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**c-Raf Ablation Induces Regression of Advanced**

**K-*Ras/Trp*53 Mutant Lung Adenocarcinomas by a Mechanism Independent of MAPK Signaling**

by

Manuel Sanclemente1,3, Sarah Francoz1,3,5, Laura Esteban-Burgos1, Emilie Bousquet-Mur1,6, Magdolna Djurec1, Pedro P. Lopez-Casas2, Manuel Hidalgo2,7, Carmen Guerra1, Matthias Drosten1, Monica Musteanu1,4,\* and Mariano Barbacid1,4,\*

1Molecular Oncology and 2Clinical Research Programmes,

Centro Nacional de Investigaciones Oncológicas (CNIO),

Melchor Fernández Almagro 3, 28029 Madrid, Spain

3 Co-first authors

4 Co-senior authors

Present Addresses:

5 Centro National de Investigaciones Cardiovasculares, 28029 Madrid, Spain

6 Institut de Recherche en Cancérologie de Montpellier, 34298 Montpellier, France

7 Beth Israel Deaconess Medical Center, Boston, MA 02215, USA

\* Correspondence: mmusteanu@cnio.es and mbarbacid@cnio.es

**SUMMARY**

A quarter of all solid tumors harbor K-*RAS* oncogenes. Yet, no selective drugs have been approved to treat these malignancies. Genetic interrogation of the MAPK pathway revealed that systemic ablation of Mek or Erk kinases in adult mice prevent tumor development but are unacceptably toxic. Here, we demonstrate that ablation of c-Raf expression in advanced tumors driven by K-*Ras*G12V/*Trp53* mutations leads to significant tumor regression with no detectable appearance of resistance mechanisms. Tumor regression results from massive apoptosis. Importantly, systemic abrogation of c-Raf expression does not inhibit canonical MAPK signaling, hence, resulting in limited toxicities. These results are of significant relevance for the design of novel therapeutic strategies to treat K-RAS mutant cancers.

**SIGNIFICANCE**

More than 30 years after the identification of K-*RAS* oncogenes in human cancer, there are no selective therapies against these tumors. Direct targeting of K-RAS has proven challenging. Hence, most efforts have focused on targeting its downstream kinases. Here, we report that targeting c-Raf expression in mice induces significant regression of advanced K-*Ras*G12V/*Trp53* mutant lung tumors by a mechanism that induces massive apoptosis without affecting canonical MAPK signaling. As a consequence, systemic ablation of c-Raf does not induce the unacceptable toxicities observed when tampering with its downstream kinases, Mek or Erk. These results should open new avenues to design effective therapeutic strategies against K-RAS mutant cancers.

# HIGHLIGHTS

* Ablation of c-Raf expression induces regression of K-*Ras/Trp53* mutant lung tumors.
* Loss of c-Raf expression does not affect MAPK signaling
* Systemic ablation of c-Raf expression in adult mice has limited toxic effects

**INTRODUCTION**

*KRAS* mutations have been identified in a quarter of all human cancers, including those with worse prognosis such as pancreatic ductal adenocarcinoma, colorectal adenocarcinoma and lung adenocarcinoma. In spite of the advances we have witnessed during the last two decades in the development of targeted therapies, there are still no selective drugs against these malignancies (Cox et al., 2014). Although K-RAS is not a trivial druggable target (Ostrem et al., 2013), most of its downstream effectors are druggable kinases. For instance, the MAPK pathway consists of a cascade of protein kinases that amplify and regulate mitogenic signals. These protein kinases come in three flavors, the RAF kinases (A-RAF, B-RAF and c-RAF), the MEK kinases (MEK1 and Mek2) and the ERK kinases (ERK 1 and ERK2). The latter diversify these mitogenic signals by phosphorylating a wide variety of substrates, including, transcription factors as well as other kinases that control cellular processes as distinct as protein synthesis and the cell cycle (Sebolt-Leopold and Herrera, 2004). K-RAS also activates a second kinase cascade via its interaction with the catalytic subunits of PI3K, a group of phophoinositol kinases responsible for the production of the second messenger PIP3. PIP3 binds and activates a wide variety of proteins including other protein kinases such as PDK1 and the AKT kinases that have been implicated in a variety of cellular activities including survival (Castellano and Downward, 2011).

One of the biggest challenges of Precision Medicine is to develop therapeutic strategies that block oncogenic signaling without affecting normal homeostasis. In mice, ablation of the Mek1/2 kinases effectively prevented K-Ras driven tumor development. Similar results were obtained upon ablation of the Erk1/2 kinases. Unfortunately, systemic ablation of the Mek1/2 or Erk1/2 kinases in adult mice led to extreme toxicities that resulted in the rapid death of the animals (Blasco et al., 2011). MEK inhibitors have proven to be effective against K-Ras driven mouse tumors, however their high toxicities have prevented their approval for their use in human patients. Ablation or inactivation of Ras-mediated PI3K activation has also shown to block progression of K-Ras driven tumors (Castellano et al., 2013). However, these strategies also induce unacceptable toxicities in adult mice (our unpublished observations). Indeed, in spite of intense efforts by the pharmaceutical industry, no PI3K inhibitors have been so far approved to treat K-RAS tumors.

Targeting of c-Raf, but not the B-Raf kinase revealed equally effective inhibition of tumor development without inducing significant toxicities (Blasco et al., 2011; Karreth et al., 2011).Thus, suggesting that these kinase isoforms are likely to play different roles in mediating K-Ras oncogenic signals. In these studies, however, target ablation was concomitant with induction of K-*Ras*G12V expression. Hence, they only interrogated the role of these Raf kinases in tumor initiation, not tumor progression, a scenario more relevant to the clinical setting. To determine whether Raf kinases may play a role in tumor development without affecting normal homeostasis, we have developed a new generation of genetically engineered mouse (GEM) tumor models that use two independent recombinase systems to temporally and spatially separate tumor induction from target inactivation. This strategy allows not only to evaluate the effect of target ablation in fully developed tumors, but also to determine their potential toxic effects on adult homeostasis when the target is ablated in a systemic fashion. These studies provide an effective approach to genetically identify effective and suitable non-toxic therapeutic strategies in mice carrying advanced K-Ras driven lung tumors.

# Results

**An Flp/frt-dependent K-*Ras*G12V oncogene driven lung adenocarcinoma model**

We have developed a new GEM strain to temporally and spatially separate tumor development from target ablation by using two independent recombinase systems. To this end, we targeted the K-*Ras* locus by inserting a Neo resistant-STOP cassette flanked by frt (F) sequences within the first intron, along with a mutated first exon encoding a Gly to Val substitution (see Experimental Procedures). The resulting strain, K-*Ras*+/FSFG12V, developed lung adenocarcinomas upon intratracheal infection with adenoviral particles expressing the Flp recombinase [Ad-Flp] (Buchholz et al., 1998) with complete penetrance and latencies similar to those observed in K-*Ras*+/LSLG12Vgeo mice infected withadenoviral particles expressing the Cre-recombinase (Blasco et al., 2011).

**c-*Raf* ablation reduces tumor burden in K-RasG12V driven lung tumors**

K-*Ras*+/FSFG12V mice were crossed to animals carrying c-*Raf* conditional floxed alleles (c-*Raf*L) (Jesenberger et al., 2001) and a ubiquitously expressed CreERT2 inducible recombinase knocked-in at the RNA polymerase II locus (*RERT*ert) (Guerra et al., 2003). To evaluate potential compensatory effects by the related B-Raf kinase, we also generated an additional strain containing B-*Raf* floxed alleles (B-*Raf*L) (Chen et al., 2006). Exposure of tumor-bearing mice to a tamoxifen (TMX) containing diet for 4 months allowed us to evaluate the effect of eliminating the c-Raf and/or B-Raf kinases in tumor-bearing mice. This strategy also allowed us to identify potential toxic effects that could result from the systemic elimination of these kinases in normal adult tissues, a key information to determine the suitability of this therapeutic strategy.

K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L, K-*Ras*+/FSFG12V;*RERT*ert/ert;B-*Raf*L/L and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L mice were infected with low titer Ad-Flp particles by intratracheal instillation to allow expression of the resident K-*Ras*G12Voncogene in lung tissue. The use of low titers of Ad-Flp particles resulted in the induction of one or very few tumors in each animal in order to facilitate their individual analysis by monthly Computed Tomography (CT) scans. Mice displaying at least one tumor larger than one millimeter in diameter were fed *ad libitum* with a TMX containing diet to ablate the conditional c-*Raf*L and/or B-*Raf*L alleles. Previous studies have shown that germ line ablation of c-Raf and B-Raf kinase was embryonic lethal (Huser et al., 2001; Mikula et al., 2001; Wojnowski et al., 1997). However, these kinases do not appear to be essential for adult homeostasis since adult mice survived well 4 months of TMX exposure, a time in which expression of these kinases was completely eliminated (Figure 1A).

All tumors present in control K-*Ras*+/FSFG12V;*RERT*ert/ert mice grew in size during this period of time leading to a significant increase in tumor burden and had to be sacrificed at humane endpoint within 6 months (Figure 1B and C). Similar results were observed in K-*Ras*+/FSFG12V;*RERT*ert/ert;B-*Raf*L/L animals, indicating that B-Raf kinase does not play a relevant role in mediating K-RasG12V oncogenic signals. In contrast, the average tumor burden in K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L decreased significantly after 4 months of TMX exposure (Figure 1B and C). At this time, analysis of tissues from these mice did not reveal any obvious alterations, indicating that systemic ablation of c-Raf does not induce significant toxicities. Moreover, all lesions present in these mice were hyperplasias or adenomas (Figure S1A and B). Concomitant ablation of c-*Raf*L and B-*Raf*L alleles led to a slightly more pronounced reduction in tumor burden (Figure 1B and C). Yet, ablation of both kinases was not well tolerated and all K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L had to be sacrificed during the first 4 months of TMX exposure (Figure 1B and C). In contrast, K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L survived for up to 9 months indicating that systemic loss of c-Raf expression is well tolerated by adult mice. Importantly, loss of c-Raf expression did not result in the development of resistance mechanisms, at least during the length (9 months) of TMX exposure.

**Elimination of c-Raf expression decreases glucose uptake in K-RasG12V driven lung tumors**

In the clinical setting, tumor progression is often diagnosed by Positron Emission Tomography (PET) scanning, a technique that detects the ability of tumors to metabolize radioactive tracers, most commonly the glucose analogue [18F]-fluoro-2-deoxy-glucose ([18F]-FDG) (Dewan et al., 1993; Scheffler et al., 2013). Comparison of PET/CT scan axial projections and 3D reconstruction images of lungs of representative mice taken before and after 2 months of TMX exposure revealed that ablation of c-Raf expression dramatically reduced [18F]-FDG uptake (Figure 2A). In contrast, tumors present in control animals displayed increased [18F]-FDG uptake. These mice were part of three cohorts of 14 K-*Ras*+/FSFG12V;*RERT*ert/ert mice, 19 K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L mice and 17 K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L mice fed with TMX for 2 months. As summarized in Figure 2B, the overall number of PET+ tumors doubled in control mice. In contrast, the number of PET+ tumors in those mice that either underwent c-*Raf* or c-*Raf+*B-*Raf* ablation decreased by 45% and 60% respectively. Similar results were obtained when we analyzed the Standardized Uptake Value (SUV) of individual tumors (Figure 2C). Most tumors expressing c-Raf (26/30, 87%) increased their [18F]-FDG uptake at the end of the 2 months TMX exposure. In contrast, only 3 out of the 46 tumors (6.5%) displayed a slightly higher PET signal after c-Raf ablation (Figure 2C). Finally, none of the tumors that underwent concomitant recombination of c-*Raf*L and B-*Raf*L alleles increased their PET signal (Figure 2C).

**c-Raf ablation prevents tumor-related deaths and increases survival**

Individual analysis of 42 tumors present in 22 K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L mice during a 4 month long TMX exposure further illustrated the therapeutic benefit that resulted from ablation of c-*Raf*L alleles. As shown in Figure 3A, most tumors present in these mice either partially regressed (12/42, 29%) or completely disappeared (14/42, 33%). Moreover, most of the 16 tumors (11/16, 69%) that progressed during the 4 month long trial did not double in size. In contrast, all tumors that could be analyzed by sequential CT scans (35/46, 76%) in control *Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*+/+ mice increased in size an average of 9.8 fold, with a significant percentage (9/35, 26%) growing more than 10-fold (Figure 3A). The remaining 11 tumors scored in this trial could not be followed by CT due to the development of pulmonary atelectasis, a bronchiolar obstruction caused by the expansion of the tumor tissue that ultimately resulted in collapse of the lungs. Finally, whereas 67 tumors appeared during the 4 months long trial in the control cohort, only 9 *de novo* tumors could be observed in K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L animals. These data are summarized in Figure 3C.

The therapeutic effect of c-*Raf* ablation resulted in a significant increase in survival. As illustrated in Supplemental Figure 1C, tumor-bearing K-*Ras*+/FSFG12V;*RERT*ert/ert mice survived an average of 37 weeks after being fed with a TMX containing diet. Ablation of B-*Raf*L alleles increased the average survival by 5 weeks in spite of not having a significant effect on tumor progression. Mice in which we eliminated c-*Raf*L alleles increased their survival to 61 weeks, a 65% increase compared to the control cohort (Figure S1C). More importantly, none of the K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L animals analyzed at humane endpoint had advanced adenocarcinomas nor sufficient tumor burden to be cause of death (Figure S1A and B). Histopathological analysis of a variety of tissues collected from these mice failed to reveal defined abnormalities. Thus, it is likely that these mice died due to a combination of factors including old age and continuous exposure to TMX for over a year. Yet, we cannot eliminate the possibility that ablation of c-*Raf*L alleles in normal tissues may also contribute to the dead of these mice (see below).

**c-*Raf* is an effective therapeutic target for advanced K-RasG12V/Trp53 driven tumors**

Human lung tumors harboring K-*RAS* mutations often lack a functional TP53 tumor suppressor (Chen et al., 2014). Ablation or inactivation of Trp53 in K-*Ras* mutant mice induces more aggressive adenocarcinomas and significantly accelerates tumor development (Jackson et al., 2005). Thus, we interrogated whether ablation of c-Raf expression in K-*Ras*G12V driven lung tumors lacking Trp53 also induced tumor regression. To this end, we added conditional *Trp53* alleles flanked by frt recognition sites (*Trp53*F) to K-*Ras*+/FSFG12V mice (Lee et al., 2012). Intratracheal infection of K-*Ras*+/FSFG12V;*Trp53*F/F mice with Ad-Flp particles resulted in accelerated tumor development and shortened survival compared to K-*Ras*+/FSFG12V animals (Figure S2A). As expected, histopathological analysis of their lungs revealed more advanced tumors of higher histological grade (Figure S2B).

Addition of conditional c-*Raf*L alleles to K-*Ras*+/FSFG12V;*Trp53*F/F mice allowed us to interrogate the therapeutic effect of ablating c-Raf expression in a more aggressive tumor environment. Unfortunately, the close proximity of the *Trp53* and RNA polymerase II loci in the mouse genome, forced us to replace the *RERT*ert alleles by an h*UBC-CreERT2* transgene in which CreERT2 expression is driven by the human ubiquitin promoter (Ruzankina et al., 2007). To determine the potential toxic consequences of eliminating c-Raf expression under the control of the CreERT2 expressing transgene (compared to the *RERT*ert alleles) we exposed 4 months old, non-tumor-bearing K-*Ras*+/+;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L mice to a TMX containing diet for 2 months. Southern blot analysis of DNA extracted from representative tissues revealed complete c-*Raf* ablation in most organs (Figure S3A). No signs of toxicity were observed in these mice during the 2-month long exposure, indicating that c-Raf ablation is well tolerated. However, 7-month old animals died after 18 months of continuous exposure to a TMX diet (Figure S3B). This long-term toxicity was also observed in mice carrying wild type c-Raf alleles indicating that this toxicity was primarily a consequence of the prolonged exposure to TMX. However, these control mice lived a bit longer (36% longer medium survival) suggesting that c-Raf ablation may also contribute to the premature dead of these mice (Figure S3B). Careful postmortem histopathological analysis of both mouse cohorts revealed no significant differences or obvious tissue damage (Figure S3C). Thus, pharmacological inhibition of c-Raf expression is likely to result in acceptable toxicities in a clinical setting.

Next, we designed an experimental trial in which we infected with low titer of Ad-Flp particles two cohorts of K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T mice carrying either c-*Raf*L or c-*Raf*+ alleles. These mice developed a total of 94 and 79 CT positive, measurable tumors, respectively, between 5 to 6 months post-infection (Figure 3B). These mice were then exposed to a TMX diet for 2 months. None of these tumor-bearing mice showed signs of toxicities during this time as determined by the lack of significant body weight loss (Figure S3D). In addition the overall appearance of the animals was comparable in both cohorts (Figure S3E). Tumor evolution was followed by CT analysis for the duration of the 2-month long trial. In the case of the control cohort, the appearance of pulmonary atelectasis prevented us from establishing the evolution of 25 lesions, thus, limiting our analysis to 54 CT+ c-Raf expressing control tumors. No such phenomena were observed in the experimental cohort carrying conditional c-*Raf*L alleles probably due to the reduced tumor burden. Indeed, the large majority of the 94 CT+ tumors present in this experimental cohort either partially regressed (65/94, 69%) or completely disappeared (9/94, 10%) (Figure 3B). Moreover, the 20 tumors (21%) that progressed during the trial grew very slowly and did not even double in size. In contrast, the 54 tumors that could be analyzed in the control mice that carried c-*Raf*+ alleles increased in size an average of 6.5-fold (Figure 3B). Moreover, whereas these control mice developed 44 *de* novo tumors during the 2-month long trial, we only detected 7 new tumors in those mice carrying conditional c-*Raf*L alleles (Figure 3C).

These results, taken together, indicate that c-Raf expression is also essential for tumor progression in the absence of the Trp53 tumor suppressor. Not surprisingly, the number of complete regressions observed in these advanced K-*Ras*G12V/*Trp53* mutant tumors was lower than in those that retained a functional Trp53 tumor suppressor (10% vs. 33%) (Figure 3C). However, we still observed a significant decrease in the number of adenocarcinomas present in these mice as compared to those scored in the control cohort (Figure S4A). This reduction in aggressive tumors is likely to be due to a significant decrease in the proliferation rate of those tumor cells lacking c-Raf expression, as determined by Ki67 immunohistochemical (IHC) staining (Figure S4B).

In summary, these results predict that inhibition of c-Raf expression/activity should have a significant therapeutic effect in the treatment of patients suffering from K-RAS mutant lung adenocarcinomas.

## Pleiotropic consequences of c-Raf ablation

Previous studies have shown that recruitment of immune cells plays a role in the regression of lung tumors (DuPage and Jacks, 2013; DuPage et al., 2012). Moreover, a recent study showed that K-RasG12D expression in resident myeloid cells can lead to mixed lung neoplasm´s (Kamata et al., 2017). We have not observed these mixed neoplasms possibly due to the fact that the limited levels of adenoviral particles used in our model resulted in the activation of K-RasG12V expression in a very small number of myeloid cells. On the other hand, tumors of TMX exposed K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L mice displayed high levels of infiltrating CD3-positive T lymphocytes, a phenomenon not observed in control tumors that retained c-Raf expression (Figure S4C). In addition, most tumors showed increased levels of CD8+ cytotoxic T cells upon c-Raf ablation (Figure S4D). Finally, c-Raf depleted tumors showed a slight decrease in the number of infiltrating macrophages when compared to control tumors (Figure S4E). To what extent these inflammatory responses play a role in tumor regression remains to be determined.

**MAPK and PI3K signaling in K-RasG12V/Trp53 driven tumors after ablation of c-*Raf* expression**

Next, we interrogated the status of the MAPK and PI3K signaling pathways in K-*Ras*G12V/*Trp53* mutant lung tumors upon ablation of the c-*Raf*L alleles (Figure 4). Western blot analysis of tissue extracts obtained from four “Progressor” tumors (P1 to P4) that increased in size during the 2 months long trial revealed that three of them (P1-P3) retained c-Raf expression levels between 50 to 100% of those tumors that carried wild type c-*Raf* alleles (C1-C4) (Figure 4A). The increase in size of these P1-P3 “Progressor” tumors is likely to result from c-Raf expression driven by un-recombined c-*Raf*L alleles. As expected, these tumors exhibited normal levels of phospho-Mek, phospho-Erk and phospho-Akt indicating the presence of active MAPK and PI3K pathways (Figure 4A). In contrast, most of the cells (>90%) in tumor P4 had lost c-Raf expression. Yet, they retained normal levels of phospho-Mek, phospho-Erk and phospho-Akt, suggesting that either the other Raf kinase isoforms or possibly other signaling pathways have compensated for the absence of c-Raf expression to sustain MAPK and PI3K activity during tumor progression.

Next, we analyzed 8 representative “Regressor” tumors that decreased in size during the trial, including 5 tumors (R4-R8) that decreased in size more than 50% of their initial volume (Figure 4B). Interestingly, tumors R7 and R8, those tumors displaying the largest regression levels (75% to 80% reduction in size, respectively) retained normal levels of c-Raf expression. Likewise, the levels of phospho-Mek, phospho-Erk and phospho-Akt were mostly comparable to those present in wild type c-Raf tumors. It is possible that these tumors represent a subpopulation of cells in which c-Raf was not efficiently eliminated. If so, we can assume that all tumor cells in which c-Raf was ablated had undergone apoptosis (Figure 4B).

Five of the 8 “Regressor” tumors (R2-R6) had minimal levels of c-Raf expression (5 to 10% of controls). Yet, four of them (R2, R4-R6) displayed enhanced activation of the MAPK pathway as determined by the increased levels of phospho-Mek and phospho-Erk proteins (Figure 4A). No significant variations were observed in the levels of phospho-Akt. Only one “Regressor” tumor, R3, failed to express detectable levels of phosphorylated Mek and Erk proteins, a result concordant with the established hypothesis that c-Raf mediates MAPK activity. The levels of phospho-Akt expression were similar to those of c-Raf expressing control tumors, suggesting that the PI3K pathway remained active in the R3 regressing tumor (Figure 4A).

These unexpected observations prompted us to examine phospho-Erk expression by IHC analysis in an additional subset of 8 tumors from the same cohort, including one “Progressor” (P5) and 7 “Regressor” tumors that underwent regression levels between 40% to 80% (R9 to R15) (Figure 4B). As illustrated in Figure 4C, all tumors displayed levels of phospho-Erk positive cells, at least comparable to those observed in control tumors that carried wild type c-*Raf* alleles (C5-C10) (Figure 4D). In addition, phospho-Erk expression did not correlate with proliferation as determined by KI67 IHC (Figure S5). The lack of c-Raf antibodies suitable for IHC analysis did not allow us to determine whether these phospho-Erk positive cells retained c-Raf expression. Likewise, the limited amount of tissue prevented us from establishing the levels of c-Raf expression in these tumors by Western blot analysis. Finally, we observed increased expression levels of K-Ras as well as of their other Ras isoforms, H-Ras and/or N-Ras in some tumors (Figure 4A). Yet, these alterations did not correlate with their therapeutic response to c-Raf ablation.

## c-Raf ablation induces tumor differentiation and apoptosis through MAPK kinase independent mechanisms

Previous studies have indicated that ablation of c-Raf in skin tumors induced by constitutively active SOS, a Ras GDP/GTP exchange factor, provokes tumor regression by a mechanism involving cell differentiation mediated by activation of the Rok-α kinase and subsequent phosphorylation of downstream targets, such as Cofilin, (Ehrenreiter et al., 2009; Niault and Baccarini, 2010). In our lung tumor model c-Raf depleted tumors also exhibited increased numbers of phospho-Cofilin positive cells compared to control lesions (Figure 5A). Since phosphorylation of Cofilin is associated with cell differentiation, it is likely that ablation of c-Raf expression might contribute to tumor regression and reduction of the number of advanced adenocarcinomas by inducing differentiation of lung tumor cells (Ehrenreiter et al., 2009).

c-Raf had also been shown to promote tumor development by inhibiting apoptosis (Matallanas et al., 2011). IHC analysis of cleaved Caspase3 expressed in tumors of K- K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L mice displayed a 3 fold increase in the number of apoptotic cells compared to control tumors that expressed c-Raf (Figure 5B). To determine whether these observations were cell autonomous, we established cell lines from tumors of K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/l mice not exposed to TMX, so we could eliminate c-Raf expression *in vitro* by adding 4-hydroxy-tamoxifen (4OHT) to the media. Indeed, Cre-mediated recombination of the floxed c-*Raf*L alleles in vitro proceeded much more efficient than *in vivo* leading to complete absence of c-Raf expression within 48 hours in three independent cell lines. As illustrated in Figure 5C, ablation of c-Raf expression triggered the rapid induction of apoptosis in a dose dependent manner. Concomitantly with loss of c-Raf expression, we observed a decrease in Caspase 3 levels along with the appearance of robust levels of cleaved Caspase 3 (Figure 5C). In contrast, loss of c-Raf expression had no effect on the levels of phospho-Mek and phospho-Erk expression, thus, confirming our *in vivo* observations that loss of c-Raf expression had no effect on the activation of the MAPK pathway (Figure 5C). These results, taken together, illustrate that c-Raf promotes tumor progression by mechanisms that do not involve the MAPK pathway.

**c-Raf expression is essential for proliferation of patient-derived xenograft tumors**

To determine whether our results could be translated to a human scenario, we established primary lung tumor cell lines from two patient-derived xenograft (PDX) tumor models. Both PDX derived cell lines (PDX-dc) express the oncogenic K-RASG12C mutation. In order to evaluate the role of c-Raf expression in proliferation, cells were infected with lentiviruses expressing either a shRNA against c-Raf or a scrambled shRNA as control. Endogenous c-Raf expression was significantly down-regulated 2 weeks after the infection with the shRNA against c-Raf but not affected by the control shRNA (Figure S6). Infection of both PDX derived cell lines with lentiviral particles expressing the shRNA against c-Raf resulted in complete (PDX-dc1) and partial (PDX-dc2) inhibition of cell growth, respectively (Figure 6A and D). Next, we injected infected PDX-dc1and PDX-dc2 cells into the lung parenchyma of immune-compromised mice. As illustrated in Figure 6B, PDX-dc1 cells expressing the c-Raf shRNA formed significantly fewer tumors than those elicited by cells containing the scrambled shRNA. Moreover, these tumors were of significantly smaller size (Figure 6C). PDX-dc2 cells expressing the c-Raf shRNA also displayed growth inhibition, albeit not as dramatic as that observed with PDX-dc1 cells (Figure 6D). These cells induced a slight decrease in tumor number and volume than control cells expressing the scrambled shRNA (Figure 6E and F).

To evaluate if the dramatic effect on growth inhibition observed in PDX-dc1 cells was specifically due to c-Raf down-regulation, we infected these cells with lentiviruses expressing either a murine c-Raf cDNA or an empty vector. Once cells resumed proliferation, they were infected with lentiviruses expressing either a shRNA against human c-Raf sequences to deplete endogenous c-Raf expression or with a scrambled shRNA as a negative control. All cells infected with the scrambled shRNA formed similar numbers of colonies regardless of the presence of the murine c-Raf sequences since they retained endogenous c-Raf expression (Figure 6G). In contrast, those PDX-dc1 cells devoid of endogenous c-Raf expression only formed significant number of colonies in the presence of the murine c-Raf sequences (Figure 6G). Taken together, these results indicate that c-Raf expression is also essential for growth of, at least some human lung adenocarcinomas.

**DISCUSSION**

In spite of intense research efforts, K-RAS mutant tumors remain intractable for targeted therapies. Pharmaceutical companies have developed selective inhibitors against most, if not all, druggable K-RAS effectors. Yet, none of them have been approved by the FDA. Genetic interrogation of the potential therapeutic value of K-Ras effectors within the MAPK signaling pathway has revealed three classes of targets (Blasco et al., 2011; Karreth et al., 2011; Puyol et al., 2010). Those that have no effect in preventing the development of K-Ras driven lung adenocarcinoma (A-Raf, B-Raf, Cdk2 and Cdk6), those that prevent tumor development but cause unacceptable toxicities if eliminated systemically (Mek1/2, Erk1/2, and Cdk1) and those that prevented tumor development (c-Raf and Cdk4) when ablated concomitantly with K-Ras oncogene expression and do not induce significant toxicities when targeted systemically (Blasco et al., 2011; Puyol et al., 2010). These observations, taken together, suggest that genetic studies may serve to guide more rational approaches to the development of targeted therapies against K-RAS driven tumors.

To this end, we have now interrogated the therapeutic effect of ablating c-Raf expression in pre-existing, advanced lung adenocarcinomas driven by K-*Ras*/*Trp53* mutations. As illustrated here, c-Raf ablation provides significant therapeutic benefit, leading to partial regression of most tumors, including some cases in which the tumor completely disappeared. As a consequence, tumor-bearing mice survived significantly longer than control animals that retain c-Raf expression. Indeed, postmortem analysis of these mice revealed that they did not die of tumor burden, suggesting the existence of subtle long-term toxicities caused by the combined effect of widespread loss of c-Raf expression, the known deleterious effects of prolonged TMX exposure and the old age of the treated animals. Yet, we did not observe significant weight loss or obvious toxic effects upon detailed examination of various tissues. Whereas these observations need to be taken into consideration when targeting c-Raf in the clinic, they are of limited concern since therapeutic strategies in a clinical setting are unlikely to result in irreversible loss of c-Raf expression.

More importantly, our results are in sharp contrast with the immediate dead caused by the systemic elimination of the Mek1/2 or Erk1/2 kinases (Blasco et al., 2011). Interestingly, pharmacological inhibition of K-Ras driven tumors in GEM models by MEK inhibitors is reasonably well tolerated and results in significant therapeutic response, highlighting the substantial differences between genetic and pharmacological inhibition of therapeutic targets (Samatar and Poulikakos, 2014). Unfortunately, MEK inhibitors have failed in the clinic, at least for the treatment of K-RAS mutant tumors, indicating that humans are more sensitive to these compounds than rodents. Thus, effective inhibition of K-RAS oncogenic signaling by blocking the MEK/ERK kinases in the clinic will depend on the existence of a narrow window between their anti-tumor effect and tolerable toxicities, a scenario not too different from that observed with classical cytotoxic compounds. In contrast, our data suggest that selective tampering with c-Raf will be better tolerated in the clinic. Moreover, we have not observed any tumor relapse after long term exposure to TMX, suggesting the absence of relevant resistance mechanisms that could bypass the therapeutic benefit provided by the absence of c-Raf expression. Yet, c-Raf ablation, by itself is not sufficient to induce complete regression of most tumors, hence suggesting that pharmacological targeting of c-Raf may not be sufficient to provide optimal therapeutic benefit to lung cancer patients and may have to be combined with other targeted therapies.

The differential toxic effects caused by ablation of c-Raf versus Mek1/2 or Erk1/2 may be due to the differential mechanisms by which they induce tumor regression. Indeed, our results suggest that the therapeutic effect observed upon c-Raf ablation is mediated by pathway(s) other than the Mek/Erk signaling cascade. Thus, the observed therapeutic activity is likely to result from the inability of c-Raf protein to interact with other partners. There is abundant evidence that c-Raf inhibits apoptosis in a kinase independent manner. At least three partners have been implicated in this activity, including the mitochondrial Bcl2 protein and the pro-apoptotic kinases Ask1 and Mst2 (Matallanas et al., 2011). In agreement with these observations, ablation of c-Raf expression in both tumor tissue and cultured tumor cells readily induces activation of Caspase3, a direct effector of apoptosis. Other c-Raf kinase independent targets such as Rok have been implicated in cell differentiation. Inactivation of Rok has been shown to play a role on the pro-tumoral effect of c-Raf in SOS-induced skin tumors (Ehrenreiter et al., 2009). Our observations that c-Raf ablation activates Rok as determined by the increased levels of phospho-Cofilin, also suggests that this kinase might be a key effector in c-Raf-mediated K-*Ras*G12V/*Trp53* driven tumor progression.

We were surprised by the differential therapeutic responses that c-*Raf* targeting induced across the battery of K-*Ras*G12V/*Trp53* mutant tumors, considering the minimal differences among the genetic background of the mice and the identical nature of the driver mutations. Some of this variability was due to the differential extent of Cre-induced recombination of the conditional c-*Raf*L alleles. Indeed, incomplete recombination was responsible for some, albeit not all, progressing tumors as well as for the remaining tumor cells in some of the regressing tumors. Several tumors, however, underwent elimination of c-Raf expression in most of their cells. Yet, most of these tumors retained significant levels of phospho-Mek and/or phopho-Erk expression. Only one tumor was completely devoid of both phospho-Mek and phospho-Erk. Similar results were observed with the tumor cell lines where c-Raf ablation did no effect MAPK activity. These results, taken together, illustrate that ablation of c-Raf in K-Ras mutant tumors can be uncoupled form MAPK signaling.

In summary, these studies demonstrate that c-Raf is a key mediator of K-*Ras*G12V/*Trp53* driven lung tumor progression by kinase-independent mechanism(s) and hence represents a suitable therapeutic target that, so far, has received little attention from the pharmaceutical industry. Targeting c-RAF in the clinic could be accomplished by inhibiting its kinase activity. However, this strategy would require certain levels of selectivity since concomitant inhibition of the B-RAF kinase will increase undesired toxicity. Moreover, complete elimination of the three Raf kinases in mice causes their rapid death (our unpublished observations), a result that suggests that additional tampering with the A-RAF kinase will further increase undesirable toxic effects. Thus, the use of degron chemistry (Raina and Crews, 2010) will provide better opportunities for selectivity. As illustrated here, c-RAF inhibition is unlike to result in complete tumor regression in the clinical setting, suggesting the need of combined therapies. Yet, targeting c-RAF will offer significant advantages over targeting other members of the MAPK pathway such as the MEK and ERK kinases due to the significantly milder toxic consequences and, possibly, the absence of resistance mechanisms.

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**AUTHOR CONTRIBUTIONS**

M.M. and M.B. designed experiments and research aims, analyzed data and wrote the manuscript with help from co-authors. M.S., S.F., L.E.-B., E.B.-M., and M.M. performed experiments and analyzed the data. M.Dj. performed experiments with hematopoietic cells. P.P.L.-C. and M.H. provided human samples. M.Dr. generated the K-RasFSFG12V allele. M.Dr., C.G., P.P.L.-C. contributed critical information and helpful discussions.

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**FIGURE LEGENDS**

**Figure 1. c-Raf ablation reduces tumor burden in K-RasG12V driven lung tumors**

(**A**) Western blot analysis of c-Raf and B-Raf expression in lysates derived from five independent tumors obtained from K-*Ras*+/FSFG12V;*RERT*ert/ert, K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L and K-*Ras*+/FSFG12V;*RERT*ert/ert;B-*Raf*L/L mice exposed for 4 months to a tamoxifen (TMX) diet. Gapdh was used as loading control. Migration of the above proteins is indicated by arrowheads.

(**B**) Computed tomography (CT) scan axial projection images and 3D reconstructions of lungs of representative K-*Ras*+/FSFG12V;*RERT*ert/ert mice carrying the indicated c-*Raf* and B-*Raf* alleles before and after 4 months of TMX exposure. Tumors are indicated by arrowheads. H: heart.

(**C**) Quantification of average tumor volume variation (indicated as percent change) during the TMX exposure for the indicated time. Tumors were measured by CT scans. K-*Ras*+/FSFG12V;*RERT*ert/ert (n=25) (open circles); K-*Ras*+/FSFG12V;*RERT*ert/ert;B-*Raf*L/L (n=16) (grey circles); K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L (n=22) (solid circles) and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L (n=8) (grey triangles) mice. Error bars indicate mean ± SEM.

**Figure 2. c-Raf ablation decreases glucose uptake in K-RasG12V driven lung tumors**

(**A**) Positron emission tomography (PET)/CT scan axial projections images and 3D reconstructions to assess metabolic activity ([18F]-FDG uptake) of representative lungs from K-*Ras*+/FSFG12V;*RERT*ert/ert, K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L mice before and after 2 months of TMX exposure. Tumors are indicated by arrowheads. H, heart. Color-scale bar represents Standardized Uptake Values (SUV) of PET.

(**B**) Quantification, indicated as percent change, of the number of tumors positive for [18F]-FDG uptake per mouse in K-*Ras*+/FSFG12V;*RERT*ert/ert (n=14) (open bar), K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L (n=19) (solid bar) and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L (n=17) (grey bar) mice after 2 months of TMX exposure.

(**C**) Quantification, indicated as percent change, of the SUV levels of [18F]-FDG uptake per tumor, measured by PET scan, in K-*Ras*+/FSFG12V;*RERT*ert/ert (n=14 mice/30 tumors) (open circles), K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L (n=19 mice/46 tumors) (solid circles) and K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L;B-*Raf*L/L (n=17 mice/37 tumors) (grey circles) animals after 2 months of TMX exposure.

**Figure 3. c-Raf is an effective therapeutic target for advanced K-RasG12V/Trp53 driven tumors**

(**A**) (Left) Waterfall plot representing the percent change in tumor volume of individual tumors from K-*Ras*+/FSFG12V;*RERT*ert/ert mice (n=25 mice/35 tumors) exposed to TMX for 4 months. (Right) Waterfall plot representing the percent change in tumor volume of individual tumors present in K-*Ras*+/FSFG12V;*RERT*ert/ert;c-*Raf*L/L mice (n=22 mice/42 tumors) exposed to TMX for 4 months. The percent change in tumor volume was calculated by CT measurements performed for each individual tumor at the beginning and end of the 4 months long trial.

(**B**) Waterfall plot representing the change in tumor volume of individual CT+ tumors present in K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*+/+ mice (n=20 mice/54 tumors) exposed to TMX for 2 months. (Right) Waterfall plot representing the change in tumor volume of individual CT+ tumors present in K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L mice (n=27 mice/94 tumors) exposed to TMX for 2 months. The percent change in tumor volumes were calculated for each individual tumor based on CT scans performed at the beginning and at the end of the 2 months long trial.

(**C**) Table resuming the numbers of regressing and de novo detected tumors in the upper mentioned mice after exposure to TMX.

**Figure 4. MAPK and PI3K signaling in K-RasG12V/Trp53 driven tumors after c-*Raf* ablation**

(**A**) Western blot analysis of K-Ras, H/N-Ras, B-Raf, c-Raf, phospho-Mek, Mek1, phospho-Erk1/2, Erk1/2, phospho-Akt and Akt, expression in lysates derived from individual tumors (C1 to C4) of control K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*+/+ mice and of K-*Ras*+/FSFG12V;*Trp53*F/F; h*UBC-CreERT2*+/T;c-*Raf*L/L mice exposed to TMX for 2 months. Progressor (P1-P4) and regressor (R1-R8) tumors analyzed are indicated. Gapdh was used as loading control. Migration of the above proteins is indicated by arrowheads.

(**B**) Waterfall plot representing the change in tumor volume of individual tumors of K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L mice previously shown in Figure 3. Tumors used for Western blot analysis are indicated in red. Tumors used for phospho-Erk immunohistochemical (IHC) analysis are indicated in blue. The scale used to represent tumor reduction is magnified 2 fold for better visualization.

(**C**) Phospho-Erk staining of representative sections obtained from the indicated tumors obtained from K-*Ras*+/FSFG12V;*Trp53*F/F; h*UBC-CreERT2*+/T;c-*Raf*L/L mice treated with TMX for 2 months. Scale bars, 1 mm (R9 to R11 tumors) and 0.2 mm (P5, R12 to R15 tumors).

(**D**) Phospho-Erk IHC staining of representative tumor sections from tumors (C5 to C10) obtained from K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*+/+ mice exposed to TMX for 2 months. IHC of the sections depicted in (c) and (d) was carried out in parallel. Scale bar, 2 mm.

**Figure 5. c-Raf ablation induces tumor differentiation and apoptosis through MAPK independent mechanisms**

(**A**) (Left) phospho-Cofilin staining and (Right) quantification of phospho-Cofilin+ cells in representative sections of paraffin embedded tumors ofK-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*+/+ (+/+,open bar) and K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L (L/L,solid bar) mice (n=6 per genotype) exposed to TMX for 2 months. Scale bar, 0.05 mm.

(**B**) (Left) cleaved Caspase3 staining and (Right) quantification of cleaved Caspase3+ cells in representative sections of paraffin embedded tumors ofK-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*+/+ (+/+,open bar) and K-*Ras*+/FSFG12V;*Trp53*F/F;h*UBC-CreERT2*+/T;c-*Raf*L/L (L/L,solid bar) mice (n=6 per genotype) exposed to TMX for 2 months. Scale bar, 0.05 mm.

(**C**) Western blot analysis of B-Raf, c-Raf, Caspase3, cleaved Caspase3, phospho-Mek, Mek1, phospho-Erk and Erk expression in lysates from K-*Ras*G12V;*Trp53*–/–;h*UBC-CreERT2*+/T;c-*Raf*L/L cells maintained in 4OHT containing media. Samples were harvested at the indicated times. Vinculin was used as loading control. Migration of the above proteins is indicated by arrowheads. 3 independent tumor cell lines from 3 different animals were evaluated.

Error bars indicate mean ± SEM. P values were calculated using the unpaired Student´s T test. \*\*\*P < 0.001.

**Figure 6. c-Raf expression is essential for proliferation of patient-derived xenograft tumors**

(**A**) Cell proliferation assay of a PDX-derived cell line (PDX-dc1 harboring K-RASG12C mutation) expressing scrambled shRNA (Scramb.) (solid circles) andshRNA against c-Raf (c-Raf) (open circles).

(**B**) Quantification of tumor number per mouse after orthotopic implantation of PDX-dc1 expressing scrambled shRNA (solid bar) andshRNA against c-Raf (open bar) in nude mice. Animals were analyzed at 5 weeks after the implantation. n=10 mice/condition.

(**C**) Quantification of tumor volume after orthotopic implantation of PDX-dc1 expressing scrambled shRNA (solid bar) andshRNA against c-Raf (open bar) in nude mice. Animals were analyzed at 5 weeks after the implantation. n=10 mice/condition.

(**D**) Cell proliferation assay of a PDX-derived cell line (PDX-dc2 harboring K-RASG12C mutation) expressing scrambled shRNA (Scramb.) (solid circles) andshRNA against c-Raf (c-Raf) (open circles).

(**E**) Quantification of tumor number per mouse after orthotopic implantation of PDX-dc2 expressing scrambled shRNA (solid bar) andshRNA against c-Raf (open bar) in nude mice. Animals were analyzed at 3 weeks after the implantation. n=7 mice/condition.

(**F**) Quantification of tumor volume after orthotopic implantation of PDX-dc1 expressing scrambled shRNA (solid bar) andshRNA against c-Raf (open bar) in nude mice. Animals were analyzed at 3 weeks after the implantation. n=7 mice/condition.

(**G**) (Left) Quantification of colonies forming in PDX-dc1 cells, PDX-dc1 cells expressing lentiviral pLVX control particles (Empty Vector) or expressing the wild type murine c-Raf (Murine c-Raf ) (as indicated). All cells indicated are expressing a scrambled shRNA. (Right) Quantification of colonies forming in PDX-dc1 cells, PDX-dc1 cells expressing lentiviral pLVX control particles (Empty Vector) or expressing the wild type murine c-Raf (Murine c-Raf ) (as indicated). All cells indicated are expressing a shRNA against human c-Raf.

Error bars indicate mean ± SEM. P values were calculated using the unpaired Student´s T test. \*\*P < 0.01 or \*\*\*P < 0.001. n.s.: not significant

## EXPERIMENTAL PROCEDURES

## Mice

The generation ofK-Ras+/FSFG12V strain is described below. *Trp53*F/F (Lee et al., 2012), *RERT*ert/ert (Guerra et al., 2003), h*UBC-CreERT2*+/T (Ruzankina et al., 2007), B*-Raf*L/L (Chen et al., 2006) and c*-Raf*L/L (Jesenberger et al., 2001) have been described. All animal procedures were evaluated and approved by the Ethical Committees of CNIO and the Carlos III Health Institute, Madrid, Spain and conducted in accordance to the recommendations of the Federation of European Laboratory Animal Science Associations.

**Generation of K-Ras+/FSFG12V mice**

The targeting vector was generated by Taconic Artemis (Cologne, Germany). Briefly, the homology arms including the first exon that contains the oncogenic G12V mutation (GGT [Gly] to GTA [Val]) were amplified by PCR using as a template a targeting vector previously developed to generate the K*-Ras*LSLG12Vgeo allele(Guerra et al., 2003). A PGK-Neo-STOP cassette was generated by PCR amplification of the 1377 bp STOP cassette derived from theK*-Ras*LSLG12Vgeo targeting vector with primers that incorporated NdeI restriction sites at the termini of the PCR product. The STOP cassette was subsequently cloned into the NdeI restriction site of pBASIC10 (Taconic Artemis) which contained a PGK-Neo cassette followed by an NdeI restriction site flanked by frt sequences. The left homology arm was cloned as a 3019 bp NotI/BstEII fragment where an internal BstEII site was previously disabled by site-directed mutagenesis. The 1867 bp right homology arm including the mutated exon 1 was PCR-amplified from theK*-Ras*LSLG12Vgeo targeting vector with primers that incorporated AscI and PmeI restriction sites to the termini of the PCR product. This fragment was finally cloned into the corresponding AscI/PmeI restriction sites to assemble the K-*Ras*FSFG12V targeting vector. All sequences amplified by PCR were verified by DNA sequencing. The resulting targeting vector was linearized with NotI and electroporated into Art4.12 (B6129S6F1) ES cells. 288 G418-resistant clones were submitted to Southern blot analysis to identify those clones that had undergone the expected recombination event (4 positive clones; recombination frequency 1%). Two independent recombinant ES cell clones were microinjected into C57BL/6J blastocysts and transplanted into pseudo-pregnant females. Chimeric mice were backcrossed to C57BL/6J mice and germ line transmission of the targeted allele was confirmed by Southern blot analysis. The resulting K*-Ras*+/FSFG12V mice were maintained in a mixed 129Sv/J x C57BL/6J background. For PCR genotyping the following primers were used: (i) forward wild type primer (5’- CCACAGGGTATAGCGTACTATGCAG-3’), (ii) forward mutant primer (5’-TAGTGCCTTGACTAGAGATCA-3’) and (iii) reverse primer (5’-CTCAGTCATTTTCAGCAGGC-3’) yielding a 358 bp DNA fragment for the wild-type allele and 507 bp DNA fragment for the targeted K*-Ras*FSFG12V allele. K*-Ras*+/FSFG12V mice are available at The Jackson Laboratory (B6.129-*Krastm3Bbd*/J, Stock No: 027010).

## Tumor induction and tamoxifen exposure

Induction of lung adenocarcinomas was carried out in anesthetized (i.p. injection of: ketamine 75 mg/kg, xylazine 12 mg/kg) 8 to 10-week old mice by intratracheal instillation of a single dose of 106 pfu of Ad-Flp virus. Activation of the inducible CreERT2 recombinase encoded by the h*UBC-CreERT2* transgene was carried out by feeding the mice with a TMX-containing diet (Teklad CRD TAM400 diet, Harlan) *ad libitum* during the duration of the experiment.

## Micro CT and PET-CT imaging

Lung images were acquired using an eXplore Vista PET-CT (GE Healthcare). MicroCT images consisted of 400 projections collected in one full rotation of the gantry in approximately 10 min. CT images were reconstructed using filtered back projection with a Shepp–Logan filter and PET images with 3D OSEM reconstruction algorithm. For PET imaging, mice were injected with 15MBq of 18F-FDG into the lateral tail vein in a volume of 0.1 cc. 60 min after radiotracer injection. MicroPET scans were performed for 15 min per bed position. PET images were reconstructed using 3D OSEM reconstruction algorithm. For PET quantification, regions of interest were drawn over tumors using MMWS/Vista software (GEHealthcare). The standardized uptake value (SUV) was calculated using the following formula: SUV = Tissue radioactivity concentration (MBq/cm3) / (injected dose (MBq) / body weight (g)).

## Histopathology and immunohistochemistry

For routine histological study, lung lobes were fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. Tumors were counted and classified according to standard histopathological grading discriminating between benign (adenoma) and malignant (adenocarcinoma) tumors. Tumor grading (stage II to V) was determined as previously described (Jackson et al., 2005). Antibodies used for immunostaining included those raised against: Ki67 (Master Diagnostica, 0003110QD), phospho-Erk (Cell Signaling, 9101), cleaved Caspase3 (Cell Signaling, 9661), phospho-Cofilin (Santa Cruz, sc-21867-R), CD3ε (Santa Cruz, sc-1127), F4/80 (ABD Serotec, MCA497). H&E and immunostained tissue slides were scanned using the Mirax scanner (Zeiss) and photos were exported using the Panoramic Viewer 1.15.3 software (3dhistech). Immunostainings were quantified using the AxioVision 4.6 software (Zeiss).

## Southern blot analysis

Genomic DNA was isolated using standard procedures and the following protocol was used for the analysis of the alleles. For c-*Raf*lox DNA was digested with PstI and probed with an 854bp DNA fragment corresponding to sequences located in intron 4 downstream from the second loxP site resulting in diagnostic bands of 3.1kbp for the c-*Raf*lox and 3.5 kbpfor the c-*Raf*– alleles respectively.

## Western blot analysis

25 μg of protein extracts obtained from tumor tissue were separated on SDS/PAGE gels (Bio-Rad), transferred to a nitrocellulose membrane and blotted with antibodies raised against B-Raf (Santa Cruz, sc-5284), c-Raf (BD Biosciences, 610151), A-Raf (Abcam, ab19880), Mek1 (Santa Cruz, sc-219), phospho-Mek (Cell Signaling, 9154), Erk-1 (BD Pharmingen, 554100), Erk-2 (BD Biosciences, 610103), phospho-Erk (Cell Signaling, 9101), Akt (Cell Signaling, 9272), phospho-Akt (Cell Signaling, 4060), Cyclin D1 (Thermo Scientific, MS-210-P1), panRas (Calbiochem, OP40), cleaved Caspase3 (Cell Signaling, 9661), phospho-Cofilin (Santa Cruz, sc-21867-R), CD3ε (Santa Cruz, sc-1127), CD8α (Santa Cruz, sc-7188), Gapdh (Sigma, G8795) and Vinculin (Sigma, V9131). Primary antibodies were detected with goat secondary antibodies directed against mouse or rabbit IgGs (HRP, Dako and Alexa Fluor 680, Invitrogen) and visualized with ECL Western Blot detection solution (GE Healthcare) or Odyssey infrared imaging system (*LI-COR* Biosciences).

**Cell proliferation and time course assays in murine cell lines**

To evaluate the effect of c-Raf ablation on apoptosis K-*Ras*G12V;*Trp53*–/–;h*UBC-CreERT2*+/T;c-*Raf*L/L tumor cells were maintained in DMEM medium containing 10%FBS and 600nM (4-hydroxy-tamoxifen) 4OHT (Sigma, H7904) for 4 days. Cells were harvested at 0, 8, 24, 48, 72 and 96 hours post exposure to 4OHT and cell lysates were analyzed by Western Blot.

**Cell proliferation assay and orthotopic implantation of human lung tumor cells**

To assess the influence of c-Raf in cell proliferation we isolated 2 PDX-derived cell lines and maintain them in DMEM with 10% FBS. These cells were infected with pLKO.1-puro lentiviral supernatants expressing a shRNA against c-Raf and a Scrambled shRNA and selected with blasticidin (4 μg/ml, Sigma) for 48h. Once selection was finished, 30.000 cells were seeded in 6-well plates and counted in triplicates by using Neubauer chambers. Scramble shRNA sequence: CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT and shRNA against cRAF sequence:

CCGGGCTTCCTTATTCTCACATCAACTCGAGTTGATGTGAGAATAAGGAAGCTTTTT. For the in vivo orthotopic tumor model 100.000 cells in 10 μl (1:1) PBS:Matrigel Matrix (Corning, 354234) infected as above were injected directly into the lungs of 6 week old nude (Foxn1nu/nu) female mice (Harlan). Each PDX-dc-injected group of mice was sacrificed at the same time. PDX-dc1 injected mice were sacrificed at 5 weeks and PDX-dc2-injected ones at 3 weeks. Tumors were counted and caliper-measured post-mortem in each case.

**Colony formation assay**

To evaluate potential effect of c-Raf in rescuing cell proliferation, PDX-dc1-derived cells were infected with pLVX-puro lentiviral supernatants expressing wild type c-Raf or an empty vector and selected by addition of puromycin (2 μg/ml, Sigma) for 48h. Then, these cells were coinfected with pLKO.1-blast lentiviral supernatants expressing a shRNA against c-Raf and a Scrambled shRNA as control and selected by addition of blasticidin (4 μg/ml, Sigma) for 72h. Once selection was finished, 10.000 cells were seeded in 10 cm diameter dishes and stained after 2 weeks by using 0.2% Crystal Violet. Colonies were counted and quantified.

## Statistical Analysis

All values are expressed as mean ±SEM. p values were calculated with the unpaired Student’s t test using the GraphPad Prism software. A P value ≤ 0.05 is considered statistically significant. Significant differences between experimental groups were: \*P < 0.05, \*\*P < 0.01 or \*\*\*P < 0.001.