RHOA-FAK Is a Required Signaling Axis for the Maintenance of KRAS-Driven Lung Adenocarcinomas

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Non-small cell lung cancer (NSCLC) often expresses mutant KRAS together with tumor-associated mutations of the CDKN2A locus, which are associated with aggressive, therapy-resistant tumors. Here, we unravel specific requirements for the maintenance of NSCLC that carries this genotype. We establish that the extracellular signal-regulated kinase (ERK)/RHOA/focal adhesion kinase (FAK) network is deregulated in high-grade lung tumors. Suppression of RHOA or FAK induces cell death selectively in mutant KRAS/INK4A/ARF-deficient lung cancer cells. Furthermore, pharmacologic inhibition of FAK caused tumor regression specifically in the high-grade lung cancer that developed in mutant Kras;Cdkn2a-null mice. These findings provide a rationale for the rapid implementation of genotype-specific targeted therapies using FAK inhibitors in patients with cancer.

**SIGNIFICANCE:** Targeted therapies are effective for only a small fraction of patients with cancer. We report that FAK inhibitors exert potent antitumor effects in NSCLCs that express mutant KRAS in association with INK4A/ARF deficiency. These results reveal a novel genotype-specific vulnerability of cancer cells that can be exploited for therapeutic purposes.

**INTRODUCTION**

Activating mutations of the proto-oncogene KRAS (mutant KRAS) promote tumorigenesis in several common human cancers such as non–small cell lung cancer (NSCLC; ref. 1). Mutant KRAS exerts its oncogenic activity through the regulation of several signaling networks. Among these, the most extensively characterized are the RAF/MAP-ERK kinase (MEK)/extracellular signal-related kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathways (1, 2).

In NSCLC, KRAS mutations occur frequently in combination with inactivating mutations or epigenetic silencing of the CDKN2A locus, which encodes 2 distinct but overlapping tumor suppressors: p19/ARF (p14 in humans, ARF hereafter) and p16/INK4a (INK4a hereafter). Both p19/ARF and p16/INK4a restrain inappropriate cellular proliferation induced by mutant KRAS by positively regulating p53 and retinoblastoma (Rb) tumor suppressors, respectively (3). Indeed, mutant KRAS in association with CDKN2A deficiency results in high-grade lung and pancreatic cancer in mouse models and has been associated with the development of aggressive NSCLC in humans (4–7).

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**ABSTRACT**

Deficiency of Cdkn2a Leads to Aberrant Activation of RHOA in Kras<sup>G12D</sup>-Induced NSCLC In Vivo

To identify the cellular networks required for the maintenance of high-grade lung cancer, we crossed tetracycline operator-regulated Kras<sup>G12D</sup> (tet-O-Kras<sup>G12D</sup>) mice with Clara cell

The genotype of cancer cells not only determines their phenotype, but also defines specific vulnerabilities that can be exploited in cancer therapy. Certain cancers are critically dependent on a single oncogenic activity, a phenomenon defined as oncogene addiction (8). For instance, continuous expression of mutant KRAS is required for the survival of NSCLC in both mouse cancer models and in human-derived cells (5, 9). However, attempts to develop direct inhibitors of mutant KRAS have been unsuccessful (10). Therefore, mutant KRAS is still a high-priority therapeutic target.

There has been a tremendous interest in identifying molecular targets that are required for the maintenance of mutant KRAS-dependent cancers (11–13). Pharmacologic inhibitors of MEK1/2, PI3K and/or mTORC1/2 led to promising antitumor effects in preclinical lung cancer models (14, 15). In addition, several compounds targeting RAF/MEK/ERK and PI3K/AKT/mTOR signaling pathways are currently under clinical investigation and hold promise for the treatment of RAS-mutant tumors (16). On the other hand, it is still unknown whether PI3K and MEK1/2 inhibitors are effective therapies in lung cancer. Thus, it is of interest to develop alternative therapeutic strategies that target mutant KRAS tumors.

The goal of this work was the identification of the vulnerabilities of mutant KRAS that can be harnessed for cancer therapy. For this purpose, we dissected the signaling pathways downstream of mutant KRAS in NSCLC developed in a genetically defined mouse model and in cellular systems. With this analysis, we determined that the RHOA-focal adhesion kinase (FAK) signaling axis is a critical vulnerability for high-grade lung tumors.

**RESULTS**

**SIGNIFICANCE:** Targeted therapies are effective for only a small fraction of patients with cancer. We report that FAK inhibitors exert potent antitumor effects in NSCLCs that express mutant KRAS in association with INK4A/ARF deficiency. These results reveal a novel genotype-specific vulnerability of cancer cells that can be exploited for therapeutic purposes.
secretory protein-reverse tetracycline transactivator (CCSP-rtTA) mice (5) in a C57Bl/6n-null background (Ink4a/Arf−/−) (17). These mice express KrasG12D in the respiratory epithelium when exposed to doxycycline.

In agreement with previous findings (5), the induction of KrasG12D combined with Ink4a/Arf deficiency resulted in increased tumor burden as shown by histologic examination and tumor volume quantification of the lungs among KrasG12D;Ink4a/Arf−/+;KrasG12D;Ink4a/Arf−/+ and KrasG12D;Ink4a/Arf−/− mice (Supplementary Fig. S1A and S1B).

After 12 weeks of doxycycline exposure, about 50% of the lungs of KrasG12D;Ink4a/Arf−/− mice were occupied by adenocarcinomas (high-grade tumors) consisting of cancer cells with atypical nuclei and cytoplasmic blebbing arranged in papillary structures. We did not detect any adenocarcinomas in the KrasG12D;Ink4a/Arf−/+ lungs, which contained only well-differentiated adenomas with some areas occupied by cells with atypical nuclei (Fig. 1A and B). Furthermore, the KrasG12D;Ink4a/Arf−/− tumors exhibited increased cell proliferation, as shown by the percentage of Ki-67–positive cells (Supplementary Fig. S1C and S1D) as well as upregulation of cyclin D1, a regulator of G1–S phase transition (Supplementary Fig. S1E). Accordingly, mice lacking Ink4a/Arf displayed a remarkable reduction in median survival compared with KrasG12D;Ink4a/Arf−/+ and KrasG12D;Ink4a/Arf−/− mice (Supplementary Fig. S1F).

To gain insights into the cellular networks that regulate high-grade lung cancer, we assessed the activation status of the main KrasG12D-regulated signaling pathways in microdissected tumors. We determined that Erk1/2 phosphorylation (p-Erk1/2) decreased in KrasG12D;Ink4a/Arf−/− lung tumors after 8 and 12 weeks of KrasG12D induction, whereas it was sustained in KrasG12D;Ink4a/Arf−/+ lung adenocarcinomas (Fig. 1C). Moreover, immunohistochemistry revealed that Erk1/2 staining was intense throughout the adenocarcinomas of KrasG12D;Ink4a/Arf−/− mice (Fig. 1D, red arrowhead) compared with adjacent adenomas (Fig. 1D, black arrowhead) and to KrasG12D;Ink4a/Arf−/+ tumors (Fig. 1D, bottom). Interestingly, other investigators reported that p-Erk1/2 is also deregulated in KrasG12D-induced high-grade lung tumors in Trp53-null mice (18, 19). However, the functional significance of this event is still unknown.

The RHO family of small GTPases (GTPs) (which comprises RAC, CDC42, and RHO) has been implicated in mutant KRAS-induced tumorigenesis. These proteins regulate the cytoskeleton, cell migration, proliferation, and survival (20). RAC1 is required for mutant KRAS-induced transformation in fibroblasts in vitro and for initiating tumorigenesis in a mouse model of lung cancer (21, 22). In addition, ERK1/2 and RHOA regulate common pathways such as cell migration and chemotaxis (23). Indeed, RHOA is also required for mutant KRAS-induced transformation in vitro (24). Finally, deregulation of RHOA occurs in a variety of cancer types (25, 26). Therefore, we interrogated the functional status of Rac1 and RhoA during induction of KrasG12D.

With glutathione S-transferase (GST) pull-down experiments, we found that the active form of Rac1 (Rac1-GTP) declined over time in KrasG12D;Ink4a/Arf−/− lung adenocarcinomas compared with KrasG12D;Ink4a/Arf−/+ tumors (Fig. 1E and Supplementary Fig. S2A). In contrast, as in the case of p-Erk1/2, the active form of RhoA (RhoA-GTP) was elevated in KrasG12D;Ink4a/Arf−/− adenocarcinomas compared with KrasG12D;Ink4a/Arf−/+ adenomas at 12 weeks after KrasG12D induction (Fig. 1E and Supplementary Fig. S2B). We did not detect differences in Ras activity (Ras-GTP) between KrasG12D;Ink4a/Arf−/− adenomas and KrasG12D;Ink4a/Arf−/+ adenocarcinomas (Fig. 1E). This finding suggests that changes in total amount of Ras-GTP are not responsible for p-Erk1/2 deregulation.

In concordance with the GST pull-down experiments, immunohistochemical (IHC) stainings confirmed that RhoA-GTP was present solely in high-grade lung tumors of KrasG12D;Ink4a/Arf−/− mice (Fig. 1F and Supplementary Fig. S2C–S2E). Furthermore, we noted no differences in Rac1-GTP or RHOA-GTP in the respiratory epithelium of Ink4a/Arf−/+ or Ink4a/Arf−/− mice in the absence of KrasG12D (Supplementary Fig. S2F).

Our observation that Rac1 and RhoA activation are mutually exclusive in vivo confirms long-standing observations obtained in tissue culture systems (27, 28) and suggests that antagonistic regulation of Rac1-RhoA signaling is of biological significance. Consistent with these results, p27/Kip1, a cyclin-dependent kinase inhibitor whose degradation is promoted by RhoA-GTP (29, 30), was undetectable in high-grade KrasG12D;Ink4a/Arf−/− tumors (Supplementary Fig. S2G).

MEK1/2–ERK1/2 Drive RHOA Activation in INK4a/ARF-Deficient Lung Cancer In Vitro and In Vivo

Our findings suggest that Erk1/2 is a positive regulator of RhoA in KrasG12D;Ink4a/Arf−/−–induced adenocarcinomas. To test this hypothesis, we conducted gain-of-function and loss-of-function experiments. Quantitative RHOA activity assays (G-LISA assays) revealed that ablation of KRAS resulted in the downregulation of RHOA-GTP in H460 and A549 human NSCLC cells (mutant KRAS, Ink4a/Arf−/−-deficient; Fig. 2A and Supplementary Fig. S3A), whereas its ectopic expression in H838 NSCLC cells (wild-type KRAS, Ink4a/Arf−/−-deficient) led to the upregulation of RHOA-GTP (Fig. 2B and Supplementary Fig. S3B). Moreover, ectopic expression of constitutively active MEK1 (MEK1Q65L) is the main regulator of ERK1/2 in H292 NSCLC cells (wild-type KRAS, Ink4a/Arf−/−-deficient), upregulated RHOA-GTP (Fig. 2C). Conversely, pharmacologic inhibition of MEK1/2 with UO126 and ARRY-142886 led to the downregulation of RHOA-GTP and the consequent upregulation of RAC1-GTP in H460 and A549 NSCLC cells (Fig. 2D and Supplementary Fig. S3C). In agreement with these findings, treatment of KrasG12D;Ink4a/Arf−/− mice with ARRY-142886 also resulted in the downregulation of RHOA-GTP in mouse lung tumors (Fig. 2E and F).

Next, we determined the mechanisms underlying p-ERK1/2-dependent upregulation of RHOA-GTP. It has been reported that p-ERK1/2 inhibits p190RHO GTPase-activating protein (p190RHOGAP), a specific GAP for RHOA, in fibronectin-stimulated cells in vitro (31). We determined whether this mechanism is functionally relevant in NSCLC induced by mutant KRAS. With pull-down assays, we detected an increased association of endogenous RHOA-GTP with p190RHOGAP after inhibition of MEK1/2 (Fig. 2G). Remarkably, knockdown of p190RHOGAP completely abolished the effect of MEK1/2 inhibition on RHOA (Fig. 2H). These findings indicate that...
Figure 1. Deficiency of Cdkn2a leads to aberrant activation of RhoA in vivo. A, hematoxylin and eosin (H&E) staining of lung tissue sections from the indicated genotype after 12 weeks of KrasG12D induction. Left, low magnification scale bars, 1 mm; right, high magnification scale bar, 40 μm. B, top, tumor number and grade/mouse in KrasG12D;Ink4a/Arf−/− and KrasG12D;Ink4a/Arf+/+ lungs after 12 weeks of KrasG12D induction. The average of 3 representative lung tissue sections/mouse were analyzed (n = 8 genotype). A, lung adenoma; AC, lung adenocarcinoma. Bottom, representative H&E image of a mixed KrasG12D;Ink4a/Arf−/− tumor (exhibiting both low- and high-grade features). Scale bar, 40 μm. C, immunoblot of microdissected lung adenomas of KrasG12D;Ink4a/Arf+/+ mice and adenocarcinomas of KrasG12D;Ink4a/Arf−/− mice obtained after 4, 8, or 12 weeks of KrasG12D induction. Each lane represents a lysate from a single mouse. D, p-Erk1/2 (Thr202/Tyr204) IHC staining of representative lung tumors of the indicated genotype after 12 weeks of exposure to doxycycline. An adjacent H&E–stained section is shown to illustrate areas of adenocarcinomas (AC) and adenomas (A). Inserts indicate area selected for higher magnification shown below. Top, scale bar: 100 μm. Bottom, scale bar, 40 μm. Red and black arrowheads indicate adenocarcinomas and adenomas respectively. E, representative immunoblots showing Ras-GTP levels along with p-Erk-Rac1-RhoA activation levels. From top to bottom: p-Erk1/2 followed by GST-PAK1, GST-RBD, and GST-RAF1 pulldown for Rac1-GTP, RhoA-GTP, and Ras-GTP respectively, from microdissected lung tumors of the indicated genotypes. Each lane represents a lysate from a single mouse. F, top, H&E staining of a lung tumor section to show areas of adenomas (A) and adenocarcinomas (AC) from KrasG12D;Ink4a/Arf−/− mice. Ovals show higher-magnification images of sections stained with H&E. Bottom, RhoA-GTP IHC staining of lung (12 weeks KrasG12D) tumors. Dotted lines demarcate A (adenomas) and AC (adenocarcinomas). Left, lower magnification; scale bars, 100 μm; right, higher magnifications; scale bars, 40 μm. Inserts and arrows indicate areas magnified further on the right panels.
**Figure 2.** MEK1/2-ERK1/2-p190RHOGAP and INK4a/ARF deficiency drive RHOA activation in NSCLC. A and B, G-LISA assay showing RHOA-GTP levels in H460, A549, and H838 NSCLC cells transduced as indicated. O.D., optical density. Mean and SEM. *, P < 0.05; **, P < 0.004; n = 3. Wt, wild type. C, G-LISA assay showing RHOA-GTP levels (top) and immunoblot (bottom) in H292 cells transduced with the indicated retroviral vectors. Mean and SEM. **, P < 0.003; n = 3. D, immunoblot of H460 and A549 NSCLC cells treated with the indicated MEK1/2 inhibitors for 24 h. UO, UO126; ARRY, ARRY-142886. RHOA-GTP and RAC1-GTP were determined by GST-RBD and GST-PAK1 pulldown respectively. E, G-LISA assay showing RhoA-GTP levels of lung tumor lysates from KrasG12D;Ink4a/Arf−/− mice (after 12 weeks of KrasG12D induction) treated with ARRY-142886. The inhibitor was given 3 times (every 12 hours) and the mice were sacrificed 2 hours after the last treatment. Mean and SEM. ***, P < 0.001; n = 3. F, immunoblot showing p-ERK1/2 inhibition upon treatment with ARRY-142886. Each lane represents a lysate from a single mouse.
p-ERK1/2 blocks the ability of p190ROHAGAP to interact with RHOA and strongly supports the conclusion that p-ERK1/2 is the major activator of RHOA in this context.

**RHOA Is a Vulnerability of Lung Cancer Cells Expressing Mutant KRAS**

To assess the functional significance of sustained RHOA-GTP in lung adenocarcinomas, we determined the effect of RHOA silencing in cell lines derived from human NSCLC specimens. Remarkably, RHOA silencing with 2 unrelated siRNAs resulted in apoptosis as shown by increased levels of cleaved PARP and cleaved caspase-3 in A549 cells (mutant KRAS, INK4a/ARF-deficient). This effect was virtually absent in HCC95 cells (wild-type KRAS, INK4a/ARF-deficient) suggesting that the apoptotic effect seen upon RNA interference (RNAi)-mediated RHOA ablation reflects a specific requirement for mutant KRAS (Fig. 3A and B). To confirm this result, we silenced RHOA in a panel of lung cancer cell lines expressing either mutant or wild-type KRAS in addition to INK4a/ARF deficiency. Indeed, we found that the presence of mutant KRAS in association with INK4a/ARF deficiency conferred vulnerability to RHOA silencing (72 hours after transfection; Fig. 3C and Supplementary Fig. S4A). RHOA silencing also resulted in loss of cell viability in NSCLC cells expressing mutant KRAS in association with mutations of TP53, although the effect was less pronounced compared with mutant KRAS, INK4a/ARF-deficient NSCLC cells (Fig. 3C and Supplementary Fig. S4A). Importantly, RHOA silencing produced similar results 120 hours after transfection (Supplementary Fig. S4B), suggesting that these effects are not due to differences in doubling time between cell lines. Taken together, these results indicate that mutant KRAS in combination with INK4a/ARF deficiency triggers a requirement for RHOA-GTP for tumor cell viability.

Next, we used human bronchial epithelial cells (HBEC3KT cells immortalized by introducing hTERT and cyclin-dependent kinase 4 (CDK4), which partially overcome the inhibitory effect of INK4a on cell-cycle progression) to test the impact of mutant KRAS expression in this context. The constitutive expression of mutant KRAS resulted in increased RHOA-GTP that became significantly higher upon additional p53 knockdown (Supplementary Fig. S4C and S4D). In both cases, the expression of mutant KRAS resulted in a considerable induction of cell death upon RHOA silencing (Supplementary Fig. S4E). Thus, HBEC3KT cells confirm our in vivo observations with transgenic mice and suggest that their susceptibility is specifically dependent on a genotype-induced activation of the ERK1/2-RHOA pathway.

To examine whether RHOA is required for the establishment of NSCLC, we conducted xenograft experiments using A549 cells, which are representative of the NSCLC cells we used in vitro. We transduced the A549 cells with a retrovirus expressing RHOA T19N, a dominant negative mutant of RHOA. Indeed, we found that RHOA T19N significantly decreased the amount of RHOA-GTP in A549 cells before implantation in mice and in tumors excised at the study end point (Supplementary Fig. S4F). We detected a more than 4-fold decrease in tumor formation of xenografts expressing RHOA T19N (Fig. 3D and E), which correlated with a dramatic difference in survival (Fig. 3F). We conclude that the activation of RHOA is critical in promoting the growth of NSCLC.

**FAK Is the Primary Target of RHOA in Mutant KRAS, INK4a/ARF, or p53-Deficient NSCLC**

To date, there are no pharmacologic drugs that target RHOA-GTP available for use in preclinical trials. Thus, we silenced the main direct and indirect downstream targets of RHOA, such as ROCK1, LIMK2, FAK, Villin 1, Contractin, Kinetin, and DIAPH1 (30, 32, 33), to identify "druggable" therapeutic targets. We found that only the silencing of FAK caused significant loss of cell viability that at least in part recapitulated the effects on cell viability of RHOA silencing (Supplementary Fig. S5A and S5B). Indeed, siRNA-mediated FAK knockdown led to significant apoptosis (72 hours after siRNA transfection) in mutant KRAS, INK4a/ARF-deficient NSCLC cells (Fig. 4A and B). Moreover, FAK silencing also triggered apoptosis in mutant KRAS and p53-deficient cells (Fig. 4A and B). Finally, assessment of cell viability at 120 hours after transfection with siRNAs against FAK revealed a more dramatic and selective cell loss (Supplementary Fig. S5C). As predicted by our previous findings regarding RHOA activation (Supplementary Fig. S4C–S4F), ectopic expression of mutant KRAS in HBEC3KT cells increased FAK activation and cell death upon FAK silencing, an effect that was amplified by the knockdown of p53 (Supplementary Fig. S5D and S5E).

FAK plays a pivotal role in focal adhesion regulation and turnover in addition to the control of cell adhesion and cancer cell survival (34–37). Moreover, it has been shown that in some cell types, FAK also contributes to actin remodeling by regulating RHOA (38). Indeed, ablation of FAK in NSCLC cells was accompanied by disruption of focal adhesions, reduced actin stress fiber formation, and remarkable upregulation of the p27/KIP1 tumor suppressor (Fig. 4C and D).

Next, we confirmed that FAK is a physiologic target of RHOA in NSCLC cells. Silencing of RHOA induced a dramatic downregulation of p-FAK Y397 in A549, H460, and H2009 cells (Fig. 4E). Conversely, forced expression of a constitutively active RHOA mutant (RHOA Q63L) led to increased p-FAK Y397 (Supplementary Fig. S5F and S5G).

**FAK and RHOA Are Coactivated in Primary NSCLC Specimens that Express Mutant KRAS in Association with INK4a/ARF or TP53 Mutations**

We predicted that, similar to RHOA-GTP, FAK activation would also be sustained in high-grade lung cancer in vivo. Indeed, immunoblot analysis conducted on microdissected lung tumors showed that p-Fak Y397 declined to almost undetectable levels after 12 weeks of doxycycline exposure in Kras G12D;Ink4a/Arf−/− lung adenomas, whereas it remained easily detectable in Kras G12D;Ink4a/Arf−/− adenocarcinomas (Fig. 5A). We found no differences in Fak activation between Ink4a/Arf−/− or Ink4a/Arf−/+ mouse lungs in the absence of Kras G12D (Supplementary Fig. S6A).

IHC staining confirmed that p-Fak Y397 was present solely in high-grade lung adenocarcinomas of Kras G12D;Ink4a/Arf−/− mice (Fig. 5B and Supplementary Fig. S6B). Finally, RHOA-GTP strongly colocalized with p-Fak Y397 in mouse lung adenocarcinomas (Fig. 5C and Supplementary Fig. S6C).

To assess whether our results are relevant to human NSCLC, we used 120 primary human NSCLC specimens and assessed the mutation status of KRAS together with FAK

**Association with Mutations of KRAS, INK4a/ARF, or p53-Deficient NSCLC**

To assess whether our results are relevant to human NSCLC, we conducted xenograft experiments using A549 cells, which are representative of the NSCLC cells we used in vitro. We transduced the A549 cells with a retrovirus expressing RHOA T19N, a dominant negative mutant of RHOA. Indeed, we found that RHOA T19N significantly decreased the amount of RHOA-GTP in A549 cells before implantation in mice and in tumors excised at the study end point (Supplementary Fig. S4F). We detected a more than 4-fold decrease in tumor formation of xenografts expressing RHOA T19N (Fig. 3D and E), which correlated with a dramatic difference in survival (Fig. 3F). We conclude that the activation of RHOA is critical in promoting the growth of NSCLC.
Figure 3. RHOA is essential for the survival of human NSCLC. A, immunoblot showing total (PARP) and cleaved PARP (ΔPARP) in HCC95 and A549 NSCLC cells 96 hours after transfection with the indicated siRNAs. B, percentage of cleaved caspase-3–positive cells after transfection with the indicated siRNAs. A total of 200 cells were scored/slide for at least 3 replicates. Mean and SEM. *, $P < 0.01$. C, histogram showing viability of the indicated NSCLC cells 72 hours after transfection with RHOA or control siRNAs. The mutation status of the cell lines is indicated. Mut, mutant; Wt, wild-type. D, tumor volume of A549 cells transduced as indicated and implanted in nude mice. Mean and SEM, $n = 5$. E, representative pictures of nude mice from panel (D), 30 days after initiation of the experiment. F, Kaplan-Meier curve of A549 xenografts [shown in (D)] transduced as indicated; $n = 5$. 

on June 27, 2017. © 2013 American Association for Cancer Research.
Figure 4. FAK is a crucial target of RHOA in mutant KRAS/INK4a/ARF-deficient NSCLC. A, histogram showing viability of NSCLC cell lines 72 hours after transfection with siRNAs against FAK. The mutation status of the cell lines is indicated. B, immunoblot showing total (PARP) and cleaved PARP (ΔPARP) in A427, H358 and HCC44 NSCLC cells 72 hours after transfection with 2 nonoverlapping siRNAs against FAK. Mut, mutant. C, siRNA control or siRNA against FAK followed by immunofluorescence staining with phalloidin (green) and vinculin (red) to detect actin stress fibers and focal adhesions, respectively, in A549 cells. 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. D, immunoblot showing p27/KIP1 upregulation 72 hours after siRNA transfection against FAK in A427 and HCC44 NSCLC cells. E, immunoblot of A549, H460, and H2009 NSCLC cells treated as indicated. Mut, mutant.
activation. This analysis revealed an association between mutant KRAS and p-FAK Y397 (Fig. 5D). In addition, we evaluated the activation status of RHOA/FAK and the mutation status of INK4a/ARF and TP53 in an independent cohort of 20 consecutive primary human NSCLC specimens expressing mutant KRAS (Table 1 and Supplementary Fig. S6D and S6E). We found that tumors carrying either INK4a/ARF, TP53 tumor-associated mutations or INK4a/ARF hemizygous deletions evidenced high levels of activated RHOA and FAK (11/20 tumors; Table 1, ID number 1–11), whereas tumors that did not show strong coactivation of RHOA/FAK carried wild-type INK4a/ARF or TP53 (8/20 tumors; Table 1, ID number 13–20). We found only one specimen that did not conform to this pattern (Table 1, ID number 12). Although there was a significant association between the activation of RHOA/FAK and deficiency of the INK4a/ARF or TP53 locus, the small sample number does not allow the establishment of firm correlations. The observation that hemizygous deletions of INK4a/ARF occur mainly in specimens that show coactivation of RHOA and FAK suggests that these samples may harbor additional mutations inactivating the INK4a/ARF or p53 tumor suppressive networks. Moreover, it seems that FAK is active regardless of INK4a/ARF or p53 status in a minority of NSCLCs. This observation suggests that in these cases FAK activation may occur through RHOA-independent mechanisms. This could be explained by the fact that several signals, other than RHOA, positively regulate FAK (36).

Pharmacologic Inhibition of FAK Leads to Lung Adenocarcinoma Regression in 
Kras<sup>G12D</sup>;Ink4a/Arf<sup>−/−</sup> Mice

Our findings suggest that Kras<sup>G12D</sup> promotes the progression of lung adenomas into adenocarcinomas through a mechanism that involves sustained activation of the Erk1/2-RhoA-FAK signaling axis in Ink4a/Arf-deficient cancer cells. Thus, we predicted that inhibition of FAK would lead to significant antitumor effects specifically in lung adenocarcinomas and not in adenomas. We evaluated the preclinical efficacy of PF562271, an ATP-competitive inhibitor, in Kras<sup>G12D</sup>;Ink4a/Arf<sup>−/−</sup>- and Kras<sup>G12D</sup>;Ink4a/Arf<sup>+/−</sup>-induced lung
Table 1. RHOA-FAK activation status associations in NSCLC

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Remarkably, treatment with PF562271 resulted in significant tumor regression in Kras\(^{G12D}\);Ink4a/Arf\(^{-/-}\) mice, whereas it had antiproliferative effects in Kras\(^{G12D}\);Ink4a/Arf\(^{+/+}\) mice (Fig. 6A). Detailed histologic evaluation determined that the treatment with PF562271 significantly suppressed the number and size of Kras\(^{G12D}\);Ink4a/Arf\(^{-/-}\) lung adenocarcinomas compared with the placebo-treated mice (Fig. 6B and C). Lung adenocarcinomas treated with PF562271 were distinguished by the presence of foamy macrophages and enrichment of reparative connective tissue (Fig. 6D, top). In addition, FAK inhibition caused localized induction of apoptosis and reduced cellular proliferation [Fig. 6D–F (bottom)].

FAK is a positive regulator of AKT (33). Indeed, we found that PF562271 not only efficiently inhibited p-FAK\(^Y397\) in vivo but also resulted in striking downregulation of p-Akt\(^S473\) (Fig. 6G). The antitumor effects observed with PF562271 were confirmed with the FAK14 inhibitor (Supplementary Fig. S7B–S7G). FAK14 is structurally unrelated to PF562271 and inhibits the autophosphorylation of FAK at Y397 (40).

Finally, we conducted studies with A427 and A549 NSCLC cells to determine the impact of the PF562271 inhibitor on the survival of athymic nude mice carrying xenografts of human cells. Notably, we detected a significant increase in the survival rate of PF562271-treated mice compared with placebo-treated mice (Fig. 6H and I). We obtained similar results with the FAK14 inhibitor (Supplementary Fig. S7H and S7I). Thus, we conclude that pharmacologic inhibition of FAK is a potent therapeutic strategy for advanced KRAS-induced lung tumors.

**DISCUSSION**

The identification of patients that may benefit from treatment with targeted cancer therapies is still a significant challenge. In this study, we show that NSCLCs characterized by mutant KRAS and loss of either INK4a/ARF or p53 are highly sensitive to inhibition of the RHOA-FAK signaling axis. The data imply that pharmacologic inhibitors of FAK are effective, genotype-specific anticancer agents. Our findings are of clinical significance because these genotypes are associated with aggressive cancers, which are refractory to conventional therapy.

Recently, several groups reported strategies to induce antitumor responses in high-grade mouse lung cancer (11, 13, 41). To the best of our knowledge, our study is the first example of an effective genotype-specific monotherapy for high-grade mutant KRAS tumors.

Consistent with the known roles of FAK in the regulation of the cytoskeleton, we determined that its inhibition resulted in the reduction of F-actin stress fibers, disruption of focal adhesions, induction of the p27/Kip1 tumor suppressor, and decreased p-AKT\(^S473\). These events occurred in conjunction with the induction of apoptosis; therefore, we propose that multiple cooperative functions of FAK contribute to its requirement for the maintenance of high-grade lung cancer. In addition, our studies show that FAK is the main effector of RHOA. However, it is still possible that other downstream targets of RHOA may contribute to its tumor-promoting ability. Future studies will be necessary to determine the mechanisms of cell death that contribute to this antitumor response.

Pharmacologic inhibition of MEK1/2 leads to compensatory upregulation of the PI3K/AKT signaling pathway (42), which in turn promotes cancer cell survival. On the contrary, we have shown that pharmacologic inhibition of FAK in vivo downregulates p-Akt. Thus, inhibition of FAK does not trigger the emergence of PI3K/AKT-dependent compensatory mechanisms. Collectively, these data show that the inhibition of an ultimate effector-arm of mutant KRAS, in this case RHOA/FAK, has detrimental antitumor effects.

It has been reported that p19/ARF and p53 restrain the progression of lung adenomas into adenocarcinomas and that their loss leads to the upregulation of MEK1/2 signaling through multiple mechanisms, including genomic amplification of mutant KRAS, inactivation of negative feedback mechanisms, or emergence of cooperative oncogenes (18, 19). We did not detect differences in the overall Ras activity (Ras-GTP) between Kras\(^{G12D}\);Ink4a/Arf\(^{-/-}\) adenomas and Kras\(^{G12D}\);Ink4a/Arf\(^{+/+}\) adenocarcinomas. Thus, we propose that in this mouse model, mechanisms other than increased Ras-GTP signaling are responsible for the deregulation of p-Erk1/2.
Preclinical efficacy of PF562271 inhibitor in advanced NSCLC. A, quantification of lung tumor burden in Kras<sup>G12D</sup>,Ink4a/Arf<sup>−/−</sup> and Kras<sup>G12D</sup>,Ink4a/Arf<sup>−/−</sup> mice treated with PF562271 inhibitor (presented as a percentage change over basal level). The treatment started after 10 weeks of Kras<sup>G12D</sup> induction (to obtain high-grade tumors) and continued for 12 days. Mean and SEM; n = 10/group. *, P < 0.05; **, P < 0.004; ***, P < 0.001. B and C, tumor number, size, and grade of individual lung tumors from Kras<sup>G12D</sup>,Ink4a/Arf<sup>−/−</sup> mice treated with PF562271 inhibitor as in (A); n = 10 per group. Mean and SEM. *, P < 0.03; ***, P < 0.003. D, top, histologic evaluation of excised lungs after treatment with placebo (left) or PF562271 inhibitor for 12 days [2 representative images to show the enrichment in reparative connective tissue (middle) or macrophage infiltration (right), during adenocarcinoma regression]. The inset indicates area magnified below. Scale bar, 40 μm. Bottom, representative images showing increased localized apoptosis (cleaved PARP) and decreased Ki-67–positive (proliferation marker) cells in the lungs of mice sacrificed 2 hours after treatment with PF562271 inhibitor; arrows indicate magnified image shown in the inset. Scale bars, 40 μm. E, the histogram reports the percentage of cleaved PARP-positive cells of the indicated treatment groups assessed at the study end point. A total of 200 cells were scored per slide for at least 3 replicates. Mean ± SEM. **, P < 0.0016. F, the histogram reports the percentage of Ki-67–positive cells of the indicated treatment groups. Mean ± SEM. *, P < 0.0029. A total of 200 cells were scored per slide for at least 3 replicates. G, immunoblot showing effective p-Fak<sup>S397</sup> and p-Akt<sup>S473</sup> inhibition with PF562271 inhibitor 2 hours after treatment. Each lane represents a lung lysate from a single mouse. H, tumor volume of A549 and A427 xenografts treated as indicated. The treatment with PF562271 inhibitor (40 mg/kg) was started when the tumors reached approximately 150 mm<sup>3</sup> and was given every day for a total of 12 days. The mice were sacrificed when the tumors reached 2,000 mm<sup>3</sup>. Mean and SEM; n = 8. I, Kaplan-Meier curves of A549 and A427 xenografts treated as in (H); n = 8.
Several questions remain to be answered regarding the role of FAK in lung cancer. As shown by our IHC data, a subset of mutant KRAS NSCLCs displayed upregulation of p-FAK in the absence of INK4a/ARF or p53 mutations/deletions. Furthermore, a subset of wild-type KRAS NSCLCs displayed either strong or moderate p-FAK staining. It will be of interest in the future to determine the significance of FAK activation in these settings. Furthermore, larger cohorts of patients will be needed to firmly establish that a correlation exists between mutant KRAS, INK4a/ARF, and/or p53 deficiency and activation of RHOA-FAK in human primary NSCLCs.

In view of the fact that NSCLCs are often comprised of heterogeneous populations of neoplastic cells, a possible mechanism of emergence of resistance to FAK inhibitors could be fueled by the persistence of neoplastic clones primarily driven by low-level oncogenic signals that are still able to develop high-grade tumors. Although this targeted therapy will have a significant benefit in cancer treatment, the elimination of less-advanced tumors is still an unmet need that must be resolved.

We conclude that the RHOA-FAK signaling axis is a novel, critical synthetic lethal partner of mutant KRAS in NSCLCs that are INK4a/ARF- or TP53-deficient. We propose that this information would serve as a biomarker for the selection of patients undergoing personalized cancer treatment protocols involving FAK inhibitors.

METHODS

Mouse Studies

The PF562271 inhibitor (Pfizer) was reconstituted in 50% dimethyl sulfoxide and 50% PEG300 and additionally diluted with saline to a final concentration of 40 mg/kg before administration to the mice twice daily for a total of 12 days by oral gavage. The FAK14 inhibitor (Tocris) was reconstituted in H2O and administered to the mice by intraperitoneal injection, once daily for a total of 10 days at the dosage of 20 mg/kg for the transgenic mice and 30 mg/kg for athymic nude mice. ARRY-142886 (AZD6244) (Selleck) was reconstituted in 0.5% methyl cellulose (Fluka) and 0.4% polysorbate (Tween 80; Fluka) and administered at 35 mg/kg by oral gavage. Xenograft experiments using A549 or A427 NSCLC cells were carried out by subcutaneous inoculation of 2 × 106 cells into 6-week-old female athymic nude mice (nu/nu). For all in vivo experiments, we used age-matched littermates. The body weight of the mice remained stable during treatment with PF562271 and decreased by less than 5% during treatment with FAK14. Tumor burden was assessed by digital quantification of the area occupied by tumors compared with unaffected tissue using FAK14. Tumor burden was assessed by digital quantification of the PF562271 and decreased by less than 5% during treatment with (nu/nu). For all

Cell Culture and Reagents

Human NSCLC cell lines H125, H441, H2087, H522, EKVX, H2030, H1264, HCC95, HCC44, H2009, H358, H460, H1563, A549, and HBECC3KT cells (HBECC3 cells immortalized by introducing CDK4 and STERT), together with the variants HBECC3KT-shp53, HBECC3KT-KRAS, and HBECC3KT-shp53+KRAS, were kindly provided by Dr. John Minna (UT Southwestern Medical Center; refs. 43–45). All NSCLC cell lines have been tested and authenticated by DNA fingerprinting using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained by American Type Culture Collection and the Minna/Gazdar lab (the primary source of the lines). See also Supplementary Methods.

RNA Interference

siRNA (siGenome) against RHOA, FAK, p190RHOGAP, or non-targeting siRNA control were purchased from Dharmacon (Thermo Scientific). See also Supplementary Methods.

PCR Amplification and Sequencing

The exonic regions of interest (NCBI Human Genome Build 36.1) were broken into amplicons of 350 bp or less, and specific primers were designed using Primer 3 to cover the exonic regions plus at least 50 bp of intronic sequences on both sides of intron-exon junctions. See also Supplementary Methods.

Mutation Detection

Mutations were detected using an automated detection pipeline at the Memorial Sloan-Kettering Cancer Bioinformatics Core (New York, NY). Bidirectional reads and mapping tables (to link read names to sample identifiers, gene names, read direction, and amplification) were subjected to a quality control filter which excludes reads that have an average phred score of <10 for bases 100 to 200. See also Supplementary Methods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: G. Konstantinidou, G. Ramadori, P.P. Scaglioni
Development of methodology: G. Konstantinidou, P.P. Scaglioni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Konstantinidou, K. Kangasniemi, R.E. Ramirez, Y. Cai, C. Behrens, M.T. Dellinger, R.A. Brekken, L.I. Wistuba, A. Heguy, J. Teruya-Feldstein
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Konstantinidou, G. Ramadori, A. Heguy
Writing, review, and/or revision of the manuscript: G. Konstantinidou, G. Ramadori, R.E. Ramirez, Y. Cai, L.I. Wistuba, P.P. Scaglioni
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.E. Ramirez, Y. Cai
Study supervision: P.P. Scaglioni

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