![Western Blot](image)

**Figure 7. C-RafD468A has decreased kinase activity.** Western Blot with specific antibodies raised against the panel of proteins indicated in the figure. Erk 1 and 2 were used as loading control. Lysates from not infected MEFs are included as controls for endogenous levels. Arrowheads indicate protein migration. *A-Raf* \( ^{lox/y} \); *B-Raf* \( ^{lox/lox} \); *C-Raf* \( ^{lox/lox} \); *ERT2* \( \text{wt/ert} \) immortal MEFs were infected with the empty pLVX lentiviral vector (\( \bigcirc \)), the pLVX-C-RafWT or the pLVX-C-RafD468A (both mouse cDNA). After puromycin selection, cells were infected with Ad-Cre viruses and treated with 4OHT containing media. Cells were collected 14 days after infection.

### 6.1.3 C-Raf kinase activity is not necessary for mouse embryo development

Different C-Raf knockout mouse models described that C-Raf antiapoptotic functions are essential for mouse embryo development (Hüser et al., 2001; Mikula et al., 2001; Wojnowski et al., 1998). On the contrary, C-Raf knocked-in mice differed in the phenotype depending on the mutation that eliminates the kinase function. Whereas the ablation of two activating residues did not impact on behaviour or homeostasis of mice, elimination of the aspartic acid in the DFG domain recapitulated the phenotype of C-Raf knockout mice (Hüser et al., 2001; Noble et al., 2008a). In order to characterize the phenotype associated to the lack of kinase activity due to the substitution of the proton acceptor, we generated C-Raf \( ^{D468A/D468A} \) homozygous mice. This was achieved by crossing C-Raf \( ^{\text{LmLD468A/LmLD468A}} \) to homozygous EllaCre
Results

mice that express the Cre transgene under the Ella promoter, that is, in two cell stage embryos (Clausen et al., 1999). The offspring was crossed to C57BL/6 mice to eliminate the Ella<sup>T</sup> transgene and to evaluate the germline transmission of the C-Raf<sup>D468A</sup> allele. Genotyping of the pups (F1) confirmed C-Raf<sup>D468A</sup> was transmitted and crosses between C-Raf<sup>+/D468A</sup> heterozygous mice were set up to generate C-Raf<sup>D468A/D468A</sup> homozygous mice in C57BL/6. Homozygous mutant mice were born at mendelian frequency; of 159 mice assessed 85 typed as C-Raf<sup>1/+</sup>, 39 as C-Raf<sup>+/D468A</sup> and 35 as C-Raf<sup>D468A/D468A</sup>.

Mutant homozygous mice were indistinguishable from wild type and heterozygous littermates at birth. However, from P18 on some C-Raf<sup>D468A/D468A</sup> mice started to differentiate due their smaller size and motility problems (Figure 8A). Some mice had to be sacrificed at one month of age (n=4) (humane end point) (Figure 8B) and H&E stainings of the tissues collected showed that the vast majority of the tissues presented hypocellularity and immaturity (Figure 8C). This phenotype was observed in 8 out 8 knocked-in mice analysed. Heterozygous animals displayed no obvious anatomical or behavioral abnormalities or increased mortality over a period of 38 weeks.

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

**Figure 8.** C-Raf<sup>D468A/D468A</sup> mice have a decreased lifespan and their tissues are hypocellular. A. Representative picture of C-Raf<sup>1/+</sup> and C-Raf<sup>D468A/D468A</sup> 1month old males. B. Survival curve. C-Raf<sup>D468A/D468A</sup> (n=22) mice and C-Raf<sup>1/+</sup> (n=14) were sacrificed at humane end point. Survival curve was stopped at 38 weeks of age. P<0.0001 indicated (***) C. Representative H&E staining of bone marrow in C-Raf<sup>D468A/D468A</sup> and C-Raf<sup>1/+</sup> mice. Scale bars represent 500 μm and 200 μm.
C-Raf \(^{+/+}\) MEFs on the 129/Sv or 129/BL6 genetic backgrounds were reported to have a reduced proliferation (Mikula et al., 2001). However, they did not show any difference in 129Ola/MF-1 genetic background regarding proliferation rate (Hüser et al., 2001). To assess if the elimination of C-Raf catalytic activity impaired growth rate in MEFS on C57Bl/6, we isolated E13.5 C-Raf \(^{D468A/D468A}\) and C-Raf \(^{+/+}\) MEFs resulting from C-Raf \(^{+/+}/^{D468A}\) intercrosses. The morphology and size was indistinguishable between mutant homozygous and wild type MEFs. Moreover, there was not detectable difference between the proliferation rates of homozygous MEFs compared to wild type controls over 10 days in culture either in standard growth factor containing media (Fetal Bovine Serum, FBS) (Figure 9A) or in restricted growth factor conditions (Calf Bovine Serum, CBS) (Figure 9B).

![Figure 9. Lack of C-Raf kinase activity does not impair MEFs growth rate.](image)

C-Raf kinase activity elimination by the substitution of the aspartic acid in the DFG domain (C-RafD486A) has been reported to undergo proteasome-mediated degradation because of the lack of autophosphorylation in S621 (Noble et al., 2008b). In order to determine if C-RafD486A kinase dead protein was also unstable, we performed a western blot with C-Raf \(^{D468A/D468A}\) and C-Raf \(^{+/+}\) MEFs and adult tissue lysates. From these results, we could conclude that C-RafD486A kinase dead protein, in contrast to the C-RafD486A, is stable in embryos and in adult tissues (Figure 10 A and B). In addition, cDNA sequencing from C-Raf \(^{LmLD468A/LmLD468A}\) and C-Raf \(^{D468A/D468A}\) E13.5 MEFs allowed us to confirm the correct C-Raf wild type and C-RafD486A mRNA transcription from these alleles respectively (Figure 10C).
Results

Figure 10. C-RafD468A protein is stable in embryonic and adult tissues. Western Blot analysis of C-Raf protein expression in (A) E13.5 MEFs and (B) adult tissues (2 months of age) extracts from the indicated genotypes. C-Raf+/− and C-Raf+/+ MEFs were used as C-Raf negative and positive controls respectively. Gapdh was used as loading control. Arrowheads indicate protein migration. C. Sequencing analysis by Sanger-style BigDye terminator chemistry of the codon codyfiying the C-RafD468A. cDNA was synthesized from E13.5 MEFs mRNA of the indicated genotypes.

As the phenotype of C-RafD468A/D468A mice (F1) was not 100% penetrant we backcrossed to C57BL/6 genetic background for 7 generations. Homozygous C-RafD468A/D468A mice in C57BL/6 (F7) recapitulated the majority of the phenotypes observed in the F1, however, all of them were milder. Both knock-in homozygous females and males had a reduced body size and weight (Figure 11) and did not produce viable matings even when they were mated to wild type mice. The histopathological analysis of these samples is under current examination.
Figure 11. **C-Raf**\textsuperscript{D468A/D468A} mice have smaller size and reduced body weight. **A.** Representative pictures of of **C-Raf**\textsuperscript{D468A/D468A} and of **C-Raf**\textsuperscript{+/+} mice at 5 months of age. **B.** Average of male body weight (upper graph). **C-Raf**\textsuperscript{D468A/D468A} (n=11), **C-Raf**\textsuperscript{+/D468A} (n=10) and **C-Raf**\textsuperscript{+/+} (n=9) mice were measured. Average of female body weight (bottom graph). **C-Raf**\textsuperscript{D468A/D468A} (n=10), **C-Raf**\textsuperscript{+/D468A} (n=10) and **C-Raf**\textsuperscript{+/+} (n=11) mice were measured. The average ±SD of body weight is represented.

### 6.1.3 C-RafD468A protein impairs tumor formation

One of the objectives of this thesis was to decipher whether the elimination of C-Raf catalytic activity in K-RasG12V driven lung tumor initiation recapitulated the effect of C-Raf protein elimination. To that end, we decided to perform the same approach followed by Blasco and collaborators and analyzed tumor burden 6 months after oncogene activation.

With this aim, we generated **K-Ras**\textsuperscript{+/LSLG12Vgeo}; **C-Raf**\textsuperscript{LmLD468A/LmLD468A} mice. **K-Ras**\textsuperscript{LSLG12Vgeo} (Guerra et al., 2003) is an inducible knock-in allele, which upon Cre recombination in the lung, it triggers adenoma (AD) and adenocarcinoma (AC) formation driven by K-RasG12V expression (**Figure 11A**). As **K-Ras**\textsuperscript{LSLG12Vgeo} and **C-Raf**\textsuperscript{LmLD468A} inducible knock-in alleles are based on the Cre mediated recombination system, expression of the K-RasG12V and substitution of the C-Raf wild type protein by C-RafD468A kinase dead was concomitant. Remarkably, in order to achieve expression of these knock-in alleles specifically in the lung, mice were intratracheally inoculated with adenovirus codifying the Cre recombinase enzyme (Ad-Cre).

As expected, no tumor was observed in H&E sections from **K-Ras**\textsuperscript{+/LSLG12Vgeo}; **C-Raf**\textsuperscript{LmLD468A/LmLD468A} mice in comparison with **K-Ras**\textsuperscript{+/LSLG12Vgeo} mice (**Figure 11B**). This fact suggests that C-Raf catalytic inhibition either impairs or delays K-RasG12V driven tumor formation. In
order to distinguish between these two possibilities a Kaplan-Meier curve with K-Ras<sup>+/LSL-G12Vgeo</sup>; C-Raf<sup>LmLD468A/LmLD468A</sup> and K-Ras<sup>+/LSL-G12Vgeo</sup> mice is currently on going.

**Figure 11. C-Raf catalytic inhibition impairs K-RasG12V driven lung tumor formation.** A. K-Ras<sup>LSL-G12Vgeo</sup> and K-Ras<sup>G12Vgeo</sup> alleles. A STOP transcriptional cassette flanked by loxP sites is placed upstream mutated exon 1 which contains the K-RasG12V substitution (*). The β-geo cassette (LacZ gene + Neomycin resistance gene) was introduced in the 3’end of K-Ras gene and was preceded by and Internal Ribosome Entry Site (IRES). The transcript of this locus is therefore a bi-cistronic mRNA. B. Representative H&E sections of K-Ras<sup>+/LSL-G12Vgeo</sup> mice (n=5) and K-Ras<sup>+/LSL-G12Vgeo</sup>; C-Raf<sup>LmLD468A/LmLD468A</sup> mice (n=7). Mice were intratracheally infected with 10<sup>6</sup> pfu/mouse and sacrificed 6 months after the infection. Scale bar represents 5mm.

### 6.1.4 C-Raf kinase activity stops tumor growth in K-RasG12V, p53 proficient lung tumors

With the aim to evaluate the C-Raf kinase activity as a potential therapeutic target in patients, we decided to express the C-RafD468A in established tumors. In order to activate the expression of K-RasG12V and C-RafD468A proteins independently we needed two different recombination systems. Due to the fact that excision of the C-Raf<sup>LmLD468A</sup> relies on the Cre-mediated recombination system, we took advantage of the K-Ras<sup>F50G12V</sup> mice (Drosten et al. unpublished) in which the K-RasG12V mutation is expressed upon Flp-mediated recombination of frt sites (**Figure 12A**).

Therefore, we generated K-Ras<sup>F50G12V</sup>; C-Raf<sup>LmLD468A/LmLD468A</sup>; Tg.hUBC-CreERT2<sup>+/T</sup> mice together with control K-Ras<sup>F50G12V</sup>; Tg.hUBC-CreERT2<sup>+/T</sup> mice. In order to follow a therapeutic approach we, first, intratracheally infected 8 weeks old mice with adenovirus codifying the Flp recombinase (Ad-Flp) to activate the K-RasG12V expression in the lung (see Material and methods). Secondly, Computed Tomography (CT) scan started to monitor tumor formation and development from 4 months after the infection. Finally, at the time when tumors were detected by CT, mice were fed a tamoxifen containing diet to replace the expression of C-Raf wild type protein by C-RafD468A. In these strains, the modified Cre recombinase (CreERT2) was introduced as a transgene and expressed under the human Ubiquitin C promoter (Ruzankina et al., 2007). Tamoxifen allowed the CreERT2 enzyme to translocate into the
nucleus and excise the minigene construct in the C-Raf<sub>LmLD468A</sub> allele. Following the initiation of the tamoxifen diet tumor size was assessed by CT once every two months and tumor burden Fold Change (FC) was represented 8 weeks after treatment (Figure 12B).

![Diagram of K-Ras alleles and timeline of therapeutic approach](image)

**Figure 12.** K-Ras<sup>F56G12V</sup> and K-Ras<sup>G12V</sup> alleles and timeline of the therapeutic approach. A. A STOP transcriptional cassette flanked by frt sites is placed upstream mutated exon 1 which contains the K-RasG12V substitution (*). Exons (squares), frt sites (black triangles). Neomycin resistance gene and STOP transcriptional cassette are indicated. B. Timeline of the therapeutic approach.

Taking references for tumor growth and regression from the clinic, we established 1.2 fold change as the value for stable state of disease. We could conclude that elimination of C-Raf kinase activity impairs tumor growth: 52% of C-RafD468A expressing tumors have a fold change below 1.2 in comparison to 32% of C-Raf wild type controls. The follow up of these tumors confirmed this trend along the time (Figure 13A). To further assess the role of C-Raf kinase activity in the FDG-Gucose uptake of these tumors, a cohort of K-Ras<sup>+/F56G12V</sup>; C-Raf<sub>LmLD468A/LmLD468A</sub>; <i>Tg.hUBC-CreERT2</i><sup>+/T</sup> and K-Ras<sup>+/F56G12V</sup>; <i>Tg.hUBC-CreERT2</i><sup>+/T</sup> mice were submitted to Positron Emission Tomography (PET) scan. This technic utilises a glucose analogue (<sup>18</sup>FDG) and allows to detect malignant tumors because of their increased metabolic rates for glucose. Similarly to the previous therapeutic approach, when tumors were PET-detectable, mice were fed a tamoxifen containing diet to activate C-RafD468A expression and tumors were monitored by PET once every two months. The southern blot performed to assess the excision of the C-Raf<sub>LmLD468A</sub> allele corroborated that in all checked tumors the minigene construct had been excised (Figure 13B).
Figure 13. Genetic inactivation of C-Raf kinase activity in p53 proficient tumors impairs growth and FDG-Glc uptake in tumor development. A. Tumor volume Fold Change (FC). **Left panel:** 8 weeks after tamoxifen diet of K-Ras<sup>+/FSG12V</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 34 tumors/12 mice) and K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 42 tumors/20 mice). p=0.007 indicated (**)(paired T-test). **Right panel:** 16 weeks after tamoxifen diet of K-Ras<sup>+/FSG12V</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 18 tumors/6 mice) and K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 24 tumors/13 mice). p=0.044 indicated (*) (paired T-test). B. FDG-Glucose uptake FC 8 weeks after tamoxifen diet of K-Ras<sup>+/FSG12V</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 10 tumors/7 mice) and K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors (n= 7 tumors/5 mice). p=0.012 indicated (*) (paired T-test). C. Southern blot analysis performed with DNA isolated from K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> tumors. Mice were fed a tamoxifen containing diet during the indicated time (weeks). DNA was digested with EcoRV. Diagnostic DNA band for each allele is indicated.

Controls are DNAs extracted from MEFs of the indicated genotypes.

Of note, due to the fact that human Ubiquitin C promoter led Cre recombinase transcription ubiquitously, C-RafD468A protein was expressed systemically. Therefore, this approach also allowed us to evaluate the possible side effects associated to a C-Raf kinase inhibitor treatment. Even though motility defects already described in C-Raf<sup>D468A/D468A</sup> mice were observed to be related to of K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>; Tg.hUBC-CreERT2 <sup>+/T</sup> mice in tamoxifen diet and therefore to C-RafD468A ubiquitous expression, there was no obvious phenotype that could cause the premature death of K-Ras<sup>+/FSG12V</sup>; C-Raf<sup>LmL468A/LmL468A</sup>;
**Results**

*Tg.hUBC-CreERT2 +/+* mice. The histopathological characterization of these mice is currently ongoing. In order to avoid toxicity associated to the systemic elimination of C-Raf catalytic activity, we are currently submitting mice to tamoxifen diet for a limited period of 2 months. As observed in the western blot from *K-Ras*^{+/FSFG12V}; *C-Raf*^{LmLD468A/LmLD468A}; *Tg.hUBC-CreERT2 +/+* tumors, 5 weeks fed on a tamoxifen containing diet is enough to efficiently excised the *C-Raf*^{LmLD468A} allele and hopefully we will abrogate toxic side effects with the diet interruption. It could be possible that an intermitent tamoxifen diet due to cells that did not undergo proper excision of the *C-Raf*^{LmLD468A} allele overgrow.

### 6.1.5 C-Raf kinase activity ablation does not stop tumor growth in K-RasG12V, p53 deficient lung tumors

p53 mutated lesions appear in 50% of human cancers and are related to a more aggressive phenotype (Hollstein et al., 1991). In order to validate C-Raf kinase inhibition to treat these type of lung malignant lesions we introduced the *p53*^{frt} allele (Lee et al., 2012) in *K-Ras*^{+/FSFG12V}; *C-Raf*^{LmLD468A/LmLD468A}; *Tg.hUBC-CreERT2 +/+* mouse strain, so that p53 deletion coincide in time and cell type with *K-RasG12V* expression.

We therefore generated *K-Ras*^{+/FSFG12V}; *C-Raf*^{LmLD468A/LmLD468A}; *p53*^{frt/frt} *Tg.hUBC-CreERT2 +/+* together with control *K-Ras*^{+/FSFG12V}; *p53*^{frt/frt} *Tg.hUBC-CreERT2 +/+* mice. Following the previous approach, CTs measured tumor volume once every two months after the initiation of tamoxifen containing diet. Unfortunately, the volume fold change of tumors lacking C-Raf catalytic activity did not decrease in comparison to the controls (Figure 14A). A preliminary southern blot of tumor samples revealed that correct excision of *C-Raf*^{LmLD468A} allele was reached from 12 weeks on tamoxifen diet (Figure 14C). Even though more tumors need to be analyzed to confirm this hypothesis, this data suggest that the lack of therapeutic effect could maybe due to the inefficient excision by the time when the fold change is measured. On the other hand H&E sections of lungs from *K-Ras*^{+/FSFG12V}; *C-Raf*^{LmLD468A/LmLD468A}; *p53*^{frt/frt} *Tg.hUBC-CreERT2 +/+* mice sacrificed at humane end point had a lower tumoral burden when compared to the wild type littermates (Figure 14B); however, mice expressing a C-Raf kinase dead protein did not have an increased median survival (13.2 weeks) compared to control mice (16 weeks) (survival curve ongoing). Nonwithstanding, tumor burden quantification at comparable time point need to be performed in order to confirm this hypothesis. Altogether, this facts suggest that *C-Raf*^{LmLD468A} allele maybe requires a longer time than 8 weeks to be completely excised and therefore monitoring tumor volume fold change 8 weeks after treatment is probably underestimating the therapeutic benefit from C-Raf kinase inhibition in this type of
Results

lung tumors. Nevertheless, toxic side effects seem to be associated to systemic C-Raf kinase inhibition and they are maybe the cause of dead of $K$-$Ras^{{+/+}}$, $C$-Raf$^{LmLd468A/LmLd468A}$; $p53^{frt/frt}$ $Tg.hUbc-CreERT2^{+/+}$ mice (currently under characterization). In order to study the effect of C-Raf kinase activity elimination in $p53$ deficient tumors and avoid side effects associated to ubiquitous elimination of C-Raf kinase activity we will follow 3 different approaches. We will take the advantage of isolated $K$-$Ras^{{+/+}}$, $C$-Raf$^{D468A/D468A}$; $p53^{-/-}$ $Tg.hUbc-CreERT2^{+/+}$ tumor derived cell lines to study the effect of a kinase inactive protein expression in vitro and in orthotopic models.

Figure 14. C-Raf catalytic activity elimination does not impair progression of $p53$ deficient tumors. A. Tumor volume Fold change of $K$-$Ras^{+/FSG12V}$; $Tg.hUbc-CreERT2^{+/+}$ tumors (28 tumors/17 mice) and $K$-$Ras^{+/FSG12V}$; $C$-Raf$^{LmLd468A/LmLd468A}$; $Tg.hUbc-CreERT2^{+/+}$ tumors (49 tumors/18 mice) were analyzed. No statistical significant (paired T-test). B. Representative H&E stainings of from $K$-$Ras^{+/FSG12V}$; $Tg.hUbc-CreERT2^{+/+}$ and $K$-$Ras^{+/FSG12V}$; $C$-Raf$^{LmLd468A/LmLd468A}$; $Tg.hUbc-CreERT2^{+/+}$ mice sacrificed at humane end point. Scale bar represents 5mm. C. Southern blot analysis performed with DNA isolated from $K$-$Ras^{+/FSG12V}$; $C$-Raf$^{LmLd468A/LmLd468A}$; $Tg.hUbc-CreERT2^{+/+}$ tumors. Mice were fed a tamoxifen containing diet during the indicated time. DNA was digested with EcoRV. Diagnostic DNA band for each allele is indicated. Controls are DNAs extracted from MEFs of the indicated genotypes.
6.2 Study of B-Raf kinase inhibition in K-RasG12V driven lung cancer

6.2.1 B-RafD594A cooperates with K-RasG12V to increase tumor burden

Even though C-Raf catalytic inhibition could potentially be used in the treatment of p53 proficient K-RasG12V driven lung cancers, most Raf inhibitors are not selective against one Raf isoform. Albeit B-Raf protein elimination in K-RasG12V driven tumors had no effect in lung tumor initiation (Blasco et al., 2011a; Ehrenreiter et al., 2009), B-Raf catalytic inhibition in a Ras oncogenic context has been proven to be deleterious either in mouse model (Heidorn et al., 2010) or in patients (Alcalá and Flaherty, 2012). However, this MAPK paradoxical activation has not been yet reported to occur in the lung. On the other hand, the different Ras oncogenic isoforms drive transformation in distinct and specific tissues; this suggest that the oncogenic threshold to be transformed must be cell type specific and on the other hand, that different oncogenes impinge distinct oncogenic levels. Therefore, we decided to elucidate the outcome of B-Raf kinase inhibition in murine K-RasG12V driven lung tumorigenesis.

To address this, we crossed the \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \) mouse (Guerra et al., 2003) to \( B-Raf^{+/LmD594A} \) (Heidorn et al., 2010) to generate a mouse strain in which the oncogene K-RasG12V and the kinase dead mutant B-RafD594A could be co-expressed at the same time. Remarkably, \( B-Raf^{LmD594A} \) allele resulted to be hypomorphic and, as \( B-Raf^{-/-} \) mice, homocytotes did not reach birth. Nevertheless, MAPK pathway paradoxical effect appeared even when only the half of the protein was in a catalytically inactive form (Heidorn et al., 2010), which corroborates the increased oncogenic potential of this synergic interaction between K-Ras oncogenic mutations and catalytic inactive B-Raf proteins.

In an effort to elucidate the outcome of B-Raf kinase inhibition in the initiation of K-RasG12V driven lung tumors, we intratracheally inoculated Ad-Cre virus to \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \), \( B-Raf^{+/LmD594A} \) mice and \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \) littermates. Therefore, the expression of both mutations in the lung was concomitant. Two months after the infection mice were sacrificed and the lungs were X-Gal stained to detect the K-RasG12V surrogate marker, the bacterial \( \beta \)-Galactosidase protein. At these times, while only few hyperplasias/adenomas were detected in \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \) mice, the number of them increased in the case of \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \). Moreover, early lesions had clearly progressed into well-defined adenomas and in contrast to \( K-Ras^{+/LSL\text{G}^{12Vgeo}} \) mice, some areas of the lung appeared also transformed in a less defined architecture (Figure 15). Therefore, the inhibition of B-Raf kinase activity in K-RasG12V
Results

lung tumors seemed to recapitulate the MAPK pathway paradoxical activation already described in melanoma (Heidorn et al., 2010).

Figure 15. B-Raf inhibition cooperates with oncogenic K-Ras to accelerate growth rate and increase tumor burden. K-Ras<sup>LSLG12Vgeo</sup>+/+; B-Raf<sup>+/LmLD594A</sup> (n=3) mice and K-Ras<sup>LSLG12Vgeo</sup>+/+; B-Raf<sup>1/2LmLD594A</sup> (n=3) littermates were infected with a high titer of Ad-Cre (see Materials and Methods) and sacrificed 2 months later. Lungs were submitted to whole mount X-Gal stainig. Sections were counterstained with Nuclear Fast Red. Representative pictures of both genotypes are shown. Scale bar represents 5mm.
6.2.2 Coexpression of K-RasG12V and B-RafD594A in the lung shortens mice lifespan

With the purpose to study whether this initial effect observed 2 months after K-RasG12V and B-RafD594A coexpression was maintained along the time, we let these mice on ageing and sacrificed them at humane end point. The resulting Kaplan-Meier curve showed that the lack of B-Raf kinase activity in K-RasG12V driven lung tumors dramatically decreased the mice life expectancy. Whereas the $t_{50}$ (time after the infection at which 50% of the mice are dead) was 71 weeks for $K$-$Ras^{+/LSL\text{G12Vgeo}}$ control mice, $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice reached $t_{50}$ at 24 weeks (Figure 17A). Lung H&E sections from mice sacrificed at humane end point suggested that $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice died because of respiratory problems due to the massive tumor burden development (Figure 17C).

Besides lung AD/AC that typically arise in intratracheally infected $K$-$Ras^{+/LSL\text{G12Vgeo}}$ mice, $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ animals displayed a high number of hyperplasias covering nearly the whole lung. In order to estimate the tumor burden developed on both genotypes at a comparable time point, we sacrificed $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ and $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice 6 months after the intratracheal infection, which was the $t_{50}$ of $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice. The quantification of the tumoral area in both groups revealed that lungs in $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice presented bigger AD/AC than $K$-$Ras^{+/LSL\text{G12Vgeo}}$ mice and that most of the lung parenchima was transformed.

The increased number and diverse morphology of lesions developed in $K$-$Ras^{+/LSL\text{G12Vgeo}}$, $B$-$Raf^{+/LmD594A}$ mice could be explained by a differential $K$-$Ras^{LSL\text{G12Vgeo}}$ and $B$-$Raf^{LmD594A}$ allele excision, a new cell type transformation, a higher number of cells being transformed, or to faster progressive lesions. Nonetheless, we could not exclude a combination of some of the hypothesis aforementioned. The concomitant expression of K-RasG12V and B-RafD594A mutations in the different lesions was assayed by X-Gal whole mount staining and PCR. On the one hand, the expression of β-Galactosidase confirmed that all lesions expressed K-RasG12V mutation. Secondly, PCR analysis of DNA extracted from microdissected lung tumors verified that B-RafD594A was also expressed in the same lesions. Therefore, the inefficient excision of one of the alleles as a cause of a morphologically different lesion development was discarted.
Results

Figure 17. Concomitant expression of K-RasG12V and B-RafDS94A in the lung decreases mice lifespan due to massive tumor burden development. A. Survival curve. K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmID594A} (n=18) mice and K-Ras^{+/LSLG12Vgeo} (n=14) littermates were intratracheally infected with Ad-Cre. P<0.0001 indicated (***) (paired T-test). B. Quantification of tumor burden/lung area per mouse. 3H&E stainings separated by 40μm were quantified per mouse. K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmID594A} (n=7) mice and K-Ras^{+/LSLG12Vgeo} (n=5) control mice were intratracheally infected with Ad-Cre and sacrificed 6 months after the infection. p=0.006 indicated (***) (paired T-test). C. Representative H&E stainings of K-Ras^{+/LSLG12Vgeo} and K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmID594A} sacrificed mice 6 months after infection. Scale bar represents 5mm.

Lung tissue is made up of several cell types, mainly basal, ciliated, goblet, secretory Clara, neuroendocrine (NE), and alveolar type I (ATI) and type II (ATII) epithelial cells (Rock and Hogan, 2011). K-RasG12V driven lung tumors have been described to express Surfactant Secretory Protein C (SPC), suggesting that they arise from alveolar type II pneumocytes (Guerra et al., 2003). In order to discriminate whether the lesions observed in K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmID594A} lungs (AD/AC and hyperplasias) were all originated from ATII cells we performed SPC and CC10 stainings. The expression of SPC protein but not CC10 marker, a protein expressed by Clara cells, suggested that all the lesions arose from the same cell type, the aTI pneumocytes
(Figure 18). On the other hand, Ki67 stainings from K-Ras<sup>+/LSLG12Vgeo</sup>; B-Raf<sup>+/LmLD594A</sup> lungs revealed that hyperplastic lesions were not highly proliferative comparing to adenomas or adenocarcinomas from the same genotype.

We can therefore ascertain that concomitant K-RasG12V and B-RafD594A raises the oncogenic threshold so that a higher number of cells is transformed, however, it is still unknown whether cells that are transformed under these conditions are a more resistant subtype of aTII pneumocytes to transformation. On the other hand, the differences that trigger AD/AC or hyperplasia morphology need to be further studied.

![Figure 18. Tumors co-expressing K-RasG12V, B-RafD594A stain positive for SPC marker and negative for CC10. K-Ras<sup>+/LSLG12Vgeo</sup>; B-Raf<sup>+/LmLD594A</sup> mice were intratracheally infected and sacrificed at humane end point. Left panels: CC10 immunohistochemistry. Only bronchioles expressed this marker. Right panels: SPC immunohistochemistry. All lesions stains positive for this protein. Scale bar represents 1mm (upper panels) and 200 μm.](image)

6.2.3 Ubiquitous co-activation of K-RasG12V and B-RafD594A leads to hyperplastic growth of certain epithelial tissues

Due to the evidence suggesting that K-RasG12V protein cooperate with B-Raf catalytic inactive proteins to raise the K-RasG12V oncogenic level, we wondered whether other tissues are susceptible of transformation under these conditions. In order to address this, we generated a strain in which we could co-express ubiquitously the K-RasG12V and B-RafD594A proteins, K-Ras<sup>+/LSLG12Vgeo</sup>; B-Raf<sup>+/LmLD594A</sup>; Tg.hUBC-CreERT2<sup>+/T</sup>, together with K-Ras<sup>+/LSLG12Vgeo</sup>; Tg.hUBC-CreERT2<sup>+/T</sup> control mice. We next submitted these mice to a single dose of tamoxifen intraperitoneal injection in order to express these two proteins in a limited number of cells so that we could detect anatomically identifiable lesions. This approach could shed light on the tissues that
having latent K-Ras mutations could undergo transformation under B-Raf inhibitor treatments.

Surprisingly, epithelial tissues such as skin, forestomach and areas subjected to abrasion (paws, ears and snout) appeared transformed with papillomas at different times after treatments, confirming the increased oncogenic potential of B-Raf inhibition in K-Ras mutant contexts (Figure 19).

Figure 19. Co-expression of K-RasG12V and B-RafD594A ubiquitously leads to transformation of some epithelial tissues. HE stainings of indicated tissues. K-Ras+/LSLG12Vgeo; B-Raf+/LmLD594A; Tg.hUBC-CreERT2/+ (n=4) and K-Ras+/LSLG12Vgeo; Tg.hUBC-CreERT2/+ (n=4) mice received a single intraperitoneal injection of tamoxifen at P21. Mice were sacrificed at humane end point.

Whereas constitutive expression of K-RasG12V in mice only yields transformation in the lung with a complete penetrance, some tissues such as gastric epithelium also develop premalignant lesions including papillomas in a fewer frequency (Guerra et al., 2003)(Johnson et al., 2001). The development of papillomas in stomach, skin and paws certainly confirms that susceptibility to K-RasG12V mediated transformation depends on cell type specificity and that it can be further increased with the inhibition of B-Raf kinase activity.

6.2.4 C-Raf kinase activity is essential for the paradoxical tumor growth acceleration

The MAPK pathway paradoxical effect has been hypothesized to be triggered by the hyperactivation of C-Raf kinase activity (Heidorn et al., 2010) and K-RasG12V and B-RafD594A co-expression in the lung seems to recapitulate this phenomenon. As a proof of concept of this paradox dependence on C-Raf kinase activity, we generated K-Ras+/LSLG12Vgeo; B-Raf+/LmLD594A; C-Raf LmLD468A/LmLD468A and K-Ras+/LSLG12Vgeo; B-Raf+/LmLD594A; C-Raf Lox/Lox mouse strains.

The quantification of K-Ras+/LSLG12Vgeo; B-Raf+/LmLD594A; C-Raf LmLD468A/LmLD468A tumor burden 6 months after Ad-Cre infection revealed that expression of a catalytic
inactive C-Raf protein impairs tumor development/growth (Figure 20B). Moreover, this reduction in tumor burden was reflected in mice lifespan. As observed in the Kaplan-Meier curve, the elimination of C-Raf protein and in particular C-Raf kinase activity in K-RasG12V and B-RafD594A expressing lung tumors seemed to cancel the MAPK pathway paradoxical activation in the lung (survival curve ongoing) (Figure 20A). Therefore, we can affirm that C-Raf kinase inhibition blocks the increased oncogenic signalling triggered by the coexpression of K-RasG12V and B-RafD594A.

**Figure 20.** C-Raf kinase activity elimination blocks the MAPK hyperactivation in lung tumors.  
A. Kaplan-Meier curve. Mice were infected with a low titter of Ad-Cre virus: K-Ras+/LSL-G12Vgeo (n=14), K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A (n=18), K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A, C-RafLmd468A/Lmd468A (n=17) and K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A, C-RafLox/lox (n=7). B. Quantification of tumor burden/lung area per mouse. H&E stainings separated by 40µm were quantified per mouse. K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A (n=7) mice, K-Ras+/LSL-G12Vgeo (n=5) and K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A, C-RafLmd468A/Lmd468A (n=5) mice were intratracheally infected with Ad-Cre and sacrificed 6 months after the infection. p=0.006 indicated (**); p=0.001 indicated (***)(paired T-test). C. Representative H&E stainings of K-Ras+/LSL-G12Vgeo, K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A and K-Ras+/LSL-G12Vgeo, B-Raf+/Lmd594A, C-RafLmd468A/Lmd468A mice sacrificed mice 6 months after infection. Scale bar represents 5mm.
6.2.5 Loss of B-Raf wild type allele in K-RasG12V and B-RafD594A co-expressing lung cells increases Clara Cell transformation and diminishes adenoma/adenocarcinoma growth

Thus far, we have demonstrated that the inhibition of B-Raf kinase activity in K-Ras mutant lung cells increases the oncogenic signal through the MAPK pathway triggering an accelerated tumor growth through a process dependent on C-Raf catalytic activity. In K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{+/LmLD594A}\) mice, two types of heterodimers co-exist: B-RafD594A/C-Raf and B-Raf/C-Raf. In order to get further insight in the MAPK signalling regulation by heterodimers in which one of the protomer is inhibited, we generated K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{Lox/LmLD594A}\) strain. Within it, we were able to eliminate the B-Raf wild type protein concomitantly to B-RafD594A and K-RasG12V expression; therefore only B-RafD594A/C-Raf heterodimers were present in K-RasG12V expressing cells. We wondered whether in this scenario the oncogenic signalling would increase to drive a more aggressive phenotype (Figure 21).

**Figure 21. Figure. Schematic representation of the loss of B-Raf wild type protein in K-RasG12V and B-RafD594A coexpressing mice.**

A. K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{+/LmLD594A}\)

B. K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{Lox/LmLD594A}\)

We infected K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{Lox/LmLD594A}\) mice and sacrificed them at humane end point. Interestingly, the Kaplan-Meier curve showing this data revealed that the life expectancy of K-Ras\(^{+/LSLG12Vgeo}\); B-Raf\(^{Lox/LmLD594A}\) mice (t\(_50\) 22 weeks after
infection) did not differ from \( K-Ras^{+/LSLG12Vgeo} \), \( B-Raf^{+/LmLD594A} \) mice lifespan (Figure 22A). However, examination of H&E sections of these mice revealed that the elimination of B-Raf wild type protein alters the pattern of arising lesions. On the one hand, the number of AD/AC was 14 times reduced in \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{\textnormal{Lox/LmLD594A}} \) mice compared to the \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmLD594A} \) cohort (Figure 22B). On the contrary, the absence of B-Raf wild type allele increased the number of the intrabronchial lesions, a type of lesions that rarely appeared in \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmLD594A} \) mice. Together these facts suggested that the loss of B-Raf wild type protein modifies the MAPK oncogenic signalling. This new oncogenic stimulus was maybe inducing Oncogene Induced Senescence (OIS) in the cells that originate AD/AC. On the other hand, and due to the location of the intrabronchial lesions, this new oncogenic input was probably inducing transformation of Clara cells.

**Figure 22.** Loss of B-Raf wild type allele did not differ from \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmLD594A} \) mice lifespan. **A.** Survival curve. \( K-Ras^{+/LSLG12Vgeo} \) (n=14), \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmLD594A} \) (n=18), and \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{\textnormal{Lox/LmLD594A}} \) (n=17) mice were intratracheally infected with Ad-Cre. **B.** Quantification of number of adenomas (AD) and adenocarcinomas (AC) in \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{+/LmLD594A} \) (n=7) and \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{\textnormal{Lox/LmLD594A}} \) (n=7) mice. p=0.009 indicated (**).

The well-conserved state of the basal membrane of the bronchioles suggested that the origin of these intrabronchial lesions might be attributed to Clara cell transformation rather than invasion from alveolar tumors. To corroborate this fact, we performed CC10 and SPC stainings from \( K-Ras^{+/LSLG12Vgeo}; B-Raf^{\textnormal{Lox/LmLD594A}} \) mice. Surprisingly, whereas adenomas and hyperplasias were uniquely positive for SPC marker, intrabronchial lesions expressed CC10 and SPC protein in a mutually
exclusive manner, from which we could infer a transdiffereniation phenomenon from CC10 to SPC expression. In an effort to classify these lesions, we performed stainings of the well-accepted makers for AD (TTF1) and SCC (p63) histopathological subtypes. Intriguingly, these lesions had a mixed AD and SCC expression patterns as both TTF1 and p63 markers were expressed in these lesions. However, whereas only CC10 positive cells expressed p63 marker in these type of lesions, we cannot confirm yet that only SPC expressing cells were positive for TTF1 (currently ongoing).

![Image of IHC staining for SPC and CC10](image)

**Figure 23. The intrabronchiolar lesions seem to undergo a transdiffereniation process from CC10 to SPC expression.** Immunohistochemistry with anti-SPC, anti-CC10, anti-TTF1 and anti-p63 was performed on paraffin lung sections of 

K-Ras$^{+/LSLG12Vgeo}$; B-Raf$^{+/LmLD594A}$ mice sacrificed at humane end point. Scale bar represents 5mm.

As a proof of concept of SPC and Clara Cell transformation giving rise to different type of lesions distinguishable by location and morphology, we performed a lineage tracing experiment. We took advantage of adenovirus that express the Cre recombinase enzyme under the SPC or CC10 promoters. This allowed us to excise Cre-recombination dependent alleles only in alveolar type II pneumocytes or Clara cells respectively. We infected a cohort of K-Ras$^{+/LSLG12Vgeo}$, K-Ras$^{+/LSLG12Vgeo}$; B-Raf$^{+/LmLD594A}$ and K-Ras$^{+/LSLG12Vgeo}$; B-Raf$^{Lox/LmLD594A}$ mice with a high titter of Ad-SPC-Cre and the same number of the corresponding genotypes with Ad-CC10-Cre virus. We sacrificed these mice two months after the infection and remarkably, Ad-SPC-Cre infected K-
Results

*Ras*+/LSLG12Vgeo; *B-Raf*+/LmLD594A mice presented bigger AD/AC in addition to hyperplastic areas, comparing to *K-Ras*+/LSLG12Vgeo littermates. This suggested that K-RasG12V driven transformation of alveolar type II cells is increased in the presence of a B-Raf kinase inactive protein. Moreover, B-Raf wild type protein elimination in this context resulted in a negative pressure for AD/AC to develop. On the contrary, Ad-CC10-Cre infections did not give rise to any lesion in *K-Ras*+/LSLG12Vgeo mice. However, the scarce number of intrabronchiolar lesions that was observed in *K-Ras*+/LSLG12Vgeo; *B-Raf*+/LmLD594A was highly increased when the B-Raf allele suffered the Loss Of Heterozygosity (LOH) monitored event.

![Image](image_url)

**Figure 24. Intrabronchiolar lesions are originated from CC10⁺ cells.** Four mice of each genotype: *K-Ras*+/LSLG12Vgeo, *K-Ras*+/LSLG12Vgeo, *B-Raf*+/LmLD594A and *K-Ras*+/LSLG12Vgeo, *B-Raf* Lox/LmLD594A mice were infected with 10⁸ pfu/mouse of Ad-SPC-Cre and Ad-CC10-Cre. Mice were sacrificed 2 months after the infection. Representative H&E stainings of each genotype/Ad-virus used are shown. Scale bar represents 5mm.

These observations raised two important issues. In the first place, molecular events when the B-Raf wild type isoform is lost results in an increased susceptibility of Clara cells to transformation and a negative pressure of AD/AC to develop. Secondly, these findings suggests that Clara cells may suffer a transdifferentiation process in which they loose the CC10 and p63 markers and start expressing SPC and TTF1 proteins.
6.2.6 Loss of B-Raf kinase activity can promote lung tumorigenesis

Even though B-RafV600E accounts for 90% of the B-Raf mutations in lung cancer, B-Raf kinase inactivating mutants have been already described in human cancers. Even though of them were coincident with Ras mutations, there are some others that were not. D594A particularly was reported not to be one B-Raf catalytically inactive mutations that did not trigger Erk or NKxβ activation. Notwithstanding, data from the Lung Adenocarcinoma database of The Cancer Genome Atlas (TCGA) highlight that B-RAF kinase inactive mutants appear in the same proportion than the widely accepted lung cancer driver B-RAFV600E. Interestingly, in these samples in which B-Raf is catalytically inactive K-Ras is not mutated, suggesting that kinase inactive B-Raf mutations could promote tumorigenesis.

With the aim to check whether B-RafD594A could initiate lung cancer we infected B-Raf$^{+/LmLD594A}$ mice (n=7) and sacrificed them 1 year later. 3 of them developed adenocarcinomas in which excision of B-Raf$^{LmLD594A}$ allele was assessed by PCR (Figure 24). Moreover, sequencing analysis of codon 12 in Ras genes confirmed the absence of Ras activating mutations that can cooperate with B-Raf kinase inactive form to drive tumorigenesis. This implies that B-Raf inhibition could be a driver event in lung tumorigenesis.

**Figure 24.** B-RafD594A can promote tumor formation originated from SPC$^+$ cells. A. H&E staining of B-Raf$^{+/LmLD594A}$ mouse intratracheally infected with $10^6$ pfu/mouse and sacrificed 1 year after the infection (Left panel). SPC immunohistochemistry of the same lungs (right panel). Scale bar represents 100 μm. B. PCR run with DNA extracted from the tumor. Expected fragments for each allele are depicted.
7 DISCUSSION
Discussion

The optimal cancer inhibitor is which abrogate an essential function for tumor growth and maintenance without affecting normal homeostasis of the rest of the tissues.

Since oncogenic K-Ras mutations were first described in human tumors, different strategies to target directly oncogenic K-RAS have been developed. However, inhibiting the protein in the clinic has resulted inefficient so far, therefore there have been many efforts to identify downstream targets that are essential for K-RASG12V driven tumor initiation and progression. Among them, in vitro experiments and different GEMMs revealed that Raf-Mek-Erk pathway has a key role in tumorigenesis. Both Mek1/2 or Erk1/2 absence impairs lung tumor initiation; however, their elimination results in an intolerable toxicity in highly proliferative tissues. Fortunately, C-Raf elimination impairs tumor initiation and progression without affecting the normal homeostasis of the rest of the tissues (Blasco et al., 2011a).

The fact that Mek1/2 or Erk1/2 elimination also impaired lung tumorigenesis suggested that the therapeutic benefit observed upon C-Raf elimination was due to the absence of its kinase activity. Even though B-Raf is considered the main Mek activator, C-Raf kinase activity is more relevant to activate the MAPK pathway in certain cell types (Rubio et al., 2006). On the other hand, elimination of C-Raf kinase independent functions has also been described to have a therapeutic benefit in other tumor types. Firstly, lack of C-Raf promotes Rockα mediated differentiation in Ras mutant keratinocytes, thus altering the proliferation/differentiation balance and impairing skin SCC initiation (Ehrenreiter et al., 2009). Secondly, C-RAF elimination in NSCLC cell lines induces the cyclinE downregulation and G1 arrest. This effect seems to be restricted to NSCLC cells with mutant K-RAS even though the depletion did not resulted in ERK phosphorylation inhibition (Pratilas et al., 2009; Takezawa et al., 2009). Therefore, there are clear tissue-specific differences in the essential C-Raf functions to drive and/or maintain tumorigenesis.

7.1 Validation of C-Raf kinase activity inhibition to treat K-RasG12V driven lung adenocarcinomas

Generation of C-Raf kinase dead protein

In order to validate the inhibition of C-Raf catalytic activity as a target for K-RasG12V driven lung adenocarcinomas we designed a mouse model that expresses a catalytically inactive C-Raf protein (C-RafD468A) to mimic the effect of a 100% selective C-Raf kinase inhibitor. The advantage of this model is that both C-Raf proteins, wild type and mutant, are expressed from its own endogenous promoter, making the transcriptional regulation as
physiological as possible. The example of oncogenic K-Ras, which when overexpressed induces senescence whereas the expression at physiologic levels triggers proliferation raises the importance of making appropriate models in which evaluate the real biologic effect of specific modifications (Tuveson et al., 2004).

Different C-Raf knockout mouse models have been reported so far. Most of them, described that C-Raf protein is essential for mouse embryo development and its absence causes embryonic lethality due to placental defects. As Erk phosphorylation was not abrogated and apoptosis was observed in all the different C-Raf knockout models and genetic backgrounds, authors affirm that C-Raf antia apoptotic functions are indispensable for mouse gestation. However, the variable penetrance and the subtle differences in the phenotype suggest that different genetic effectors modulate the C-Raf protein deficiency (Table 1) (Hüser et al., 2001; de Iriarte Rodriguez et al., 2015; Mikula et al., 2001; Wojnowski et al., 1998).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Effect</th>
<th>Background</th>
<th>Phenotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Craf-1 hapl/haplo</td>
<td>Expression of 10% of C-Raf kinase activity</td>
<td>CD1</td>
<td>Placenta defects and developmentally arrested embryos. Die hours after birth due to lungs fail to inflate. MEFs have a reduced proliferation rate</td>
<td>Wojnowski et al. 1998</td>
</tr>
<tr>
<td>Craf-1 hapl/haplo</td>
<td>Expression of 10% of C-Raf kinase activity</td>
<td>C57Bl/6 and 129sv</td>
<td>Embryonic lethal ([E]10.5-12.5). Embryo developmental arrest</td>
<td>Wojnowski et al. 1998</td>
</tr>
<tr>
<td>Raf-1-/-</td>
<td>C-Raf knockout</td>
<td>MF1</td>
<td>Die shortly after birth (around P20-30) No liver apoptosis Hypocellularity and anaemia</td>
<td>Hüser et al. 2001 Karnata et al. 2004</td>
</tr>
<tr>
<td>Raf-1-/-</td>
<td>C-Raf knockout</td>
<td>129Ola/C57Bl.6</td>
<td>Developmentally arrested and anaemic embryos and abnormal blood vessel formation in embryo and placenta from E9.5</td>
<td>Hüser et al. 2001</td>
</tr>
<tr>
<td>Raf-1-/-</td>
<td>C-Raf knockout</td>
<td>129Ola/MF-1</td>
<td>Some developmentally arrested embryos at E9.5 Blood vessel formation defects and reduced size of placenta Hypocellularity in liver Die hours after birth</td>
<td>Hüser et al. 2001</td>
</tr>
<tr>
<td>Raf-1 Y340/Y341 to Phe</td>
<td>Knockin mutation</td>
<td>129Ola/C57Bl.6 and 129Ola/MF-1</td>
<td>Survive 1 year No behavioural and weight differences</td>
<td>Hüser et al. 2003</td>
</tr>
<tr>
<td>c-Raf-1-/-</td>
<td>C-Raf knockout</td>
<td>129/5v 129Bl/6</td>
<td>Embryonic lethal (E11.5-E16.5) Smaller and poorly vascularized placenta and embryo Hypocellular liver with numerous apoptotic hepatoblasts Impaired survival of hematopoietic precursor cells MEFs have an increased in apoptosis</td>
<td>Mikula et al. 2001</td>
</tr>
</tbody>
</table>

Table 1. C-Raf knockout and knocked-in mouse models and their associated phenotype.

Apart from knockouts, three knock-in mouse models which express C-Raf kinase dead mutations have been also generated: C-RafY340/341FF (C-RafYFF hereafter), C-RafD486A and C-RafD468A. C-RafYFF is mutated in two C-Raf activating residues, C-RafD486A is
Discussion

mutated in an aspartic acid localized in DFG domain and C-RafD468A is mutated in the proton acceptor, the aspartic acid of the HRD domain. Homozygous mice expressing C-RafD486A, died during midgestation recapitulating the phenotype associated to C-Raf knockout mice. This was reported to be due to the C-RafD486A proteasome-mediated degradation because of the lack of autophosphorylation in Ser621. Likewise, the C-Raf kinase dead mutation C-RafK375M was also degraded when overexpressed (Noble et al., 2008b). Even though we were not able to obtain any conclusive result from the western blot raised against pSer621 in C-Raf D468A/D468A and C-Raf "+" MEFs lysates, the protein stability was verified by western blot in adult tissues and MEFs. Notwithstanding, the kinase assay that we performed in Rafless MEFs revealed that C-RafD468A catalytic activity was impaired. Therefore, we could affirm that C-RafD468A protein lacked kinase activity without being affected in terms of protein stability. These results were in accordance to in vivo observations. As expected, whilst C-Raf D486A/D486A mice recapitulated the phenotype associated to the lack of C-Raf protein (Mikula et al., 2001; Wojnowski et al., 1998), C-Raf D468A/D468A mice were born at expected mendelian frequency. Consistent with these results, C-RafYYFF kinase dead mutation was not associated to embryonic lethality, being homozygous mice alive for more than one year (Hüser et al., 2001).

As previously seen and inferred from the C-Raf knockout models, there are phenotypic differences among the three C-Raf kinase dead mouse models. I will try to discuss in the different hypothesis that could explain them.

Regarding the discrepancies between C-RafD468A and C-RafD486A, the in silico predictions suggest that whereas C-RafK375M mutations may affect the correct folding of the protein, neither C-RafD468A nor C-RafD486A variations destabilize C-Raf protein tertiary structure. However, C-RafD486A has been reported to have an increased ability to bind the chaperone HSP90 compared to the wild type isoform. This indicates that C-RafD486A is misfolded, nonetheless, the rationale for that, rather than the mutation itself, could be that lack of pS621 impairs C-Raf activation and targets the protein for this interaction. On the other hand, C-RafD468A and C-RafD486A homozygous mice were bred in the C57BL/6 genetic background; therefore the variability that unknown strain-specific genetic modulators confer was reduced. However, the genetic drift to which C57BL/6 strains are subjected in different laboratories may account for subtle differences in some alleles. In line with this, we have observed that the expression of C-RafD486A in the same C57Bl/6 varies depending on the number of generation. Finally, the last possible explanation is the different levels of residual kinase activities of both proteins, accounting for S621 phosphorylation to levels compatible with life in the case of C-RafD468A and incompatible in C-RafD486A protein. Surprisingly, as in the case of C-RafD486A, in the experiments in which C-RafD468A and wild type protein were
overexpressed we consistently observed a faster migration of the mutant protein in SDS-PAGE. As reported by Noble et al. phosphorylation play a role in protein migration, therefore, we will have to analyze in detail which of the residues, included S621, are probably not being phosphorylated in the case of C-RafD468A protein (Noble et al., 2008b).

Even though the phenotype associated to C-RafYYFF and C-RafD468A imply that C-Raf kinase activity is not essential for mouse embryo development, the features characteristic of each of them differs. Contrary to rafFF/FF homozygous C-RafD468A/D468A mice developed behavioural problems and developmental retardation. Differences in protein structure leading to variations to bind to substrates or scaffold proteins could be the possible explanations of these differences. Further backcrossing of these two mutants may confirm phenotypic heterogeneity between strains as it has been revealed for knockout mice.

Interestingly, C-RafD468A/D468A mice showed hypocellularity in most of the tissues, which resembles the hypocellularity observed in some of the C-Raf knock out mouse models. In these last models, hypocellularity was reported to be related to antiapoptotic functions as proliferation was not affected by the absence of C-Raf. The presence of this phenomenon when a catalytically inactive protein is expressed implies that C-Raf kinase activity regulates some of these antiapoptotic processes, such as the phosphorylation of Bcl2, BAD, or that hypocellularity is controlled by the MAPK pathway. As the levels of pErk did not vary in C-Raf knockout MEFs, the first hypothesis is more probable. However, as I will discuss later, pErk is not the optimal read-out for MAPK activation.

These results suggest that C-Raf function is regulated by genetic modifiers. Candidates for this C-Raf modulation are not known yet but their identification would be very interesting due to their high influence in C-Raf associated phenotype.

The role of C-Raf kinase activity in K-RasG12V lung adenocarcinomas

In order to decipher the impact of C-Raf kinase inhibition in lung tumor initiation we quantified lesions 6 months after the infection in K-Ras+/LSLG12Vgeo, C-RafLmLD468A/LmLD468A and K-Ras+/LSLG12Vgeo mice. This revealed that even though inhibition of C-Raf kinase activity, concomitantly in “space” (cell type) and time together with K-RasG12V mutation, delayed tumor formation. Even though the Kaplan-Meier curve is ongoing, preliminary data suggest that expression of C-RafD468A does not increase control mice lifespan. However, tumors were observed in K-Ras+/LSLG12Vgeo, C-RafLmLD468A/LmLD468A dead mice. PCR Analysis of DNA extracted from these tumors revealed that 16% of them had completely excised C-RafLmLD468A allele and 16% scaped Cre-mediated recombination. Even though, more samples need to be analysed
and a Southern blot is needed to quantify the percentage of excision of 68% mixed excised
tumors, we can already conclude that there are compensatory mechanisms that bypass C-Raf
catalytic activity inhibition to drive lung tumorigenesis. Although we could argue that C-Raf
antitumoral effect might be exerted by more than one of C-Raf functions, more analysis need
to be done in order to confirm these observation.

Notwithstanding, our aim was to validate the inhibition of C-Raf kinase activity for the
treatment for human K-RasG12V driven lung adenocarcinomas. Therefore, we tried to mimic
the real scenario in which a patient is diagnosed from K-Ras mutant lung cancer and is given a
selective inhibitor against C-Raf kinase activity. To that end, we expressed C-RafD468A in
already established tumors. Whilst elimination of C-Raf protein from tumors impaired
proliferation or even induced regression independently of p53 status (Francoz & Musteanu,
unpublished data), elimination of its kinase activity only impaired tumor growth and 18FDG
uptake of tumors in a p53 proficient context. The lack of response in p53 deficient tumors needs
to be further study. However, and even though more tumors need t be analysed, it seems that
K-RasF557SfG12V, C-Raf LmlD468A/LmlD468A, p53frtf Tg.hUBC-CreERT2 +/T tumors need at least 12
weeks on tamoxifen diet to excise C-Raf LmlD468A allele completely. Therefore, one possibility for
the lack of response in these p53 deficient tumors is that by the time that the fold change in
tumor volume is being evaluated (8 weeks after tamoxifen diet) the C-Raf LmlD468A allele has not
been totally excised. The different excision timing that C-Raf LmlD468A allele requires could be
attribution to proliferation rate of tumors in the absence of p53 protein. If the expression of a
C-Raf kinase dead protein impairs cell growth, there will be a positive pressure for the
proliferation of cells that have not undergone C-Raf LmlD468A allele excision so that they take
over hiding the therapeutic benefit of inhibiting C-Raf kinase activity in these tumors. The H&E
sections of control mice showed a higher tumoral burden when compare to mice of interest,
what suggest an impairment of tumor growth probably due to C-Raf kinase activity
elimination. However, preliminary data suggest that this effect is not reflected in the lifespan
of these mice; maybe due to the toxic side effects that the lack of C-Raf kinase activity have in
other organs. Notwithstanding, inhibition of C-Raf kinase activity could be a proper therapy
for the 50% of patients with K-RASG12V driven lung adenocarcinomas in which p53 remains
in its wild type state that are still missing a targeted therapy. The mechanism in which the
therapeutic benefit of C-Raf kinase activity elimination relies has not yet been studied. The
different outcome between C-Raf protein elimination and lack of kinase activity evidences that
C-Raf kinase dependent and independent functions sustain lung tumorigenesis.
Study of the mechanism of the therapeutic benefit of C-RafD468A expression

Erk phosphorylation levels in C-Raf^{+/−} embryo lysates are indistinguishable from those of control mice, however, MEFs proliferation is impaired although not abrogated. (Blasco et al., 2011b; Mikula et al., 2001; Wojnowski et al., 1998). This fact reflects that pErk is not a good readout to asses MAPK activation. Even though the therapeutic benefit observed in mice lacking Mek or Erk proteins suggested that the C-Raf essential function for tumor initiation and maintenance was the kinase activity, pErk staining of tumors did not vary in K-Ras^{{V12G}}; C-Raf^{L_{ox}/L_{ox}}; p53^{ft/ft}; Tg.hUBC-CreERT2^{+/-} on tamoxifen diet in which the C-Raf^{L_{ox}} allele had been excised compared to C-RafD468A or C-Raf wild type expressing tumors. Three facts have to be taken into account:

- Erk protein elimination impairs lung tumor formation.
- pErk and MEFs proliferation in C-Raf knockout mice reveal that Erk activation is not disrupted under these circumstances.
- Ablation of C-Raf protein does impair lung tumor formation as well without affecting pErk levels either in a mouse model as in NSCLC cell lines.
- pErk staining as a readout of MAPK pathway activation seems not be accurate (Pratilas et al., 2009). Moreover the intensity and duration of Erk signalling and more importantly, the upstream mode of activation can specify different biological programs, such as proliferation or differentiation.

Erk specific cluster of targets must be analysed by RT-PCR to determine if the MAPK is still activated in tumors that express the C-RafD468A mutant. In addition C-Raf kinase dependent effectors must be analysed in order to know which is the pathway involved in sustain and inicinate tumorigenesis. C-Raf can phosphorylate other proteins apart from Mek. Rb C-Raf dependent phosphorylation in the nucleus is necessary for cell cycle entry. C-Raf:Rb complex quantification in K-RasG12V; C-RafD468A expressing tumors need to be done and compared to K-RasG12V; C-Raf wild type and K-RasG12 tumor samples in which C-Raf protein has been eliminated.

C-Raf protein has been described to regulate Yap antiapoptotic effect by two different processes. First of all, C-Raf can bind Mst1/2 and impede its homodimerization and activation. On the second hand, C-Raf can trigger Yap phosphorylation and activation in a Erk-dependent process (Zhang et al., 2014). To elucidate if YAP C-Raf kinase activity affects is mediating tumorigenesis in the lung, Yap Erk-mediated phosphorylated residues should be checked.

Myc protein family is a downstream target of C-Raf regulated by Rockα. C-Raf activation promotes Myc transcription, which appear upregulated in many cancer types. However, it is
not very probable that Myc proteins will be downregulated in tumors that express a catalytically inactive form of C-Raf. Mts2 activates Rockα, however, protein MTS2 dimerization, and hence, activation is prevented by C-Raf interaction. However, we will check the mRNA levels of Myc proteins because as we have already affirmed, C-Raf functions are modulated by genetic alleles in which interaction could mediate C-Raf kinase activity.

Moreover, it would be interesting to decipher if there are some C-Raf functions implicated in the metastatic process. However, the models that we have been using in this work rarely metastatize to other organs, therefore, this research cannot be address in this mouse model.

Moreover, apart from K-Ras driven lung cancer, there have been reported many cancers with C-Raf amplifications as bladder (Simon et al., 2001), NSCLC and urothelial cancers. So, this mouse model could be used also to determine if the progression of these cancers rely on C-Raf kinase activity or not and therefore to predict if patients with C-Raf amplifications would benefit from ATP-competitive inhibitors.

Side effects associated to the Expression of C-RafD468A systemically

Even though it seems that the lack of C-Raf kinase activity impairs the growth and $^{18}$FDG-uptake of K-Ras G12V lung adenocarcinomas, the lifespan of these mice is not increased. In order to determine if the expression of a C-Raf kinase dead isoform systemically is associated with toxic side effects we will perform Southern blots to check the percentage of excision of C-Raf$^{LmLD468A}$ allele together with histopathological analysis of the main tissues of K-Ras$^{+/FSG12V}$, C-Raf$^{LmLD468A/LmLD468A}$, Tg.hUBC-CreERT2$^{+/T}$ that were fed on tamoxifen containing diet. Germline expression of C-RafD468A in homozygosis triggered trembling movements and immaturity in most of the tissues analysed. Surprisingly, K-Ras$^{+/FSG12V}$, C-Raf$^{LmLD468A/LmLD468A}$, $p53^{int/int}$ Tg.hUBC-CreERT2$^{+/T}$ mice fed on a tamoxifen containing diet also showed trembling movements, suggesting that the expression of C-RafD468A in adult mice recapitulated at least one of the phenotypes associated to the germline expression and therefore, that the C-Raf$^{LmLD468A}$ allele was being excised properly. Conversely, the elimination of C-Raf protein systemically in mice following the same approach (fed on a tamoxifen containing diet upon tumor detection) did not alter the homeostasis in the main tissues (Blasco et al., 2011b). This counterintuitive fact can be explained by the excision rate of both alleles as Cre recombinase in these two models was expressed under different promoters. On the contrary, C-RafD468A may have a dominant negative effect in substrate titration.
Moreover, as Rushworth and collaborators reported, either C-Raf or B-Raf kinase dead proteins can confer high activity in B-/C-Raf heterodimers, which have been reported to hyperactivate the MAPK pathway (Rushworth et al., 2006). However, this seems not be recapitulated in our model, as this would imply the development of more aggressive tumors. On the contrary, if a catalytically inactive C-Raf protein could induce the hyperactivation of the MAPK pathway, the fact that 6 months after the coexpression of K-RasG12V and C-RafD468A no tumor was detected in the lung would suggest that this coexpression could trigger OIS.

To avoid these side effects that difficult the analysis of C-Raf kinase elimination in K-RasG12V driven tumors different approaches have been mentioned in Results section.

### 7.1 7.2 Study of the effect of B-Raf kinase inhibition in K-RasG12V driven lung adenocarcinomas

C-Raf protein was the first RAF isoform to be described and the best studied until BRAF mutations spectrum in human cancers was reported. In 2002, together with the well-known B-RAFV600E, B-RAF kinase inactivating mutations were surprisingly described in human colorectal tumors (Davies et al., 2002; Yuen et al., 2002). Even though they had reduced or abolished kinase activity, the fact that they occurred at the activation segment in conserved residues, as B-RAFV600E, suggested that they were not passenger mutations selected by serendipity. Importantly, this fact implied that other mechanisms apart from the increased ERK kinase activity were involved in tumorigenesis. Among these catalytically inactive B-Raf mutants, D594V in the DFG domain was first described. Mutations in this residue, as well as other catalytically inactive mutants, were coincident with K-RasG12D in many human cancer samples (Garnett and Marais, 2004; Ikenoue et al., 2003). Later in time, several B-Raf kinase dead mutants were reported to heterodimerize with C-Raf and activate endogenous ERK in COS and Xenopus cells. However, B-RafD594V failed to induce this Erk activation in cells nor activate C-Raf protein (Wan et al., 2004).

In 2010, Heidorn and colaborators reported that expression of B-RafD594A concomitantly with K-RasG12V in melanocytes triggered MAPK pathway paradoxical hyperactivation (Heidorn et al., 2010). This effect consists on the hyperactivation of the pathway due to the formation and stabilization of B-/C-Raf heterodimers, in which one protomer is catalytically inactive, in the presence of upstream activating mutations. Remarkably, even though kinase activity is impaired, Raf isoforms can hyperactivate the MAPK because of the transactivation of the protomer to which it is bound due to allosteric interactions. This effect has been been reported to be one of the resistance mechanisms in
Discussion

melanoma patients treated with B-Raf inhibitors, who initially respond to B-Raf inhibition therapy but develop different types of Ras mutated tumors after some time (Abdel-Wahab et al., 2014).

As Raf inhibitors are not 100% selective, one objective of this thesis was to describe the effect of B-Raf kinase inhibition in the lung in the presence of oncogenic K-Ras. Therefore, at the very same time we were analysing the possible C-Raf kinase inhibition associated therapeutic effect we wanted to check if a non-selective Raf inhibitor could be detrimental in these tumors in case of inhibiting B-Raf isoform.

**B-RafD594A and K-RasG12V coexpression in lung is detrimental for mice lifespan**

Our results confirmed that coexpression of a B-Raf kinase inactive protein and K-RasG12V in lung is deleterious for mice lifespan. These mice develop bigger AD/ADC than control mice at comparable time points, suggesting a faster progression of these lesions when K-Ras was coexpressed with a B-Raf kinase dead protein. Intriguingly, hyperplasias that never evolved to full-blown tumors were covering the majority of lung parenchima at humane end points. This phenotype resembled the MAPK pathway paradoxical activation that has been already mentioned. Hence, and even though we have not decipher yet if this phenotype is led by the stabilization of B-/C-Raf heterodimer formation and the transactivation of C-Raf, I will refer to the MAPK paradox hereafter in reference to the acceleration in tumor growth and the hiperplasia development observed in the lung.

These hyperplasias could have been developed due to different reasons. Firstly, the different morphology could imply a different cell of origin. K-RasG12V expression in the lung has been reported to trigger proliferation in both CC10\(^{+}\) Clara and SPC\(^{+}\) ATII cells. However, Clara cells only progressed to benign adenomas whereas ATII pneumocytes gave rise to full-blown tumors (Mainardi et al., 2014). The SPC and CC10 marker stainings of these hyperplasias confirmed that they were originated from SPC\(^{+}\) ATII cells. However, we cannot exclude the possibility of the existence of different epigenetic states or subtypes of ATII cells in terms of precursor or mature ATII pneumocytes, which would require further studies. On the other hand, all the lesions that we checked (AD/ADC and hyperplasias) had excised the \(K-Ras^{+/LS1G12Vgeo}\) and \(B-Raf^{+/-Mld594A}\) knocked-in alleles, therefore we could exclude that difference in morphology was due to partial locus excision and differential expression of K-RasG12V oncogene and B-RafD594A. Finally, Ki67 stainings revealed that these lesions were not highly proliferative in contrast to the AD/ADC. Therefore, together these facts suggested that a higher number of cells was being transformed upon K-RasG12V and B-RafD594A coexpression.
To stimulate proliferation, the level of Erk signaling must be carefully modulated, because if it is too strong, cells will stop cycling and differentiate or senesce (Marshall, 1995; Kerkhoff and Rapp, 1996; Sewing et al., 1997; Woods et al., 1997). This is the case of B-RafV600E in nevi or in benign lung tumors, which will only progress upon the loss of tumor suppressor genes (Dankort et al., 2007; Mercer et al., 2005). Likewise, the different catalytically inactive B-Raf mutants trigger different levels of Erk phosphorylation and the fact that some B-RAF kinase dead mutations are coincident with oncogenic Ras suggest that they cooperate to carefully modulate Erk pathway (Davies et al., 2002; Yuen et al., 2002). According to this, the inhibition of B-Raf kinase activity may raise the oncogenic threshold so that it triggers proliferation in a higher number of ATII pneumocytes or in a subtype of ATII cells that was reluctant to K-RasG12V driven transformation.

**Ablation of C-Raf kinase activity in the lung impairs K-RasG12V; B-RafD594A tumorigenesis**

MAPK paradoxical activation in melanoma has been reported to be dependent on C-Raf kinase activity (Heidorn et al., 2010). As a proof of concept, we generated K-Ras<sup>+/LSLGrvgeo</sup>; B-Raf<sup>+/LmL594A</sup>; C-Raf<sup>LmLD468A/LmLD468A</sup> mice, which after Ad-Cre intratracheal infection reverted the phenotype associated to B-Raf D594A and K-RasG12V coexpression (lifespan was increased and only few and small tumors were detected in the lung). Even though we cannot assume C-Raf kinase activity is driven this tumor growth acceleration, we can affirm that C-Raf kinase inhibition is impairing MAPK paradoxical activation in the lung. This implies that a pan-Raf inhibitor that completely block B- and C-Raf kinase activities would avoid MAPK hyperactivation. In this sense, maybe inhibitors targeting the dimer interface could be effective as well. However, we will have to check the excision of all the inducible alleles to determine if they have scaped C-Raf<sup>LmLD468A</sup> Cre-mediated excision or, if on the contrary, there are some cells that express K-RasG12V and B-RafD594A and can grow without the C-Raf catalytic activity.

There is some controversy in the field about the need of C-Raf kinase activity to form heterodimers. Whereas C-Raf5621A and C-RafK375M kinase dead proteins have been reported to diminish their interaction with B-Raf (Rushworth et al., 2006), C-RafSS338/339 catalytic inactive mutant showed no alteration in heterodimer formation (Diaz et al., 1997). We will have to test whether C-RafD468A protein binds B-Raf with a reduced affinity accounting this effect for the possible mechanism of therapeutic benefit. Binding of 14-3-3 to the site in the catalytic domain has a positive function, at least in C-Raf; autophosphorylation of this site in cis and the ensuing 14-3-3 binding are required to prevent proteasome-mediated C-Raf degradation (Noble et al., 2008a).
Discussion

B-Raf loss of heterozygosity

As I have mentioned before, Raf-Mek-Erk pathway is tightly controlled and subtle variations can modify the outcome. MAPK pathway paradox effect in lung and skin was accomplished by the inactivation of catalytic activity 50% of the B-Raf proteins. Therefore, in this scenario B-Raf/C-Raf and B-RafD594A/C-Raf heterodimers coexisted. We tried to describe the result of kinase activity inhibition in 100% of the B-Raf proteins, so that only B-RafD594A/C-Raf heterodimers are present in the cell. The lifespan of these mice was very similar to \( K-Ras^{+/LSL12V^geo}, \text{ B-Raf}^{+/LmD594A} \); however, mice developed less AD/ADC and the infrequent intrabronchiolar lesions that were observed in \( K-Ras^{+/LSL12V^geo}, \text{ B-Raf}^{+/LmD594A} \) mice were the most prominent type of lesion when the B-Raf wild type was eliminated. Among the different hypothesis Oncogene Induce Senescence observed in many cell types in response to certain oncogenes (Serrano et al., 1997) could be impairing AD/ADC formation if the LOH phenomenon in B-Raf increases the MAPK pathway. We will check morphologic as well as histopathologic markers (heterochromatin foci, p16, Senescence-Associated \( \beta \) Galactosidase staining,...) in these samples to elucidate if OIS is mediating the absence of AD/ADC.

Intrabronchiolar lesions arising from bronchioles maybe obstruct bronchioles and cause the death of \( K-Ras^{+/LSL12V^geo}, \text{ B-Raf}^{Lox/LmD594A} \) mice. The localization of the intrabronchiolar lesions suggested they originated from Clara cells rather than an invasive alveolar lesion. Moreover, these lesions presented a mix pattern already in the early stages in which some areas in the bronchiole lost the CC10 marker and invading areas expressed the SPC protein. Interestingly, SPC expressing cells seemed to be the origin of the intrabronchiolar lesion. Therefore, it seems that the increased MAPK signalling induces the Clara cell transdifferentiation to a new SPC positive a higher proliferative cell.

Surprisingly, in some \( K-Ras^{+/LSL12V^geo} \) mice, which were intratracheally infected with Ad-CC10-Cre virus, some foci were observed in distal areas which further confirmed that there must be either cells that expresses both CC10 and SPC protein at levels that are not detected by normal immunohistochemistry techniques but are detected by RT-PRC. On they other hand, they can be progenitor cells from which further differentiation events commit a an alveolar type II or Clara cell.

Maybe the human process is the other way around. All the papers claim that there are pre-existing quiescent Ras mutant lesions in which B-Raf inhibition promotes proliferation. However, in B-Raf inhibited cells, a mutation of Ras proteins can trigger proliferation. Maybe, because these cells are inhibiting another pathway or raising the levels needed to be transformed. We do not know if the sequence of the factors is important.
However, there are physiologic mechanisms that naturally protect cells from transformation. Therefore, it is thought that only when these “guardians” are swicht off” mutations in K-RAS can trigger oncogenesis. The identification of these alterations is not always trivial as they can be epigenetically silenced or mutated.

Lung targeted expression of either C-Raf wild type protein or a C-Raf isoform lacking the NH2-terminal regulatory sequences has been shown to promote the formation of lung adenocarcinomas in transgenic mice (Kerkhoff et al., 2000).

The different levels of Erk activation triggered by B-Raf catalytically inactive mutants were proposed as the rationale for B-Raf kinase dead mutations coexistence with oncogenic Ras (Garnett and Marais, 2004). However, our results demonstrate that B-RafD594 can trigger tumorigenesis in the absence of K-RasG12V mutations in Ras proteins. However, we cannot exclude mutations in other driver or tumor suppressor gene. Surprisingly, mutations in the aspartic residue of the DGF domain were reported to not activate C-Raf or Erk activity (Wan et al., 2004).

Together these facts suggest that MAPK pathway is tightly modulated and it fits different variations on its effectors to trigger unpredicted responses.
8 CONCLUSIONS/
CONCLUSIONES
THE CONCLUSIONS OBTAINED IN THIS WORK WERE THE FOLLOWING:

1. C-Raf catalytic activity is dispensable for life. Its absence is associated to smaller weight and size and motility problems. Hypocellularity and immaturity are observed in most of the tissues.


3. The coexpression of a B-Raf catalytically inactive protein (B-RafD594A) and K-RasG12V oncogene in the lung diminishes mice lifespan due to an increased tumor burden. This seems to recapitulate the MAPK pathway paradoxical activation reported in other tissues.

4. The expression of a C-Raf kinase inactive protein concomitantly with K-RasG12V and B-RafD594A impairs tumor initiation and progression.

5. The ubiquitous coexpression of B-RafD594A and K-RasG12V induces transformation in other epithelial tissues.

6. The loss of B-Raf wild type allele contemporary to K-RasG12V and B-RafD594A expression in the lung triggers tumorigenesis in Clara cells leading to the formation of intrabronchiolar lesions.

7. Expression of a B-Raf catalytic inactive protein in the lung can drive tumorigenesis independently of K-Ras mutations.
LAS CONCLUSIONES DE LA PRESENTE TESIS HAN SIDO:

1. La actividad catalítica de C-Raf es dispensable para la vida. Su ausencia está asociada a menor tamaño, peso y problemas de movilidad de los ratones. La mayoría de los tejidos presenta hipocelularidad e inmadurez.

2. La eliminación de la actividad quinasa de C-Raf impide el crecimiento y la toma de $^{18}$FDG en tumores de pulmón inducidos por K-RasG12V. Sin embargo su eliminación tiene un efecto reducido en los tumores de pulmón inducidos por K-RasG12V en los que p53 está mutado.

3. La coexpresión de una proteína B-Raf catalíticamente inactiva (B-RafD594A) y del oncogene K-RasG12V en el pulmón disminuye la esperanza de vida de los ratones debido a un aumento de la carga tumoral. Esto parece recapitular el efecto paradójico de activación de la vía MAPK ya descrita en otros tejidos.

4. La expresión de una forma de C-Raf catalíticamente inactiva de forma concomitante a la expresión de K-RasG12V y B-RafD594A en el pulmón impide la iniciación y progresión tumoral.

5. La pérdida de la forma silvestre de B-Raf en células que coexpresan las proteínas K-RasG12V y B-RafD594A induce la transformación de las células Clara en los bronquios, induciendo la formación de lesiones intrabronquiolares.

6. La inactivación de la actividad quinasa de la proteína B-Raf puede inducir la formación de tumores en el pulmón en ausencia de mutaciones de K-Ras.
9 REFERENCES
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93


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10 APPENDIX/
PUBLICATIONS