C-Raf Is Required for the Initiation of Lung Cancer by K-Ras

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**ABSTRACT**

The Ras/Raf/MEK/ERK (extracellular signal–regulated kinase) pathway is primarily responsible for mitogenesis in metazoans, and mutational activation of this pathway is common in cancer. A variety of selective chemical inhibitors directed against the mitogen-activated protein kinase pathway are now available for clinical investigation and thus the determination of the importance of each of the kinases in oncogenesis is paramount. We investigated the role of two Raf kinases, B-Raf and C-Raf, in Ras oncogenesis, and found that although B-Raf and C-Raf have overlapping functions in primary mesenchymal cells, C-Raf but not B-Raf is required for the proliferative effects of K-Ras G12D in primary epithelial cells. Furthermore, in a lung cancer mouse model, C-Raf is essential for tumor initiation by oncogenic K-Ras G12D, whereas B-Raf is dispensable for this process. Our findings reveal that K-Ras G12D elicits its oncogenic effects primarily through C-Raf and suggest that selective C-Raf inhibition could be explored as a therapeutic strategy for K-Ras–dependent cancers.

**SIGNIFICANCE:** Ras is one of the most prevalent oncogenes in human cancer; however, it is considered “undruggable.” Therefore, increasing our understanding of the importance of Ras effectors, including the Raf/MEK/ERK pathway, will create novel avenues for therapeutic intervention.

**INTRODUCTION**

Activating mutations in Ras genes are observed in approximately 30% of human cancers (1). Ras stimulates multiple downstream effector pathways, which are constitutively activated in a growth factor-independent fashion in cancer cells expressing oncogenic Ras (2). Despite extensive efforts, targeted therapies against Ras were largely unsuccessful in clinical trials (3), and thus pharmacological inhibition of Ras effector pathways may represent a more feasible approach to eradicate Ras-mutant cancers.

The contribution of Ras effector pathways to Ras-mediated transformation has been studied in detail in human and murine cells (4–6). These studies found that the Ras effectors required for transformation depend on the species, the cell type, and the tumor stage. However, these findings may be confounded because they were mainly conducted in cultured cells and by using H-Ras, the least commonly mutated Ras gene, to transform cells. K-Ras is the predominantly mutated Ras family member, and activating mutations in K-Ras are associated primarily with malignancies of the lung, pancreas, and colon. An in vivo evaluation of the role of Ras effectors in these cancer types is limited and has only been studied in the lung thus far. For example, the importance of the Ras/Rac axis has been validated in a K-Ras–mutant mouse model of lung cancer using conditional Rac knockout mice. Rac deficiency strongly impaired Ras tumorigenesis in the lung and developing tumors invariably retained wild-type Rac (7), suggesting an essential contribution of Rac to K-Ras G12D–initiated lung cancer. In addition, the Ras/phosphoinositide 3-kinase (PI3K)/AKT pathway has been demonstrated to be critical for Ras-mediated tumorigenesis. Mice expressing a Ras-binding–deficient form of the p110α subunit of PI3K were insensitive to oncogenic K-Ras–driven lung tumorigenesis (8).

However, the contribution of the Ras/MAPK (mitogen-activated protein kinase) axis, a critical regulatory pathway that is often perturbed in cancer, to K-Ras–mediated transformation has been insufficiently addressed. The potential...
Raf in K-Ras<sup>G12D</sup> Oncogenesis

In this study, we genetically ablated B-Raf or C-Raf expression in K-Ras<sup>G12D</sup>-mutant primary cells in vitro as well as in lung epithelial cells in vivo to evaluate the contribution of individual Raf proteins to K-Ras<sup>G12D</sup>-mediated transformation. Our genetic analysis reveals that C-Raf is uniquely required for K-Ras<sup>G12D</sup>–driven transformation of epithelial cells in vitro and in vivo.

RESULTS

Effect of Raf Loss on K-Ras<sup>G12D</sup>-Mediated Transformation of Mouse Embryonic Fibroblasts

Endogenous expression of oncogenic K-Ras<sup>G12D</sup> partially transforms mouse embryonic fibroblasts (MEF) as indicated by immortality, elevated proliferation rates, and the ability to form foci under confluent culture condition (14). To analyze the role of B-Raf and C-Raf in transformation by oncogenic K-Ras, B-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup> (BBK) and C-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup> (CCK) MEFs were infected with Cre recombinase–expressing adenovirus (Ad-Cre) to produce B-Raf<sup>Δ/Δ</sup>, K-Ras<sup>G12D</sup> and C-Raf<sup>Δ/Δ</sup>, K-Ras<sup>G12D</sup> cells, respectively. First, proliferation was assessed in 10% and 2% fetal calf serum. Similar to K-Ras<sup>G12D</sup> (K) cells, both B-Raf<sup>Δ/Δ</sup>, K-Ras<sup>G12D</sup> and C-Raf<sup>Δ/Δ</sup>, K-Ras<sup>G12D</sup> MEFs displayed increased proliferation compared to empty adenovirus (Ad-Mock)–infected isogenic control cells under normal and low-serum conditions (Fig. 1A, Supplementary

Figure 1. Effect of Raf deficiency on K-Ras<sup>G12D</sup>-mediated transformation of MEFs. A, increase in cell number of Ad-Cre–infected MEFs of the indicated genotypes relative to Ad-Mock–infected isogenic control cells. Cells were analyzed in 10% fetal calf serum (FCS). The fold increase of proliferation of Ad-Cre–infected isogenic control cells compared to Ad-Mock–infected isogenic control cells is shown. B, quantification of Western blot analysis of MAPK activation in response to K-Ras<sup>G12D</sup> expression in 2% and 10% FCS. pERK was normalized to total ERK; fold change compared to Ad-Mock–infected isogenic control cells. C, proliferation of C-Raf<sup>Δ/Δ</sup>, K-Ras<sup>G12D</sup> (Ad-Mock) or C-Raf<sup>fl/fl</sup>, K-Ras<sup>G12D</sup> (Ad-Cre) MEFs transfected with 100 pmol siRNA against C-Raf, B-Raf, or scrambled (scr) control. D, Western blot for ERK phosphorylation and cyclin D1 expression in MEFs analyzed in C. BBK: B-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup>; BK: B-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup>; CCK: C-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup>; CK: C-Raf<sup>fl/fl</sup>, K-Ras<sup>LSL</sup>; K: K-Ras<sup>LSL</sup>. Error bars represent ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figs. 1A, 2). Moreover, focus formation of K-RasG12D-mutant MEFs was not diminished by loss of either B-Raf or C-Raf (Supplementary Fig. 1C), indicating that these Raf proteins are dispensable for several characteristics of oncogenic Ras biology in MEFs.

Next, we analyzed MAPK signaling downstream of Ras/Raf in K-RasG12D, B-RafΔ3, K-RasG12DΔ, and C-RafΔ3, K-RasG12DΔ MEFs. Western blot analysis confirmed recombination of the floxed Raf alleles as well as induction of K-RasG12D expression upon infection with Ad-Cre (Supplementary Fig. 1C). Interestingly, under low and normal serum conditions deletion of B-Raf or C-Raf slightly decreased or elevated, respectively, steady-state phosphorylated ERK (pERK) levels (Fig. 1B). However, only the increase in pERK in C-Raf-depleted cells grown in 2% serum was statistically significant. Hence, modest differences in steady-state pERK levels did not correlate with induction of proliferation in response to oncogenic K-Ras. To unequivocally investigate whether Raf deficiency alters MAPK activation, ERK phosphorylation in response to serum stimulation was examined. Notably, the kinetics of MAPK activation of serum-starved and re-stimulated K-RasG12D, B-RafΔ3, K-RasG12DΔ, and C-RafΔ3, K-RasG12DΔ MEFs were comparable (Supplementary Fig. 1D). These findings indicate that loss of either B-Raf or C-Raf alone does not abrogate oncogenic transformation by K-RasG12DΔ and that B-Raf and C-Raf may have overlapping functions in MEFs.

Because genetic ablation of either B-Raf or C-Raf alone had no impact, the effect of combined depletion of B-Raf and C-Raf on K-RasG12DΔ-mediated transformation of MEFs was assessed. Accordingly, we utilized siRNA against B-Raf in C-RafΔ3, K-RasG12DΔ MEFs infected with Ad-Cre or Ad-Mock. Proliferation of C-RafΔ3, K-RasG12DΔ control cells transfected with either B-Raf siRNA or control siRNAs (scrambled or C-Raf siRNA) was comparable. In contrast, RNA interference (RNAi)-mediated B-Raf depletion by approximately 50% to 60% in C-RafΔ3, K-RasG12DΔ MEFs reduced proliferation to levels comparable to C-RafΔ3, K-RafΔ3 control cells (Fig. 1C). Moreover, this rescue of the proliferation defect in K-RasG12DΔ-mutant MEFs was accompanied by a modest decrease in pERK and a more substantial decrease in cyclin D1, a known target of the MAPK pathway (Fig. 1D). Knockdown of C-Raf in BBK MEFs similarly rescued proliferation (data not shown). These data suggest that K-RasG12DΔ signals through both B-Raf and C-Raf in MEFs, and combined deletion of both paralogues is necessary to suppress K-Ras-driven hyperproliferation of MEFs and activation of downstream targets.

Depletion of Raf Proteins in K-RasG12D-Mutant Epithelial Cells In Vitro

MEFs are a commonly used experimental system to assess the oncogenicity of mutant K-Ras; however, the number of conclusions that can be drawn from experiments using MEFs is limited concerning human cancer because K-Ras is typically mutated in epithelial cells. We therefore investigated whether B-Raf or C-Raf are critical for K-RasG12DΔ-induced transformation in primary epithelial cells. To address this question, we examined signaling downstream of K-RasG12DΔ and proliferation of K-RasG12DΔ, B-RafΔ3, K-RasG12DΔ, and C-RafΔ3, K-RasG12DΔ-mutant baby mouse kidney (BMK) epithelial cells. BMK cells have previously been shown to exhibit increased proliferation in response to K-RasG12DΔ activation (Fig. 2A; ref. 7). Similar to MEFs, BMK cells were infected with either Ad-Cre to produce K-RasG12DΔ, B-RafΔ3, K-RasG12DΔ, and C-RafΔ3, K-RasG12DΔ cells or with Ad-Mock. Ablation of B-Raf expression in otherwise wild-type BMK cells had no effect on proliferation (Fig. 2B) or pERK and cyclin D1 expression (Supplementary Fig. 3A). Similar to K-RasG12DΔ-mutant BMK cells, activation of K-RasG12DΔ in the absence of B-Raf elevated proliferation of B-RafΔ3, K-RasG12DΔ BMK cells (Fig. 2B). These data indicate that B-Raf is dispensable for proliferation of wild-type and K-Ras–mutant epithelial cells in vitro.

In contrast, C-RafΔ3, K-RasG12DΔ BMK cells proliferated less than wild-type control cells (Fig. 2C), and accumulated in the G0 to M-phase of the cell cycle (Supplementary Fig. 3A). No changes in pERK and only modest and heterogeneous changes in cyclin D1 levels were observed (Supplementary Fig. 3B). Reduced cell numbers could not be attributed to apoptosis in the absence of C-Raf because the levels of cleaved caspase 3 and cleaved PARP did not increase upon C-Raf deletion (Supplementary Fig. 3C), implying that C-Raf may have additional functions in BMK cells. Interestingly, C-RafΔ3, K-RasG12DΔ BMK cells did not display an increase in proliferation compared to control cells infected with Ad-Mock (Fig. 2C), suggesting that oncogenic K-RasG12DΔ could not override the proliferative defect of BMK cells elicited by the absence of C-Raf. Cell-cycle analysis of BMK cells revealed that expression of oncogenic K-RasG12DΔ resulted in a larger proportion of cells in G0 and S-phase and fewer cells in G2 to M phase (Fig. 2D), similar to endogenous expression of K-RasG12DΔ in MEFs (14). The absence of B-Raf had no impact on the cell-cycle profile of K-RasG12DΔ-mutant BMK cells, whereas the C-RafΔ3, K-RasG12DΔ BMK cells displayed cell-cycle distribution similar to wild-type control cells (Fig. 2D), thus corroborating the proliferation analysis (Fig. 2A–C).

Expression of K-RasG12DΔ in MEFs induced the expression of several cell-cycle regulatory proteins (14). We determined the expression levels of cell-cycle proteins in BMK cells to address if the absence of C-Raf alters the induction of such proteins, thereby rescuing the proliferation defect of K-RasG12DΔ–expressing cells. K-RasG12DΔ modestly increased phosphorylation of ERK and expression of CDK4, which was negated by depletion of either B-Raf or C-Raf (Fig. 2E and F, and data not shown). Moreover, induction of cyclin D1 expression in response to K-RasG12DΔ was diminished in the absence of C-Raf but not B-Raf (Fig. 2D and E). Other cell-cycle regulators were not induced by K-RasG12DΔ or affected by Raf deficiency (data not shown). In addition, RNAi-mediated depletion of cyclin D1 in K and BBK BMK cells resulted in a markedly reduced number of cells in S-phase, as measured by bromodeoxyuridine incorporation (Supplementary Fig. 3D). Thus, impaired upregulation of cyclin D1 in the absence of C-Raf may diminish the proliferative response of BMK cells to expression of K-RasG12DΔ. Taken together, C-Raf is a crucial mediator of proliferation in K-RasG12DΔ-expressing epithelial cells, whereas B-Raf is not required.
**Lung Tumor Development and Survival of K-RasLSL Mice in the Absence of B-Raf or C-Raf**

To investigate the functions of Raf proteins in K-RasG12D-mediated transformation *in vivo*, we generated compound mutant mice harboring conditional B-Raf (15) or C-Raf (16) knockout alleles (B-Raffl/fl or C-Rafl/fl, respectively) in combination with an endogenous, conditional K-RasLSL allele in which oncogenic K-RasG12D is expressed upon Cre-mediated excision of a lox-stop-lox (LSL) element (12). Compound mutant B-Raffl/fl; K-RasLSL and C-Raffl/fl; K-RasLSL mice and compound mutant mice bearing heterozygous conditional Raf alleles (B-Raffl/w; K-RasLSL and C-Raffl/w; K-RasLSL) as well as K-RasLSL mice on the same genetic background were infected with Ad-Cre via intranasal instillation at 4 to 6 weeks of age to induce lung tumorigenesis. This route of Cre delivery has been demonstrated to successfully recombine floxed DNA sequences, including the K-RasLSL allele (12). Mice were aged for 6, 9, and 12 weeks, and lung tumorigenesis examined. B-Raffl/w; K-RasLSL (BBK) mice displayed similar overall tumor burden as B-Raffl/w; K-RasLSL (BK) and K-RasLSL (K) mice (Fig. 3A). In contrast, C-Raffl/w; K-RasLSL (CCK) mice demonstrated drastically reduced lung tumor burden compared to C-Raffl/w; K-RasLSL (CK) and K-RasLSL (K) mice (Fig. 3A). In addition, C-Raffl/w; K-RasLSL mice exhibited a significant reduction in bronchiolar hyperplasia (BH), adenomatous alveolar hyperplasia (AAH), and adenomas (Fig. 3B), although the size of neoplasms was not affected by the absence of C-Raf (Fig. 3C), suggesting that the lower number of neoplasms results in reduced lung tumor burden. Thus, K-RasG12D-induced formation of alveolar and bronchiolar tumors is impaired in the absence of C-Raf (Fig. 3D). Interestingly, Although B-Raffl/w; K-RasLSL mice displayed a specific reduction in the number of BHs but not AAHs or adenomas (Fig. 3B), the size of all neoplasms was reduced (Fig. 3C). Thus, B-Raf plays a role in the biology of K-RasG12D expressing alveolar and
bronchiolar neoplasms but its function is less prominent than that of C-Raf.

To assess whether loss of either C-Raf or B-Raf affects survival of K-Ras^{G12D}-mutant mice, cohorts of K-Ras^{L53} mice harboring floxed Raf alleles were aged. Heterozygous genetic deletion of B-Raf in K-Ras^{G12D}-mutant lung epithelia resulted in a short, albeit statistically insignificant increase in survival compared to K-Ras^{L53} control mice (200.5 versus 167 days median survival; Fig. 4A). However, the lifespan of B-Raf^{flox/flox}; K-Ras^{L53} mice was extended compared to K-Ras^{L53} or B-Raf^{+/−}; K-Ras^{L53} mice (249 versus 167 or 200.5 days, respectively; Fig. 4A). Heterozygous loss of C-Raf in C-Raf^{flox/flox}; K-Ras^{L53} mice resulted in a slight prolongation of survival compared to K-Ras^{L53} mice (211 versus 167 days, Fig. 4B). Moreover, homozygous deletion of C-Raf extended survival even further (268 days, Fig. 4B). Hence, lower tumor burden in K-Ras^{L53}-mutant mice deficient for C-Raf correlates with extended survival. The increased lifespan of C-Raf^{flox/flox}; K-Ras^{L53} mice was unexpected because these mice displayed a similar tumor burden compared to K-Ras^{L53} mice at early time points (data not shown). It suggests, however, that loss of only one copy of C-Raf is disadvantageous to K-Ras^{G12D}-mutant tumors. Similarly, C-Raf heterozygosity

Figure 3. Lung tumor development in the absence of Raf proteins. A, tumor burden in B-Raf^{flox/flox}, K-Ras^{G12D} (BBK), B-Raf^{+/−}, K-Ras^{L53} (BK), C-Raf^{flox/flox}, K-Ras^{L53} (CCK), C-Raf^{−/−}, K-Ras^{L53} (CK), and K-Ras^{L53} (K) mice at 6, 9, and 12 weeks post Ad-Cre infection. Burden was determined as percent disease area per total lung area and is significantly decreased in the absence of C-Raf. B, characterization of the spectrum of pulmonary preneoplasms in BBK, BK, CCK, CK, and K mice at 12 weeks post Ad-Cre infection. C-Raf loss reduced the number of BH, AAH, and adenomas, whereas B-Raf deficiency reduced only the number of BH. C, size of neoplasms was unchanged in BK, CCK, and CK mice but was decreased in BBK mice at 12 weeks post Ad-Cre infection. D, H&E staining of representative lung sections from BBK, CCK, and K mice 12 weeks post Ad-Cre infection. *P < 0.05, **P < 0.01, ***P < 0.001.
strongly impairs the development of SOS-driven epidermal tumors (17).

**MAPK Activity and Proliferation of Lung Neoplasms**

To evaluate Raf/MEK/ERK pathway activation, immunohistochemistry was employed to measure phosphorylated ERK in lung tumors of K-RasG12D mice harboring conditional Raf knockout alleles. Premalignant lesions of the lung have been previously reported to lack increased levels of pERK, whereas advanced tumors harboring concomitant loss of the tumor suppressor gene p53 demonstrated augmented activation of the MAPK pathway (18–20). Similarly, AAH, BH, and adenomas at 12 weeks post Ad-Cre infection showed no elevation of pERK levels compared to surrounding normal lung epithelia in all genotypes (data not shown). Thus, we examined pERK expression in lung tumors of the survival cohorts, as these tumors presumably had progressed to more advanced stages. Although the majority of tumors at the time of death of these animals displayed no increase in pERK staining (~80%; Fig. 4C, Supplementary Fig. 4A), a fraction of tumors exhibited elevated levels of pERK. We devised a grading system to assess the intensity of ERK phosphorylation in lung tumors. Weak pERK staining in less than 25% of cells in a tumor was considered low-intensity staining. Weak to moderate staining in more than 25% of cells or strong pERK positivity in less than half of the tumor was counted as medium-intensity staining. Tumors with high-intensity pERK staining displayed strong staining for pERK in more than 50% of cells. Intriguingly, although the lack of C-Raf had no effect on the percentage of tumors with increased ERK phosphorylation (Supplementary Fig. 4A), B-Raf−/−; K-RasG12D mice displayed fewer lung tumors with pERK positivity (Fig. 2C). Thus, K-RasG12D may activate the MAPK pathway in advanced lung tumors through B-Raf signaling.

Because the MAPK pathway is a major regulatory pathway of cell proliferation, we examined proliferation of lung neoplasms at 12 weeks post Ad-Cre infection by Ki-67 immunohistochemistry. The percentage of Ki-67-positive neoplastic cells in C-Raf−/−, K-RasG12D mice was unchanged (Supplementary Fig. 4B). In contrast, proliferation of AAH and early grade adenomas was reduced in B-Raf−/−; K-RasG12D mice (Fig. 4D), suggesting that reduced MAPK pathway activation in these tumors may result in impaired proliferation. Thus, although B-Raf is dispensable for initiation of alveolar neoplasms, B-Raf deficiency impairs proliferation of K-RasG12D mutant lung tumors, correlating with an extended survival of B-Raf−/−; K-RasG12D mice. These data support the notion that both B-Raf and C-Raf are important for K-RasG12D-driven lung tumorigenesis, albeit at different stages.

**Recombination of Raf Alleles In Vivo**

To confirm that decreased MAPK activation and proliferation in conditional B-Raf knockout tumors is due to B-Raf deficiency, tumor tissue was isolated from mice at end point by laser capture microdissection and genotyping PCRs were performed on the DNA isolated from these tissues. Notably, in 50% of tumors (5 of 10) a recombined floxed B-Raf product...
could be amplified, but the intact flox allele was absent (Fig. 4E), indicating that these tumors had recombined both floxed B-Raf alleles and are deficient for B-Raf. Examination of the remaining 5 tumors revealed that 4 (T1, T2, T4, and T6) had deleted at least 1 B-Raf<sup>ΔΔ</sup> allele and retained a variable but usually reduced content of unrecombined B-Raf<sup>ΔΔ</sup> allele, potentially representing the contribution of non-neoplastic cells. Only 1 tumor retained both wild-type copies of B-Raf (T8, Fig. 4E). Thus, B-Raf deficiency does not prevent tumor formation but correlates with impaired MAPK activation and proliferation. Tumor tissue was similarly isolated from C-Raf<sup>fl/fl</sup>, K-Ras<sup>G12D</sup>-<sup>fl/fl</sup> mice and recombination of floxed C-Raf alleles evaluated. Strikingly, any presence of a recombined C-Raf allele could only be detected in 2 of the 10 isolated tumors (T8, T9, Fig. 4F). However, these tumors displayed an incomplete loss of C-Raf, supporting the notion that K-Ras<sup>G12D</sup>-induced tumor formation is severely compromised in the absence of C-Raf. Interestingly, even heterozygous loss of C-Raf in C-Raf<sup>fl/wt</sup>, K-Ras<sup>G12D</sup>-<sup>fl/wt</sup> mice was minimally detected, further supporting the importance of C-Raf in K-Ras<sup>G12D</sup>-mediated oncogenesis (Fig. 4B). The fact that tumors that develop in C-Raf<sup>fl/wt</sup>, K-Ras<sup>G12D</sup>-<sup>fl/wt</sup> mice are indistinguishable from control tumors in terms of proliferation and MAPK activation (Supplementary Fig. 1A, B) substantiates the notion that these lesions are “escaper” tumors with normal C-Raf expression. Similar results have been obtained in Ras-driven epidermal tumors (17). Although excision of the conditional B-Raf and C-Raf alleles is easily accomplished in cell culture, there may be differential recombination efficiencies in vitro, or simply the outgrowth of Raf<sup>ΔΔ</sup>-, K-Ras<sup>G12D</sup>-lung epithelial “escaper” cells that have a fitness advantage in comparison to C-Raf<sup>ΔΔ</sup>-, K-Ras<sup>G12D</sup>-cells but not in comparison to B-Raf<sup>ΔΔ</sup>-K-Ras<sup>G12D</sup>-cells. Nonetheless, this experiment highlights the unique importance of intact C-Raf expression for the genesis of lung adenocarcinoma.

**DISCUSSION**

In this study, we described the role of B-Raf and C-Raf in K-Ras<sup>G12D</sup>-mediated transformation. In primary MEFs, a cell system commonly utilized to assess the oncogenicity of mutant Ras, simultaneous ablation of B-Raf and C-Raf is required to prevent the proliferative effects of K-Ras<sup>G12D</sup>. In contrast, K-Ras<sup>G12D</sup> promotes proliferation of cultured epithelial cells through C-Raf but not B-Raf. Furthermore, lung tumor initiation by K-Ras<sup>G12D</sup> is completely prevented by the absence of C-Raf.

Oncogenic K-Ras<sup>G12D</sup> is one of the most common cancer-associated genetic lesions; however, devising therapeutic approaches to target K-Ras<sup>G12D</sup> has been unsuccessful thus far and inhibition of Ras effector pathways may yield better results. In this study, we sought to determine the contribution of the Raf/MEK/ERK pathway to K-Ras<sup>G12D</sup>-mediated transformation in vitro and in vivo. Conditional A-Raf knockout mice have not yet been generated; therefore, we limited our analysis to B-Raf and C-Raf, the two best characterized Raf family members. K-Ras<sup>G12D</sup> has been demonstrated to partially transform primary MEFs (14), and we assessed the contribution of B-Raf and C-Raf to this phenotype. Individual ablation of either B-Raf or C-Raf did not obviously alter the cellular effects conferred by K-Ras<sup>G12D</sup>. Curiously, the levels of steady-state ERK phosphorylation were influenced by the absence of both B-Raf and C-Raf. Recently, a negative feedback loop was described in which ERK-dependent phosphorylation of C-Raf results in inactivation of the latter (21), and ERK-dependent phosphorylation of B-Raf reduces its binding to activated Ras and disrupts heterodimerization with C-Raf (22). Thus, feedback regulation of the MAPK pathway may be perturbed in the absence of B-Raf or C-Raf, leading to alterations in steady-state levels of pERK. However, the absence of either B-Raf or C-Raf does not impinge on the kinetics of MAPK activation upon serum stimulation, and steady-state pERK levels do not correlate with proliferation rates of these MEFs. Decreased pERK levels upon B-Raf ablation and elevated pERK upon C-Raf ablation have also been observed in fibroblasts expressing wild-type K-Ras as well as in other cell types (reviewed in ref. 23). Whether these differences in ERK activation have cell biological consequences may depend upon the cell type and/or the mutational status of MAPK-activating genes. In K-Ras<sup>G12D</sup>-mutant MEFs, B-Raf and C-Raf collectively relay the oncogenic signals from K-Ras<sup>G12D</sup>. Simultaneous ablation of B-Raf and C-Raf was required to negate the hyperproliferative effect of K-Ras<sup>G12D</sup>, supporting the notion of overlapping functions of B-Raf and C-Raf in this context.

To determine whether B-Raf and C-Raf are similarly redundant in epithelial cells, the cell type most commonly affected by K-Ras mutations in humans (9), we employed BMK cells. Kissil and colleagues (7) have previously shown that BMK cells are hyperproliferative following expression of K-Ras<sup>G12D</sup>. Indeed, we found that levels of pERK and the cell-cycle regulator cyclin D1 are elevated upon K-Ras<sup>G12D</sup> expression, resulting in higher proliferation rates (Fig. 2). Ablation of C-Raf impaired induction of cyclin D1, which correlated with a complete abrogation of the K-Ras<sup>G12D</sup>-mediated elevation of proliferation. These data show that C-Raf and B-Raf have unique functions in BMK cells and suggest that induction of at least cyclin D1 may be critical for the higher proliferative rates of epithelial cells upon K-Ras<sup>G12D</sup> expression.

In a lung cancer mouse model of oncogenic K-Ras<sup>G12D</sup>, we showed that C-Raf is crucial for lung tumorigenesis. Unexpectedly, none of the tumors analyzed displayed homozygous deletion of C-Raf. In fact, the vast majority of tumors retained both alleles of C-Raf, indicating that loss of only one allele is detrimental to tumor development. This is further supported by the observed extension of survival in C-Raf<sup>fl/wt</sup>, K-Ras<sup>G12D</sup>-<sup>fl/wt</sup> mice. C-Raf appears to be important at the early stages of tumor development, potentially tumor initiation, as suggested by the reduction in tumor burden and tumor number at early time points. We were unable to detect any increase in apoptosis immediately following Ad-Cre delivery or at later time points, thus the fate of such cells remains unclear. Homozygous deletion of B-Raf was tolerated by lung epithelial cells; however, it resulted in reduced MAPK activation and decreased proliferation of B-Raf-deficient low-grade tumors and correlated with an extended lifespan of B-Raf<sup>ΔΔ</sup>, K-Ras<sup>G12D</sup>-<sup>ΔΔ</sup> mice. Due to the viral titer used to deliver Ad-Cre, mice in this study succumbed to the overwhelming lung tumor burden and respiratory distress. Whether B-Raf affects proliferation or progression to high-grade...
adenocarcinoma could not be addressed in this study and remains to be determined. Our findings support the model that K-Ras<sup>G12D</sup> signals through C-Raf in both bronchiolar hyperplasia and adenomatous alveolar hyperplasia cells or a common progenitor cell type and that the loss of C-Raf impinges on the initiation of both of these preneoplasms. B-Raf, on the other hand, may participate once a cell fate decision has been made. Indeed, germline and conditional B-Raf and C-Raf knock-out mice revealed that these Raf proteins serve different functions in distinct cell types (24). Alternatively, the absence of B-Raf could influence this cell fate decision, leading to fewer bronchiolar hyperplasias. Interestingly, it has been noted that the importance of individual Raf proteins changes depending on the transformation status of a cell (25). Thus, it is plausible that B-Raf is dispensable for tumor initiation but becomes more important once an epithelial cell has been transformed by K-Ras<sup>G12D</sup>.

Recently, three studies reported that ATP-competitive Raf inhibitors have unexpected adverse effects in Ras-mutant cancer cells, where they promote Raf dimerization and MAPK hyperactivation (26–28). These pharmacological results argue that treatment of patients with Raf inhibitors could be detrimental if the Ras mutation status is unknown. However, our genetic analysis supports Raf proteins, and especially C-Raf, as potential therapeutic targets in K-Ras–mutant lung cancer. Because genetic ablation is not equivalent to pharmacological inhibition, future experiments are required to determine whether our genetic data are accurately predictive of a pharmacological response to complete Raf inhibition. In addition, once the genetic tools become available, the effect of Raf ablation in established tumors can be addressed. Patients treated with currently available Raf inhibitors often develop therapy-induced squamous cell carcinoma, potentially due to increased MAPK activation as mentioned above. Thus, our finding of the critical role of C-Raf in K-Ras<sup>G12D</sup>–mediated lung cancer development should stimulate further research to validate C-Raf as a therapeutic target and prompt the development of Raf inhibitors without detrimental side effects. Given the importance of C-Raf during tumor initiation, such agents may be useful not only for the treatment of advanced cancer but also as chemopreventive agents.

Previously, B-Raf was considered the main activator of the MAPK pathway because deletion of B-Raf, but not A-Raf or C-Raf, abrogated ERK phosphorylation in several cell types (23). However, our data show that in the context of K-Ras<sup>G12D</sup>–expressing mutant epithelial cells, C-Raf plays a more prominent role than B-Raf. Interestingly, two other reports implicated C-Raf as the major Raf member downstream of oncogenic Ras in different cancer types. In melanoma cells, oncogenic N-Ras signals to MEK/ERK via C-Raf (25). Moreover, C-Raf was required for the development and maintenance of squamous cell carcinomas induced by either an activated SOS transgene or 7,12-dimethylbenz(a)anthracene (DMBA)–12-O-tetradecanoylphorbol-13-acetate (TPA) carcinogenesis protocol (17). Thus, in several oncogenic contexts C-Raf appears to be the most critical Raf family member, providing an attractive target to inhibit during chemoprevention and intervention studies.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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