**Skin Tumorigenesis Stimulated by Raf Inhibitors Relies Upon Raf Functions That Are Dependent and Independent of ERK**

Eszter Doma, Christian Rupp, Andrea Varga, Florian Kern, Bettina Riegler, and Manuela Baccarini

**Abstract**

RAF inhibitors achieve unprecedented but mainly transient clinical responses in patients with melanoma whose tumors harbor an activating BRAF mutation. One notable side-effect of RAF inhibitors is the stimulation of cutaneous skin tumors, arising in about 30% of patients receiving these drugs, which are thought to develop as a result of inhibitor-induced activation of wild-type Raf in occult precursor skin lesions. This effect raises the possibility that less manageable tumors might also arise in other epithelial tissues. Here we provide preclinical evidence supporting this disquieting hypothesis by showing that the RAF inhibitors PLX-4032 (vemurafenib) and GDC-0879 precipitate the development of cell-autonomous, Ras-driven tumors in skin and gastric epithelia. The magnitude of the effects correlated with the inhibitors’ relative abilities to induce ERK activation. Epidermis-restricted ablation of either B-Raf or C-Raf prevented PLX-4032–induced ERK activation and tumorigenesis. In contrast, GDC-0879 induced ERK activation and tumorigenesis in B-Raf-deficient epidermis, whereas C-Raf ablation blocked GDC-0879–induced tumorigenesis (despite strong ERK activation) by preventing Rokα–mediated keratinocyte dedifferentiation. Thus, inhibitor-induced ERK activation did not require a specific Raf kinase. ERK activation was necessary, but not sufficient for Ras + Raf inhibitor-induced tumorigenesis, whereas C-Raf downregulation of Rokz was essential even in the face of sustained ERK signaling to prevent differentiation and promote tumorigenesis. Taken together, our findings suggest that combination therapies targeting ERK-dependent and -independent functions of Raf may be more efficient but also safer for cancer treatment.

**Introduction**

RAF inhibitors have revolutionized the treatment of metastatic patients with melanoma whose cancers harbor the BRAF mutation V600E, which increases kinase activity and leads to the constitutive activation of the MEK/ERK pathway (1–3). The side effects of the drugs are mild but include the development, in up to 30% of the patients, of keratoacanthomas or cutaneous squamous cell carcinomas (cSCC). In cultured cells, Raf inhibitors can paradoxically stimulate Raf kinase activity and MEK/ERK activation, particularly in the presence of activated Ras. The precise mechanism of action of the Raf inhibitors is yet unclear; activation/dimerization induced by the direct binding of the drugs to the Raf kinase domain (4, 5), relief of autoinhibitory P-loop phosphorylation (6), and a more complex mechanism involving reactivation of desensitized B-Raf (7) have been proposed to explain inhibitor-induced ERK activation. Inhibitor-induced ERK activation could potentially account for the development of therapy-related tumors in the patients with melanoma. These tumor harbor Ras mutations with variably increased frequency (from 17% to 60% in different studies; refs. 8–10). In addition, inhibitor treatment accelerates tumor development in a mouse model of chemical skin carcinogenesis (8), which partially depends on H-Ras and involves a strong inflammatory response (11). It is thus yet unclear whether Ras activation, in the absence of other mutations or of inflammation, is enough to license Raf inhibitor-driven tumorigenesis and whether B-Raf or C-Raf (Raf-1) is essential for the development of drug-induced epithelial tumors.

To test the effect of Raf inhibitors on tumor-prone epidermis, we used a transgenic mouse model in which the expression of a dominant active, farnesylated Son of Sevenless (SOS-F) in keratin 5 positive basal keratinocytes leads to the development of epidermal tumors (K5-SOS-F transgenic mice; ref. 12). This system has the advantage of being cell autonomous and not dependent on inflammation, which is absolutely necessary to support the growth of chemically induced skin tumors (11). These 2 features allow us to focus on the effect of the inhibitors on the signaling operating in premalignant cells, reducing the impact of other cell compartments (inflammatory cells) supporting tumorigenesis. In addition, the transgenic model mimics the situation of spontaneous human cSCC, in that it induces Ras and Erk activation in the absence of somatic Ras mutations (13), which are comparatively rare event in drug-related tumors (9).
Materials and Methods

Mouse strains and inhibitor treatment

Mouse strains and genotyping have been previously described (14). Strains were maintained on a 129Sv/Bl6 (F1) background. Animal experiments were authorized by the Austrian Ministry of Science and Communications, following the approval by the Ethical Committee for Animal Experimentation. GDC-0879, PLX-4032, synthesized and formulated according to published procedures (WO2006/084015 and WO2007/002325), and, in selected experiments GSK1120212 (trametinib) and Y-27632 were applied daily by gavage (100 mg/kg GDC-0879 in 0.5% Natrosol, citric acid pH 3.0: 100 mg/kg PLX-4032, high-bioavailability formulation in 5% Klucel, HCl pH 3.2, WO2010/114928; 0.5 mg/kg GSK1120212 in DMSO/1% Natrosol; 2 × 15 mg/kg Y-27632 in 0.5% Klucel). The Rok inhibitor Fasudil was added to the drinking water (exchanged every 3–4 days) at a concentration of 2 mg/mL.

Histological analysis

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed as described (14). For BrdU incorporation, mice were injected with BrdU (12.5 mg/g body weight) 1 hour before tissue isolation.

Cell culture

Mouse keratinocytes were isolated and maintained as described (14). HaCaT cells (15) were obtained from the DFKZ and SCC cell lines (16) were kindly provided by Gian-Paolo Dotto and maintained as previously described (16). COS7 cells were bought from Genlanitis and maintained according to the manufacturer’s instruction. The cell lines were not authenticated in our lab but were used shortly after resuscitation of preexisting tumors and the thickening of the epidermis of tail, eyelids, and ears (Fig. 1B and C). These phenotypes were already evident after 5 to 7 days of inhibitor treatment. The mice rapidly lost weight (around 20% in 1 week; Fig. 1D) and had to be euthanized. Necropsy revealed papilloma development in the squamous epithelia of esophagus and forestomach in 13 of 13 animals/inhibitor (Fig. 1E), likely causing weight loss and death. These epithelia express K5, but they do not develop tumors in the absence of inhibitor treatment. All phenotypes were most marked in GDC-0879-treated animals and correlated with an increase in proliferation (% BrdU+ cells) and a decrease in differentiation (% of epidermal layers positive for K10, expressed by suprabasal keratinocytes) in the affected tissues, and with a strong increase in ERK phosphorylation (Fig. 2A–C). Neither inhibitor caused tumor development, toxicity, or any other overt adverse effects in WT mice, indicating that the phenotypes depended on concomitant Ras activation. Some thickening of the tail, accompanied by an increase in proliferation (% of epidermal layers positive for K10, expressed by suprabasal keratinocytes) in the affected tissues, and with a strong increase in ERK phosphorylation (Fig. 2A–C). Neither inhibitor caused tumor development, toxicity, or any other overt adverse effects in WT mice, indicating that the phenotypes depended on concomitant Ras activation. Some thickening of the tail, accompanied by increased ERK phosphorylation and proliferation but not by dedifferentiation, could be observed at late time points in GDC-0879-treated WT animals (Supplementary Fig. S1). ERK activation in vivo was paralleled by MEK/ERK activation in cultured wild type and K5-SOS-F+ premalignant mouse keratinocytes treated with low concentrations of the inhibitors, as previously described (18) using α-B-Raf (Santa Cruz), α-C-Raf (Cell Signaling), or Rokα antibodies (BD Biosciences) coupled to ProteinG-Sepharose beads (Invitrogen). Immunoprecipitation of FLAG-tagged C-Raf was carried out using Anti-FLAG M2 affinity gel (Sigma-Aldrich).

Ras activation assay

Ras-GTP was pulled down from keratinocyte lysates using immobilized C-Raf RBD according to the manufacturer’s recommendation (Cytoskeleton Inc.).

Statistical analysis

Values are expressed as mean (±SD) of at least 3 independent experiments. P values were calculated using the 2-tailed Student t test. A P value <0.05 is considered statistically significant.

Results

We have treated the K5-SOS-F transgenic mice with 2 selective Raf inhibitors: PLX-4032 (vemurafenib; refs. 1–3, 19), currently used in the clinical treatment of melanoma; and GDC-0879 (20, 21), which is not used in the clinic but has higher affinity for Raf and reportedly increases proliferation in mouse epidermis (5). The estimated IC50 values of the inhibitors at 1 mmol/L ATP concentration are similar for wild-type B-Raf and C-Raf; in both cases, GDC-0879 is more potent with an IC50 of 34 or 180 nmol/L against B- or C-Raf, respectively, compared with the 530 and 1100 nmol/L IC50 of the tool compound PLX-4720, the precursor of PLX-4032 (5). The inhibitors, applied daily by gavage, could be detected in mouse plasma (Fig. 1A); in particular, the plasma exposures to PLX-4032 were comparable to those necessary to achieve melanoma regression in patients (1, 2). Both drugs caused a range of skin phenotypes in K5-SOS-F transgensics, including the growth of preexisting tumors and the thickening of the epidermis of tail, eyelids, and ears (Fig. 1B and C). These phenotypes were already evident after 5 to 7 days of inhibitor treatment. The mice rapidly lost weight (around 20% in 1 week; Fig. 1D) and had to be euthanized. Necropsy revealed papilloma development in the squamous epithelia of esophagus and forestomach in 13 of 13 animals/inhibitor (Fig. 1E), likely causing weight loss and death. These epithelia express K5, but they do not develop tumors in the absence of inhibitor treatment. All phenotypes were most marked in GDC-0879-treated animals and correlated with an increase in proliferation (% BrdU+ cells) and a decrease in differentiation (% of epidermal layers positive for K10, expressed by suprabasal keratinocytes) in the affected tissues, and with a strong increase in ERK phosphorylation (Fig. 2A–C). Neither inhibitor caused tumor development, toxicity, or any other overt adverse effects in WT mice, indicating that the phenotypes depended on concomitant Ras activation. Some thickening of the tail, accompanied by increased ERK phosphorylation and proliferation but not by dedifferentiation, could be observed at late time points in GDC-0879-treated WT animals (Supplementary Fig. S1). ERK activation in vivo was paralleled by MEK/ERK activation in cultured wild type and K5-SOS-F+ premalignant mouse keratinocytes treated with low concentrations of the inhibitors.
Figure 1. Raf inhibitors accelerate tumor formation. A, the concentration of GDC-0879 or PLX-4032 in the plasma of mice was determined at the indicated time points after application of the inhibitors via gavage and used to determine AUC. The plasma exposures to PLX-4032 were comparable to those necessary to achieve melanoma regression in patients (1; 2). The total plasma exposure was higher for PLX-4032 than for GDC-0879. B and C, thickening of the epidermis of the tail and eyelids in K5-SOS-F+ mice treated with vehicle, GDC-0879, or PLX-4032 for 8 consecutive days. D, inhibitor-treated K5-SOS-F+ mice rapidly lose weight. Mice (n = 6) were weighed every day and euthanized as soon as the weight loss reached 20%. **, P < 0.01. E, massive hyperproliferation in the esophagus of inhibitor-treated K5-SOS-F+ mice. Top, H&E-stained sections; middle, expression of the basal keratinocyte marker K5 in the suprabasal layers of the epithelia; bottom, increased proliferation in the esophagus of inhibitor-treated mice. Proliferation was assessed by BrdU incorporation. Positive cells are stained in brown. Scale bars, 100 μm.
whereas high concentrations reduced phosphorylation (Fig. 3A). Inhibitor treatment similarly increased ERK phosphorylation in human keratinocytes and squamous cell carcinoma cell lines (Supplementary Fig. S2; refs. 15 and 16). GDC-0879 and, to a lesser extent, PLX-4032 induced B-Raf-C-Raf heterodimerization in primary keratinocytes (Fig. 3B). More importantly, heterodimerization could be clearly detected in epidermal lysates of inhibitor-treated animals (Fig. 3C and D). GDC-0879 was more effective than PLX-4032 in inducing ex vivo MEK/ERK activation and Raf heterodimerization, in good correlation with the more pronounced effects achieved in vivo at lower GDC-0879 plasma exposures (Fig. 1A).

Figure 2. Raf inhibitor treatment is associated with increased proliferation, ERK phosphorylation, and dedifferentiation in vivo. A–C, thickening of the tail epidermis in K5-SOS-F+ mice treated with vehicle, 100 mg/kg GDC-0879, or 100 mg/kg PLX-4032 orally for 8 consecutive days. A, H&E-stained sections. B, increased proliferation and ERK phosphorylation. C, reduced differentiation in the epidermis of inhibitor-treated K5-SOS-F+ mice. Proliferation was assessed by BrdU incorporation. Keratin 10 (K10) was used as a marker of differentiating, suprabasal keratinocytes. BrdU, pERK, and K10 positive cells are stained in brown. The plots show the percentage of BrdU- and pERK-positive cells and the percentage of K10+ layers in vehicle and inhibitor-treated mice (mean ± SD; n = 3; *, P < 0.01). Dotted lines, the boundary between epidermis and dermis. Scale bars, 100 µm.
Importantly, the MEK inhibitor trametinib reduced both Ras and Ras\(^+\)Raf inhibitor-induced tail tumor formation, indicating that activation of the ERK pathway is necessary for this process (Supplementary Fig. S3).

The existing literature predominantly indicates that inhibitor-induced ERK activation in cultured cells depends on C-Raf (4, 5, 7). Raf inhibitors might simply induce conformational changes that promote the folding of the Raf kinase domain and therefore dimerization (4, 5), or preclude autoinhibitory P-loop phosphorylation (6). Alternatively, there might be a more complex mode of action, involving the inhibition of B-Raf activity and release of inactive B-Raf from a cytosolic complex, which, in Ras-transformed cells, leads to B-Raf plasma membrane localization and formation of a Ras/B-Raf/C-Raf complex (7). To investigate which of these models is operating \textit{in vivo} during Ras- and Raf inhibitor-driven tumorigenesis, we combined the \textit{K5-SOS-F} transgenic mice with epidermis-restricted B-Raf or C-Raf ablation (\textit{K5-SOS-F;\textcolor{red}{b-raf}KOep} and \textit{K5-SOS-F;\textcolor{red}{c-raf}KOep}, knockout in the epidermis). Using these mice, we have previously demonstrated that both the development and the maintenance of Ras-driven tumors absolutely require the presence of C-Raf, not as an Erk activator but rather as an endogenous inhibitor of the cytoskeleton-based kinase Rok\(\alpha\) (Rock2), which induces keratinocyte differentiation (14). The effect of B-Raf ablation is milder, but it still leads to an impressive reduction of tumor number and size that correlates with reduced ERK activation in the epidermis (22). Both \textit{K5-SOS-F;\textcolor{red}{c-raf}KOep} and \textit{K5-SOS-F;\textcolor{red}{b-raf}KOep} mice were more resistant to treatment with Raf inhibitors. Neither strain experienced dramatic weight loss or death. Epidermis-restricted B-Raf or C-Raf ablation protected \textit{K5-SOS-F\textcolor{red}{+}} animals from the effects of PLX-4032 (Supplementary Fig. S4). However, treatment with GDC-0879, a

![Figure 3](https://www.aacrr.org/)

**Figure 3.** Effect of the inhibitors on mouse primary keratinocytes and epidermis. A, immunoblot analysis of lysates from primary wild type (WT) and \textit{K5-SOS-F\textcolor{red}{+}} keratinocytes treated with different concentrations of GDC-0879 or PLX-4032. B, GDC-0879 and, to a lesser extent, PLX-4032 promote Raf heterodimerization in WT and \textit{K5-SOS-F\textcolor{red}{+}} primary keratinocytes. C, differential centrifugation for the production of K5/K10\textcolor{red}{+}-enriched epidermal lysates. Epidermal lysates were centrifuged at progressively increasing \(g\), and the pellets were subjected to immunoblotting. The pellets of the first two centrifugation steps were enriched in K5\textcolor{red}{+} basal cells and K10\textcolor{red}{+} suprabasal cells. Active transglutaminase 1 (Tgase1), a marker of progressive differentiation, was present in increasing amounts in the pellets of the last three centrifugation steps. All the proteins at study were present in the first two pellets, which were therefore pooled and used to determine the effect of inhibitor treatment on signaling \textit{in vivo}. D, GDC-0879 and, to a lesser extent, PLX-4032 promote Raf heterodimerization in \textit{K5-SOS-F\textcolor{red}{+}} epidermis from systemically inhibitor treated mice. The presence of A-, B-, and C-Raf, total (tMEK), phosphorylated MEK (pMEK), total (tERK), and phosphorylated ERK (pERK) was detected by immunoblotting. GAPDH is shown as a loading control. D, DMSO. \textcolor{red}{+}, longer exposure.
more potent Raf activator, induced the development, albeit delayed, of large tail tumors in 8 of 8 K5-SOS-F;b-rafKOep mice (Fig. 4A and B). Thus, B-Raf is required for Ras-induced tumorigenesis (22), and specifically tumorigenesis induced by Ras + PLX-4032, but this requirement is alleviated by treatment with GDC-0879. In contrast, 22 of 22 K5-SOS-F;c-rafKOep mice were resistant to GDC-0879 treatment and merely showed moderate thickening of the tail epidermis and lengthening of claws (Fig. 4A and B and Supplementary Fig. S5A). Importantly, Ras is activated at similar levels in K5-SOS-F, K5-SOS;c-rafKOep, and K5-SOS-F;b-rafKOep primary keratinocytes (Fig. 4C).

Spontaneous regression of therapy-related cutaneous SCC upon discontinuation of inhibitor treatment has been observed in the clinic (9). In the K5-SOS-F transgenic mice treated with PLX-4032 (clinically relevant situation), tail tumors continued to develop after inhibitor treatment stopped (Supplementary Fig. S6A). This result was expected because the SOS-F transgene can drive the tumors independently of inhibitor treatment. However, the mice regained some weight (Supplementary Fig. S6B) and the thickening of the esophageal epithelia brought about by the Raf inhibitor stopped (Supplementary Fig. S6B). In addition, the interruption of GDC-0879 treatment, which induces tail tumors in

Figure 4. C-Raf, but not B-Raf ablation, prevents tumor development in inhibitor-treated K5-SOS-Fþ mice. A, tail tumors developed in K5-SOS-F; b-rafKOep mice treated with GDC-0879, but not with PLX-4032 for 21 days. K5-SOS-F;c-rafKOep mice displayed slight epidermal thickening, but did not develop any tumors. B, the plot shows the volume of tumor-affected tails (mean ± SD; n = 8/genotype; P < 0.01). C, the K5-SOS-F transgene drives Ras activation in f/f and KOep primary keratinocytes. Ras-GTP was pulled down from keratinocyte lysates using immobilized C-Raf RBD. The presence of SOS, SOS-F, B-Raf, C-Raf, and Ras was detected by immunoblotting. D (top), H&E-stained sections of the tail of GDC-0879–treated mice. Middle and bottom, proliferation (% of BrdU positive cells) and ERK phosphorylation in the epidermis of inhibitor-treated K5-SOS-Fþ mice of different genotypes. Note the increased proliferation and ERK activation in the epidermis of GDC-0879–treated K5-SOS-F; c-rafKOep mice. E, reduced differentiation (% of K10 positive epidermal layers) and cofilin phosphorylation (pCofilin) in K5-SOS-F; b-rafKOep treated with GDC-0879. The plots in F show a quantification of tissues obtained from GDC-0879 and PLX-4032–treated mice of the indicated genotypes (mean ± SD; n = 3; P < 0.01). Scale bars, 100 μm.
the otherwise tumor-refractory K5-SOS-F; b-rafKOep mice, stopped the growth of inhibitor-induced tumors by reducing proliferation and increasing differentiation (Supplementary Fig. S6C and S6D).

Interestingly, GDC-0879 induced proliferation and ERK phosphorylation to similar extents in B- or C-Raf–deficient K5-SOS-F+ epidermis (Fig. 4D and F). Moreover, GDC-0879 maintained ERK signaling in cells deficient in both B-Raf and

Figure 5. GDC-0879 treatment activates ERK signaling in K5-SOS-F+ B-Raf− or C-Raf− deficient epidermis. A, immunohistochemical analysis of the phosphorylation of a cytosolic (pRSK) and a nuclear (pELK) target of ERK, as well as of the expression of ERK-inducible gene products (cFOS, cyclin D1). Positive cells are stained in brown. B, the plots show the percentage of positive cells observed in mice of the indicated genotypes (mean ± SD; n = 3; *, P < 0.05; **, P < 0.01). Scale bars, 100 μm.
C-Raf, likely because of stimulation of signaling through A-Raf (Supplementary Fig. S7). C-Raf-deficiency, however, was sufficient to reverse Ras+GDC-0879 induced de-differentiation. K10-negative cells were confined to the basal layer, and reinstatement of differentiation correlated with the presence of pCofilin (Fig. 4E and F) and the absence of pSTAT3 and nuclear Myc staining (Supplementary Fig. S5B), a sign that Rokα signaling was activated in the epidermis of inhibitor-treated K5-SOS-F;c-rafKOep mice (14). Proliferation, differentiation, ERK, and cofilin phosphorylation were indistinguishable in vehicle and PLX-4032-treated K5-SOS-F;c-rafKOep and K5-SOS-F;b-rafKOep mice (Supplementary Fig. S4A and S4B). To exclude the possibility that the ablation of B- or C-Raf has different effects on downstream ERK signaling, we tested the phosphorylation of cytosolic (RSK) and nuclear (ELK1) ERK targets, as well as the expression of ERK-induced gene products (cFos, cyclin D1) in the epidermis of vehicle- or inhibitor-treated K5-SOS-F;c-rafKOep and K5-SOS-F;b-rafKOep mice (Fig. 5). In all cases, GDC-0879-induced ERK downstream signaling was activated at comparable levels in the B- and C-Raf-deficient epidermis. To obtain a more quantitative measure of these changes, we monitored signaling in K5+/K10+ epidermal lysates (Fig. 6A). This method revealed a reduction in the amount of C-Raf, B-Raf, and Rokα in inhibitor-treated mice, irrespective of their genotype; in contrast, the amount of cofilin was increased. The basis of these changes is currently unknown. Irrespective of this, the experiment shows similar ERK downstream signaling in inhibitor-treated B- and C-Raf-deficient epidermis, although there was a tendency toward increased ERK signaling in vehicle-treated C-Raf KO tissue (Figs. 5 and 6A; ref. 22); in contrast, phosphorylation of the

![Diagram](https://example.com/diagram.png)

**Figure 6.** GDC-0879 activates ERK signaling without interfering with the C-Raf/Rokα interaction. A, immunoblot analysis of epidermal lysates (K5+/K10+ cells) from mice of the indicated genotypes treated with GDC-0879 for 8 days. B-and C-Raf, phosphorylated ERK (pERK), total (tERK), phosphorylated RSK (pRSK), cFos p62 and p40, cyclin D1, Rokα, phosphorylated cofilin (pCofilin), and total (tCofilin) were detected by immunoblotting. GAPDH is shown as a loading control. The upregulation of cFos is most clearly seen in the p40 product. B, complex formation between C-Raf and Rokα is increased in B-Raf KO epidermis and is not affected by GDC-0879 treatment in vivo. Rokα (top) or C-Raf (bottom) were immunoprecipitated from epidermal lysates obtained from mice of the indicated genotypes, untreated or treated with GDC-0879 for 8 days. The presence of Rokα, B-Raf, and C-Raf was visualized by immunoblotting. Note the reduced amount of C-Raf i.p. loaded in lane 2 of the top panel. C, Raf dimerization is dispensable for the C-Raf/Rokα interaction. FLAG-tagged wild-type C-Raf or a dimerization mutant incapable of Raf dimerization was expressed in COS-7 cells. GDC-0879-treated or control cells were lysed and the presence of Rokα and B-Raf in the FLAG-C-Raf immunoprecipitates as well as ERK and cofilin phosphorylation in the lysates was determined by immunoblotting.
Rok downstream target coflin was abolished by GDC-0879 in B-Raf–deficient epidermis, but was maintained in C-Raf–deficient epidermis (Fig. 6A).

We next investigated whether Raf inhibitors had any influence on the interaction between Rokα and C-Raf by immunoprecipitating Rokα from epidermal lysates of GDC-0879 or vehicle-treated mice. We found that inhibitor treatment did not affect the amount of C-Raf co-precipitating with Rokα, but that more C-Raf bound to Rokα in B-Raf–deficient lysates; B-Raf could not be detected in Rokα immunoprecipitates from epidermal lysates of vehicle- or inhibitor-treated mice (Fig. 6B). This result was confirmed by assessing the amount of Rokα and B-Raf in C-Raf immunoprecipitates from the same epidermal lysates. In addition, a Raf dimerization-deficient C-Raf mutant (4) failed to form heterodimers with B-Raf but it still bound to Rokα in a manner similar to wild-type C-Raf in both untreated and Raf inhibitor-treated cells. Consistently, ERK but not coflin phosphorylation was affected by GDC-0879 treatment or by the expression of the Raf dimerization-deficient C-Raf mutant (Fig. 6C). These results show that C-Raf homodimer formation is not required for Rokα binding or inhibition. They are in line with our previous data showing that C-Raf/Rokα interaction is a nonkinase function of C-Raf mediated by the N-terminal regulatory domain of C-Raf (17, 23, 24) and further support the hypothesis that Ras + Raf-inhibitor–induced tumors requires both Raf-mediated ERK activation and a kinase-independent Rokα inhibition by C-Raf.

To causally link blockade of tumorigenesis and maintenance of differentiation to the lack of C-Raf's inhibition of Rokα, we treated K5-SOS-F;Ec-rafKOep mice with both Raf and Rokα inhibitors (Figs. 7 and S8). Medium-term (18 days) treatment with the selective and potent Raf inhibitor Y-27632, which reactivates tumor development in K5-SOS-F;Ec-rafKOep epidermis (14), promoted proliferation in the GDC-0879 treated K5-SOS-F;Ec-rafKOep forestomach (Supplementary Fig. S8A); and longer treatment with another Rokα inhibitor, Fasudil, inhibited differentiation and induced proliferation as well as the development of squamous cell carcinomas of the forestomach. This was accompanied by loss of coflin phosphorylation (Fig. 7A–C). Surprisingly, cotreatment with Fasudil did not induce tumor formation in the epidermis. This seems to be because of a side-effect of the inhibitor in the epidermis, namely the concomitant inhibition of Rokα signaling and ERK phosphorylation (Supplementary Fig. S8B), sporadically observed in other tissues (25–27). ERK inhibition by Fasudil is not observed in the forestomach (Fig. 7B), reinforcing the concept that both ERK activation and the dimming of Rokα signaling are required for the formation of Ras plus Raf inhibitor-driven epithelial tumors.

Discussion

Our results provide formal proof that Raf inhibitors increase Ras-dependent ERK activation in vivo, and show that Ras activation suffices, in the absence of other mutations and tumor-promoting inflammation, to render keratinocytes more prone to forming tumors upon treatment with Raf inhibitors. The development of drug-induced tumors in the K5-SOS-F+ esophagi and forestomachs, where cells express K5 but do not give rise to tumors unless treated, indicates that Raf inhibitors expand the spectrum of epithelial tumors induced by Ras activation. Relevant for drug use in patients, both KRAS and BRAF mutations can be detected in high-grade Barrett's intraepithelial neoplasias and in Barrett's adenocarcinoma (28).

Our results support a model in which inhibitor-driven Raf and ERK activation are necessary and sufficient for hyperproliferation of the epidermis; in the presence of SOS-F, differentiation is suppressed, leading to tumor formation (Supplementary Fig. S9). This is consistent with the assumption that the level of ERK activation is rate limiting for tumorigenesis (1), a concept that advocates the combination therapies using Raf and MEK inhibitors (29) currently being tested in clinical trials (30, 31). In support of this, PLX-4032, a weaker promoter of Raf activation and ERK phosphorylation, does not lead to increased proliferation in wild-type epidermis (Supplementary Fig. S1); similarly, reducing ERK activation by cotreatment with Trametinib (Supplementary Fig. S3) or by ablation of either B-Raf or C-Raf (Supplementary Fig. S4) abrogates SOS-F– and PLX-4032–induced ERK activation and tumorigenesis; thus, B- and C-Raf are equally important in the context of PLX-4032–induced pathway activation.

In contrast to PLX-4032, GDC-0879 can induce sustained ERK activation and proliferation in WT epidermis, and increases Ras-driven tumorigenesis in the absence of B-Raf (Fig. 4). Taking into account that GDC-0879–induced ERK activation is reduced in cells expressing a C-Raf dimerization mutant (Fig. 6C; ref. 4), the most likely explanation for this finding is that GDC-0879, but not PLX-4032, can facilitate Raf activation in the context of C-Raf homodimers or of heterodimers containing A-Raf, as previously reported (5). In support of this, GDC-0879 but not PLX-4032 is able to induce ERK activation in keratinocytes lacking both B-Raf and C-Raf (Supplementary Fig. S7).

Mechanistically, the fact that GDC-0879 sustains ERK activation in the absence of both B- or C-Raf in vivo indicates that B- and C-Raf are redundant in this context and therefore that conformational changes leading to kinase maturation/dimerization involving A-Raf (4, 5), rather than a more complex mechanism involving reactivation of desensitized B-Raf (7), are responsible for Raf/ERK activation and keratinocyte proliferation. Because Raf heterodimerization is not readily detected in other cell types (5, 7), these data suggest a potential explanation for the selective effects of the drugs on the squamous epithelia.

The increase in ERK activation and proliferation is necessary but not sufficient for the development of tumors; concomitant dedifferentiation, induced by SOS-F and requiring C-Raf as endogenous inhibitor of the Rokα signaling pathway, is also indispensable (Supplementary Fig. S9). This concept is clearly illustrated by the lack of tumor development in SOS-F–C-Raf–deficient epidermis treated with GDC-0879, despite increased ERK activation and proliferation (Figs. 4 and 5); and by the induction of squamous cell carcinomas in the forestomach of K5-SOS-F;Ec-rafKOep mice treated with both Raf and Rokα inhibitors (Fig. 7).
Rokα inhibition is a nonkinase function of C-Raf, which can be carried out both by a kinase-dead mutant (17) and by truncated C-Raf lacking the kinase domain (23, 24). Inhibition is mediated by the physical interaction of the C-Raf regulatory domain with the Rokα kinase domain (23). This interaction is not affected by Raf-inhibitor–induced Raf hetero-
homodimerization, but it is increased in SOS-F+, B-Raf-deficient epidermis; whether B-Raf ablation frees up an additional pool of C-Raf for Rok interaction or whether more indirect mechanisms are responsible for this effect is currently unclear. Collectively, our results verify the disquieting hypothesis that Raf inhibitors dramatically exacerbate Ras-driven tumorigenesis *in vivo*, and extend the range of target tissues to gastric epithelia. Drug-induced epidermal tumors develop in mice with epidermis-restricted B-Raf ablation, whereas C-Raf ablation prevents tumorigenesis despite increased ERK activation by enforcing keratinocyte differentiation. These data suggest that combination therapies targeting kinase and non kinase functions of Raf may be more efficient and safer for the treatment of skin tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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