Kinase-Dead BRAF and Oncogenic RAS Cooperate to Drive Tumor Progression through CRAF

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SUMMARY

We describe a mechanism of tumorigenesis mediated by kinase-dead BRAF in the presence of oncogenic RAS. We show that drugs that selectively inhibit BRAF drive RAS-dependent BRAF binding to CRAF, CRAF activation, and MEK–ERK signaling. This does not occur when oncogenic BRAF is inhibited, demonstrating that BRAF inhibition per se does not drive pathway activation; it only occurs when BRAF is inhibited in the presence of oncogenic RAS. Kinase-dead BRAF mimics the effects of the BRAF-selective drugs and kinase-dead BRAF and oncogenic Ras cooperate to induce melanoma in mice. Our data reveal another paradigm of BRAF-mediated signaling that promotes tumor progression. They highlight the importance of understanding pathway signaling in clinical practice and of genotyping tumors prior to administering BRAF-selective drugs, to identify patients who are likely to respond and also to identify patients who may experience adverse effects.

INTRODUCTION

The RAS–ERK (extracellular-signal regulated protein kinase) MAPK (mitogen-activated protein kinase) signaling pathway regulates cell responses to environmental cues (Marshall, 1995) and plays an important role in human cancer (Gray-Schopfer et al., 2007). The pathway comprises the RAS small guanine-nucleotide binding protein and the protein kinases RAF, MEK (mitogen and extracellular-regulated protein kinase kinase), and ERK. RAS is attached to the inner face of the plasma membrane and is activated downstream of growth factor, cytokine, and hormone receptors. Active RAS recruits RAF to the membrane for activation through a complex process involving changes in phosphorylation and binding to other enzymes and scaffold proteins (Kolch, 2000). RAF phosphorylates and activates MEK, which phosphorylates and activates ERK.

The complexity of this pathway is increased by the multiplicity of its components. There are three RAS (HRAS, NRAS, and KRAS), three RAF (ARAF, BRAF, and CRAF), two MEK (MEK1 and MEK2), and two ERK (ERK1 and ERK2) genes that encode proteins with nonredundant functions. Furthermore, the pathway is not linear. BRAF binds to and activates CRAF in a RAS-dependent manner that appears to require CRAF transphosphorylation by BRAF (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001), providing subtle pathway regulation that is not fully understood. ERK phosphorylates many substrates and the duration and intensity of its activity affects how cells respond to extracellular signals (Marshall, 1995). Thus, the pathway must be carefully controlled to ensure appropriate responses to environmental cues. In normal cells, outcomes include survival, proliferation, senescence, and differentiation, but in cancer the constitutive pathway activation favors proliferation and survival.

RAS–ERK signaling is particularly important in melanoma. Somatic mutations occur in BRAF, NRAS, and KRAS in 43%, 20%, and 2% of melanomas respectively (www.sanger.ac.uk/genetics/CGP/cosmic/). The mutations in RAS trap it in a GTP-bound, active conformation and mostly involve glycine 12 (G12), glycine 13 (G13), and glutamine 61 (Q61). A glutamic acid substitution for the valine at position 600 (V600BRAF) accounts for over 90% of the mutations in BRAF in cancer. However, over 100 other rare mutations have been described, most of which cluster to the glycine-rich loop and activation segment in the kinase domain. These regions normally trap BRAF in an inactive conformation by forming an atypical intramolecular interaction, and it is thought that the mutations disrupt this interaction, thereby allowing the active conformation to prevail (Wan et al., 2004).

Functional studies have shown that most of the mutations in BRAF are activating and enhance its ability to directly phosphorylate MEK (Wan et al., 2004; Garnett and Marais, 2004). Curiously however, some mutants have impaired activity and
Raf Inhibitors Induce BRAF Binding to CRAF in RAS Mutant Cells

Wild-type BRAF binds to CRAF in a RAS-dependent manner and although this binding is weak, it leads to CRAF activation (Garnett et al., 2005). Since RAS and CRAF are required for ERK activation by PLX4720 and 885-A, we investigated if these drugs induce BRAF binding to CRAF. Endogenous BRAF was immunoprecipitated from melanoma cells and western blotted for endogenous CRAF. We show that CRAF did not bind to BRAF in untreated or PD184352 treated WM852, D04, MM415, or MM485 cells (Figure 2A), demonstrating that MEK inhibition does not induce binding. In contrast, sorafenib and 885-A induced strong binding of BRAF to CRAF in all four lines (Figure 2A). We also performed the experiment in the inverse manner, immunoprecipitating CRAF and showing that BRAF binding was strongly induced by sorafenib and 885-A (Figure 2A). Curiously, PLX4720 did not appear to induce BRAF binding to CRAF, but previous studies have shown that ERK phosphorylates BRAF in a negative-feedback loop that destabilizes its binding to CRAF (Rushworth et al., 2006). We show that PD184352 stabilizes BRAF binding to CRAF in the presence of PLX4720 (Figure 2B), demonstrating that PLX4720 does induce binding, albeit less strongly than the other drugs. In addition to inducing BRAF binding to CRAF in NRAS mutant cells, 885-A and sorafenib also induce this binding in WM1791c melanoma cells and in SW620 and HCT116 colorectal carcinoma cells (Figure 2C), all of which express mutant KRAS (Table S2). Importantly, no strong binding of BRAF to CRAF
was seen in A375 cells even in the presence of PD184352 and the drugs did not induce strong BRAF binding to CRAF in two other BRAF mutant melanoma cell lines (Figure 2D and Figure S2).

**BRAF Binding to CRAF Is Mediated by RAS**

Thus, sorafenib, 885-A and PLX4720 all induced BRAF binding to CRAF in NRAS or KRAS mutant cells, but not in BRAF mutant cells, showing that BRAF inhibition per se did not induce this binding; it only occurred when BRAF was inhibited in the presence of oncogenic RAS. To confirm the essential role of RAS, we show that a CRAF mutant (R89LCRAF) that cannot bind to RAS (Fabian et al., 1994) did not bind to BRAF (Figure 3A and Figure S3A) and the corresponding mutant of BRAF (R188LBRAF) did not bind to CRAF (Figure 3B and see Figure S3B). We also prepared membrane/cytosol fractionations of RAS mutant cells and show that under normal conditions over 40% of CRAF is in the membrane, whereas BRAF is largely cytosolic (Figure 3C). Notably, 885-A treatment leads to strong recruitment of BRAF to the membrane fraction, whereas CRAF is only weakly affected (Figure 3C). We also show that under normal conditions, EGF did not induce BRAF binding to CRAF in PMWK cells and this resulted in sustained pathway activation (Figure 3D). This shows that BRAF binding to CRAF is induced in the presence of both oncogenic RAS and activated wild-type RAS.

We note that sorafenib and 885-A induce a mobility shift in BRAF in SDS-gels (Figure 2A). BRAF also undergoes a mobility shift in PLX4720 treated cells in the presence of PD184352 (Figure 2B). This mobility shift is reduced when immunoprecipitated BRAF is treated with calf intestinal alkaline phosphatase (CIP; Figure 3E) and PD184352 pretreatment reduced, but did not ablate the magnitude of the shift induced by 885-A (Figure 3F). Importantly, in vitro CIP treatment and cell pretreatment with PD184352 did not prevent BRAF binding to CRAF (Figures 3E and 3F). Together, these data suggest that the BRAF bound to CRAF is hyperphosphorylated through MEK–ERK-dependent and MEK–ERK-independent mechanisms, but that this phosphorylation is not required for BRAF binding to CRAF.

**BRAF Inhibition Activates CRAF**

To test directly if BRAF binding to CRAF is driven by 885-A binding to BRAF, we mutated the so-called “gatekeeper threonine” (T529) of BRAF to asparagine (T529N). Since BRAF is not active in RAS mutant melanoma cells (Figure 1E), we measured T529NBRAF activity using transient expression in COS cells (Wan et al., 2004). The results show that T529NBRAF is still activated by G12VHRAS, G12VRAS and G12VKRAS (Figure 4A and Figure S4A). Importantly, T529NBRAF is ~170-fold less sensitive to 885-A than wild-type BRAF (17 nM versus 2669 nM; Figure 4B) and 885-A

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**Figure 1. BRAF Inhibitors Activate CRAF, MEK, and ERK in RAS Mutant Cell Lines**

(A) A375, D04, MM415, MM485, and WM852 cells were treated with DMSO (−), PD184352 (PD; 1 μM), sorafenib (SF; 10 μM), 885-A (1 μM) and PLX4720 (PLX; 0.3 μM) for 4 hr. Cell extracts were western blotted for phospho-ERK (ppERK) and total ERK2 (loading control).

(B and C) D04 cells were transfected with siRNA against NRAS or CRAF, or control (Mock) as indicated. After 48 hr the cells were treated with DMSO (−), 885-A (1 μM) or PLX4720 (PLX; 0.3 μM) for 4 hr. Cell lysates were western blotted for NRAS, CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK) and tubulin (loading control).

(D) D04 cells were treated with 885-A for various times and endogenous CRAF kinase activity was measured. Data show fold activation of experimental triplicates compared to untreated cells with error bars to represent standard deviations from the means.

(E) Endogenous BRAF kinase activity was measured in A375 or D04 cells. The results (arbitrary units per μg of cell protein) are the mean of an assay performed in triplicate with error bars to represent standard deviation from the mean.
Figure 2. BRAF Inhibitors Induce CRAF Binding to BRAF
(A) WM852, D04, MM415 and MM485 cells were treated with DMSO (−), PD184352 (PD; 1 μM), sorafenib (SF; 10 μM), 885-A (1 μM) or PLX4720 (PLX; 0.3 μM) for 4 hr. Endogenous BRAF (IP: BRAF) or endogenous CRAF (IP: CRAF) were immunoprecipitated and the immunocomplexes were western blotted (WB) for BRAF or CRAF. BRAF, and CRAF levels in the cell lysates are also shown.

(B) D04 cells were treated with DMSO (−), PD184352 (PD; 1 μM), sorafenib (SF; 10 μM) and PLX4720 (PLX; 0.3 μM) for 4 hr. Endogenous CRAF (IP: CRAF) was immunoprecipitated and the immunocomplexes were western blotted (WB) for BRAF or CRAF. BRAF and CRAF levels in the cell lysates are shown.

(C) SW620, HCT116 and WM1791c cells were treated with DMSO (−), PD184352 (PD; 1 μM), sorafenib (SF; 10 μM) or 885-A (1 μM) for 4 hr. Endogenous BRAF (IP: BRAF) or endogenous CRAF (IP: CRAF) were immunoprecipitated and the immunocomplexes were western blotted (WB) for BRAF or CRAF. The cell lysates were also blotted for BRAF, CRAF, phospho-ERK (ppERK) and total ERK2 (loading control).

(D) A375 cells were treated with DMSO (−), PD184352 (PD; 1 μM), sorafenib (SF; 10 μM), 885-A (1 μM) or PLX4720 (PLX; 0.3 μM) for 4 hr. CRAF (IP: CRAF) was immunoprecipitated and the immunocomplexes were western blotted (WB) for BRAF or CRAF. BRAF and CRAF levels in the cell lysates are shown.
did not stimulate its binding to CRAF (Figure 4C), proving that drug binding to BRAF drives BRAF binding to CRAF.

Next, we expressed a kinase-dead version of BRAF (D594ABRAF) in D04 cells and show that it forms a constitutive complex with CRAF (Figure 4D) and that it activates MEK constitutively (Figure 4E, compare lanes 1, 4, and 7). Notably, 885-A does not further enhance MEK activation driven by D594A-BRAF (Figure 4E, compare lanes 4, 6 to 7, 9), presumably because it cannot further inhibit this already inactive kinase. Two other kinase-dead BRAF mutants, the classical catalytic lysine mutant (K483MBRAF), and D594A-BRAF, a mutant found in human cancer (Wan et al., 2004), also activate MEK in D04 cells (Figure 4F). Thus, it is BRAF inhibition and not drug binding that drives BRAF binding to CRAF. This experiment also shows that MEK activation driven by kinase-dead BRAF is inhibited by sorafenib (Figures 4E and 4F). Indeed, cell responses to sorafenib appear to be paradoxical. We show that although sorafenib inhibits ERK (Figure 1A), it induces BRAF binding to CRAF (Figure 2A), CRAF activation (Figure 4G) and CRAF phosphorylation on S338 (Figure 4G, inset), a critical event in CRAF activation (Mason et al., 1999). To test directly the role of BRAF in cells when BRAF is inhibited, we mutated its gatekeeper threonine to asparagine (T421NCRAF). Notably, T421NCRAF still binds to BRAF and drives CRAF activation, but without activating ERK (see Figure S4D).

Oncogenic Ras and Kinase-Dead Braf Cooperate to Induce Melanoma in Mice

Our data establish that inhibition of BRAF in the presence of oncogenic RAS hyperactivates CRAF, MEK, and ERK. To investigate the consequences of this in vivo, we used conditionally targeted alleles of oncogenic Kras (KrasLSL-G12D) and kinase-dead Braf (BrafLSL-D594A) in transgenic mice. These alleles use Cre-recombinase/LoxP-Stop-LoxP (LSL) technology to regulate inducible expression of mutant proteins from the endogenous mouse genes to ensure normal levels of protein expression. The KrasLSL-G12D allele has been described (Jackson et al., 2001), and we recently developed the BrafLSL-D594A allele. Briefly, exon 15 of endogenous Braf was targeted to mutate D594 to alanine (D594A; see Figure 5A). To prevent expression of D594A-Braf in all cells, an LSL cassette was inserted between exon 14 and the mutated exon 15. This contains a minigene for exons 15–18 of WT Braf, a transcription terminator and a NeoR selection marker to ensure that only WT Braf is expressed. Removal of the LSL cassette by Cre-recombinase reveals the mutated exon 15 and D594A-Braf is expressed. These mice were crossed to Tyr::CreERT2 mice (Yajima et al., 2006), in which the tyrosinase promoter is used to express tamoxifen-activated Cre-recombinase (CreERT2) in the melanocytes. Since CreERT2 is activated by tamoxifen, this approach provides exquisite spatial and temporal control over G12D Kras and D594A-Braf expression.

KrasLSL-G12D, BrafLSL-D594A, and Tyr::CreERT2 mice were crossed to generate KrasLSL-G12D; Tyr::CreERT2 mice, and this model, V600EBraf induces skin hyperpigmentation, nevus formation, and melanoma (Dhomen et al., 2009). In contrast, D594A-Braf did not induce skin hyperpigmentation, nevi (data not shown) or tumors (Figure 5C). G12D Kras induced weak tail darkening after 5–6 months (Figure 5B) but did not induce either nevi (data not shown) or tumors (Figure 5C). However, when D594A-Braf and G12D Kras were combined, they induced a conspicuous skin phenotype. Within 2–3 months the ears (data not shown), tails (Figure 5B), and paws (Figure 5D) darkened visibly. The mice did not develop nevi, but within 6 months, they all developed large, rapidly growing oligo-pigmented tumors (Figures 5C and 5E). The tumors displayed evidence of ulceration (Figure 5F) and were composed largely of spindle cells that exhibit features of malignancy, including cellular atypia, nuclear pleomorphism, and conspicuous nucleoli (Figure 5G). They were highly proliferative as evidenced by large numbers of mitotic figures in the superficial and deep aspects of the lesions (~6 mitosis/10HPF; Figure 5H) and positive staining for Ki67 throughout (Figure 5I).

The tumors were strongly and diffusely positive for S100 (Figure 6A) and expressed the melanocyte markers tyrosinase, Dct, Pax3, and silver (Figure 6B), consistent with a diagnosis of melanoma. Genomic DNA analysis of the tumors and cell lines derived from them confirmed that BrafLSL-D594A had been recombined to BrafLSL-D594A (Figure 6C). However, for technical reasons we could not detect KraLSL-G12D recombination (data not shown), so used RT-PCR to amplify and sequence Kras mRNA. We show that only wild-type Kras is expressed in the kidneys, whereas the tumors expressed both wild-type Kras and G12D Kras (Figure 6D). Importantly, we show constitutive binding of Braf to Craf in cells from the G12D Kras, D594A-Braf tumors (Figure 6E). As a control, we used cells from melanoma induced by G12V Kras overexpression. Briefly, when G12V Kras was overexpressed in melanocytes in mice using the β-actin promoter (β-actin::LKS; G12V Kras; Meuwissen et al., 2001), it induced rapid onset melanoma (median time to onset 2 months, 100% penetrance within 3 months) in the absence of D594A-Braf (manuscript submitted). Importantly, in cells from these tumors, Braf does not bind to Craf (Figure 6E). Thus, it is only kinase-dead Braf and not wild-type Braf that binds to Craf in the presence of oncogenic Kras.

DISCUSSION

In this study, we show that inhibition of BRAF by chemical or genetic means in the presence of oncogenic or growth-factor activated RAS induces BRAF binding to CRAF, leading to CRAF hyperactivation and consequently elevated MEK and
Figure 3. BRAF Binding to CRAF Requires RAS

(A) Myc-epitope tagged CRAF or R89L-CRAF (R89L), or an empty vector control (EV) were transfected into D04 cells. After 48 hr, the cells were treated with DMSO (−) or 885-A (1 μM) for 4 hr. Myc-tagged CRAF was immunoprecipitated (IP) and the immunocomplexes were western blotted (WB) for endogenous BRAF or myc-CRAF. Endogenous BRAF and myc-CRAF levels in the cell lysates are also shown.

(B) Myc-epitope tagged BRAF or R188L-BRAF (R188L) or an empty vector control (EV) were transfected into D04 cells. After 48 hr the cells were treated with DMSO (−) or 885-A (1 μM) for 4 hr. Myc-tagged BRAF was immunoprecipitated (IP) and the immunocomplexes were western blotted (WB) for myc-BRAF or endogenous CRAF. Myc-BRAF and endogenous-CRAF levels in the cell lysates are also shown.

(C) Membrane or cytosol fractions were prepared from untreated (−) or 885-A (1 μM) treated D04 cells. BRAF, CRAF, Tubulin (cytosol control) and HRAS (membrane control) were western blotted in the total lysate (TL), cytosolic fraction (CYT) and membrane fraction (MEM). The graph shows the quantification of the relative levels of BRAF and CRAF in the membrane and cytosol fractions.
ERK signaling. The mechanism we describe is another paradigm of RAF activation downstream of RAS and based on our findings, we propose the following mechanism by which this occurs. We posit that in RAS mutant cells, BRAF maintains itself in an inactive conformation through its own kinase activity, either through auto-phosphorylation, or by phosphorylating a partner protein that then keeps it inactive (Figure 7A). We are currently using mass-spectrometry and mutagenic approaches to elucidate the underlying mechanism. We propose that when BRAF is inhibited, it escapes this auto-inhibited state and is recruited to the plasma membrane by RAS, where it forms a stable complex with CRAF. Critically, we posit that because it is inhibited, BRAF does not directly phosphorylate MEK, but rather it acts as a scaffold whose function is to enhance CRAF activation, thereby allowing CRAF to hyperactivate the pathway (Figure 7B). We do not know the stoichiometry of the components in these complexes, but since BRAF and CRAF must both bind to RAS for complex formation, it seems likely that at least two RAS proteins are needed to stimulate formation of the complex (Figure 7B).

It is unclear why PLX4720 only induces weak binding of BRAF to CRAF, but this may stem from its unique property of displacing the α-C helix of BRAF when it binds (Tsai et al., 2008) and suggests that this helix is important for BRAF binding to CRAF, something that will only be resolved when the BRAF-CRAF crystal structure is solved. We have attempted to identify other proteins that may be required to stabilize the BRAF-CRAF complexes. Our unpublished mutagenesis data suggests that 14-3-3 is required to stabilize these drug-induced complexes (data not shown) and this is consistent with previous observations demonstrating that 14-3-3 mediates BRAF binding to CRAF (Garnett et al., 2005; Rushworth et al., 2006). Although this appears to contradict our observation that dephosphorylation does not disrupt the complex, because 14-3-3 binds to BRAF and CRAF in a phosphorylation-dependent manner, we presume that 14-3-3 protects these sites from dephosphorylation. We have also used RNAi to examine the potential role of other proteins implicated in BRAF-CRAF complex formation or pathway activation, including the scaffold proteins KSR, Sprouty2 and RKTG and the small G protein RHEB, but our preliminary results have not revealed obvious roles for these proteins. Our studies have parallels to the recently described heterodimers between DRAF and KSR in Drosophila (Rajakulendran et al., 2009). Notably, flies have only one RAF isoform and can heterodimerize with CRAF. Our study also highlights the critical difference between BRAF-selective and pan-RAF drugs. Whereas BRAF-selective drugs cause pathway activation in a RAS-dependent manner, this does not occur with pan-RAF drugs.

Our results provide important insight into the genetics of human cancer. Excluding V600 mutants, D594 mutants are the third most common in BRAF in cancer (34 out of 443 cases or 7.7%; www.sanger.ac.uk/genetics/CGP/cosmic/). Furthermore, as mentioned in the Introduction, while BRAF and RAS mutations are generally mutually exclusive, 4 of the 34 (11.8%) tumors with D594 mutations also have mutations in RAS. This is a highly significant enrichment for the coincidence of these mutations (p < 10⁻⁶; Fisher’s Exact Test) and suggests a functional interaction. We now provide strong circumstantial evidence of such an interaction using transgenic mice. By themselves, D594A-Braf and G12D-Kras do not induce melanoma, but they cooperate to induce rapid onset melanoma. This highly significant result (p < 0.0002) provides a rational explanation
Figure 4. BRAF and Not CRAF Inhibition Drives CRAF Binding to BRAF and CRAF Activation

(A) COS cells were transiently transfected with myc-epitope tagged BRAF, or T529NBRAF (T529N) in the presence of G12VHRAS (RAS) and their kinase activity was measured. The data represent one assay performed in triplicate, with error bars to represent standard deviations from the mean. Activity (%) is relative to wild-type BRAF activated by G12VHRAS.

(B) As in (A) but immunocomplexes were treated with DMSO (−) or 885-A for 10 min prior to measuring their kinase activity. The data represent one assay performed in triplicate, with error bars to represent standard deviations from the mean. Activity (% control) is relative to the untreated kinase.

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for the coincidence of these mutations in human cancer. Furthermore, we show that the BRAF inhibitors also hyperactivate this pathway in growth factor stimulated cells, providing an explanation of why kinase dead BRAF mutations are not always coincident with RAS mutations; presumably in some tumors the cooperating mutation is upstream of RAS.

Our results also suggest several potential mechanisms by which resistance to RAF targeting drugs could develop in

Figure 5. Oncogenic Kras and Kinase-Dead Braf Cooperate to Drive Tumorigenesis

(A) Diagrammatic representation of targeted conditional Braf$_{L560F}$ allele used for D594A-Braf expression in mouse melanocytes. The endogenous mouse Braf gene from exons 14–15 is represented. Exon 15 is mutated to express D594A-Braf (15’). LoxP sites are represented by triangles. The relative position of the wild-type BRAF minigene (M3) comprising exons 15–18 of Braf, the transcription terminator (term) and the Neo$^R$ cassette are shown. Cre recombinase mediated removal of these regions results in Braf$_{M3}$-D594A, allowing expression of D594A-Braf.

(B) Photographs of the tails of tamoxifen-treated wild-type (WT Braf, WTKras/WTBraf), or Kras$_{L560F}$/Braf$_{L560F}$ mice.

(C) Kaplan-Meier plots showing disease free progression of study mice. The controls consisted of 12 tamoxifen-treated Tyr::CreERT2$^{+/o}$;Braf$_{LSL-D594A};Tyr::CreERT2^{+/o}$ mice, 10 ethanol-treated Braf$_{LSL-D594A};Tyr::CreERT2^{+/o}$ mice, and 6 ethanol-treated Kras$_{LSL-G12D};Tyr::CreERT2^{+/o}$ mice. The experimental groups consisted of 12 tamoxifen-treated Kras$_{LSL-G12D};Tyr::CreERT2^{+/o}$, 24 tamoxifen-treated Braf$_{LSL-D594A};Tyr::CreERT2^{+/o}$, 1Kras$_{LSL-D594A};Tyr::CreERT2^{+/o}$ and 3 tamoxifen-treated Kras$_{LSL-G12D};Braf_{LSL-D594A};Tyr::CreERT2^{+/o}$ mice.

(D) Photographs of the feet of tamoxifen-treated wild-type (WT Braf, WTKras/WTBraf), or Kras$_{L560F}$/Braf$_{L560F}$ mice.

(E) Photograph showing a large tumor on the back of a tamoxifen-treated Kras$_{LSL-G12D};Braf_{LSL-D594A};Tyr::CreERT2^{+/o}$;Kras$_{LSL-D594A};Braf_{LSL-D594A};Tyr::CreERT2^{+/o}$;Braf$_{LSL-D594A}$ mouse. The fur was removed to reveal the lesion.

(F) Photomicrograph of a tumor from the back of a G12DKras/D594ABraf mouse. An area of ulceration is highlighted by the arrow.

(G) High magification photomicrograph of a section of tumor showing atypical cells, conspicuous nucleoli (arrowheads) and nuclear pseudo-inclusions (arrows).

(H) High magification photomicrograph of a section of tumor showing mitotic figures (arrows).

(I) Photomicrograph of a section of tumor subjected to immunohistochemical analysis with antibodies against Ki67 (MIB1).
patients. BRAF mutant tumors could become resistant to BRAF-selective drugs, if they acquire a mutation in RAS or an upstream component that activates RAS, or if the drugs select a population of cells harboring pre-existing mutations in RAS. Theoretically, this would cause BRAF-mediated CRAF activation, which may not only induce resistance, but could potentially promote tumor growth. In line with this, increased expression of CRAF can mediate acquired resistance to pan-RAF drugs in BRAF mutant cancer cells in vitro (Montagut et al., 2008), establishing that CRAF can mediate resistance under some circumstances. Our in vitro studies also suggest that a potential mechanism of resistance in patients with RAS mutant tumors being treated with pan-RAF drugs is acquisition (or selection for cells with pre-existing mutations) of a CRAF mutation such as a gatekeeper mutation that prevents drug binding. Again this would potentially result in BRAF-mediated activation of CRAF (Figure 7D) and possibly accelerated tumor growth.

Although our studies are restricted to cell lines and transgenic mice, they do have important immediate clinical implications. They strongly argue that BRAF-selective inhibitors should not be administered to patients with RAS mutant tumors, because long-term use could accelerate tumor growth. Intriguingly, 10%–15% of patients treated with BRAF-selective drugs develop squamous cell carcinoma (SCC) (Flaherty et al., 2009; Schwartz et al., 2009). Although MEK–ERK signaling has not yet been implicated in this response, 22% of SCCs harbour oncogenic mutations in RAS (9% HRAS, 8% NRAS, 5% KRAS: www.sanger.ac.uk/genetics/CGP/cosmic/), raising the intriguing possibility that the BRAF-selective drugs act as tumor promoters in premalignant skin cells harboring existing mutations in RAS and/or activation of upstream components that activate RAS.

While sorafenib is equipotent for wild-type and V600E BRAF (Wilhelm et al., 2004), the BRAF inhibitors we used are approximately 10-fold more active against V600E BRAF (King et al., 2006; Tsai et al., 2008). Nevertheless, our data establish that they target wild-type BRAF in RAS mutant cells. The problem of mutant v.s. wild-type protein specificity is likely to be difficult to resolve, because whereas full inhibition of V600E BRAF may be necessary for clinical response in BRAF mutant tumors, activation of only a small proportion of wild-type BRAF could be sufficient to activate the pathway in RAS mutant cells. Thus, to achieve efficacy against V600E BRAF but avoid activation of wild-type BRAF in RAS mutant cells, the drugs will need to be exquisitely selective for the mutant protein. Alternatively, pan-RAF drugs may be effective because they will target both V600E BRAF and CRAF activated by BRAF in RAS mutant tumors.

Furthermore, our data suggest that CRAF or MEK selective drugs should be used in RAS mutant tumors, because they do not induce BRAF-CRAF complexes and will not activate the pathway if the tumors acquire mutations such as T429A CRAF that block drug binding. Perhaps RAF and MEK inhibitors should be combined to provide the best responses and prevent emergence of resistance, but these issues need to be balanced against the urgency of the clinical problem being addressed.

In summary, we show that inhibition of BRAF in RAS mutant cancer cells leads to MEK hyperactivation through CRAF. We have elucidated another mechanism by which BRAF activates MEK–ERK signaling, not only to drive tumorigenesis and tumor progression, but also potentially to allow development of de novo or acquired resistance to RAF-targeted therapies. Clearly, BRAF is a remarkably versatile oncogene that can promote MEK–ERK activation and tumor progression through several mechanisms and these will require different therapeutic strategies for effective disease management. Notably, many of the mutations that occur in other kinases in cancer are also predicted to cause inactivation (www.sanger.ac.uk/genetics/CGP/cosmic/). Our data raise the possibility that these could also act as idiosyncratic gain-of-function mutations that drive tumorigenesis. This study also raises important clinical questions and highlights the importance of fully understanding how signaling
networks function to fully comprehend how patients may respond to targeted drugs. They also highlight the importance of genetic screening for patients, not only to identify those who are likely to respond, but to exclude those who could experience adverse effects and thereby ensure successful implementation of personalized medicine.

**EXPERIMENTAL PROCEDURES**

**Reagents**
Expression vectors for epitope-tagged BRAF and CRAF have been described (Wan et al., 2004). For western blotting the following antibodies were used: rabbit anti-p-pMEK1/2 and mouse anti-myc 9B11 (Cell Signaling Technology); mouse anti-NRAS (C-20), rabbit anti-ERK2 (C-14), rabbit anti-A RAF (C-20), mouse anti-BRAF (F-7) (Santa Cruz Biotechnology); mouse anti-Tubulin, and mouse anti-p-pERK1/2 (Sigma; mouse anti-CRAF (for western blotting) (BD Transduction Laboratories). For immunoprecipitation, the following antibodies were used: rabbit anti-myc (Abcam); rabbit anti-CRAF (C-20; Santa Cruz Biotechnology); mouse anti-BRAF (F-7) (Ab from Santa Cruz Biotechnology); mouse anti-Tubulin, mouse anti-NRAS (C-20), rabbit anti-ERK2 (C-14), rabbit anti-AR AF (C-20), mouse anti-BRAF (F-7) (Santa Cruz Biotechnology); mouse anti-p-pMEK1/2 (Sigma); mouse anti-CRAF (for western blotting) (BD Transduction Laboratories). For immunoprecipitation, lysates were incubated with 2 μg BRAF F-7, 5 μg CRAF C-20 or 2 μg rabbit anti-myc antibodies, captured on Protein G sepharose 4B beads (Sigma) and analyzed by western blotting using standard protocols. Specific bands were detected using fluorescent-labeled secondary antibodies (Invitrogen; Li-COR Biosciences) and analyzed using an Odyssey Infrared Scanner (Li-COR Biosciences). For CIP treatment, immunoprecipitates were washed twice with NP40 lysis buffer, once in CIP buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, and 1 mM EDTA), and incubated with CIP with or without 0.2 mM Na3VO4 and 7 mM EDTA. The immunoprecipitates were washed in CIP buffer and western blotted. Coupled RAF kinase assays were performed with immunoprecipitated CRAF or BRAF as described (Wan et al., 2004). Membrane fractionation was as described (Garnett et al., 2005).

**Cell Culture Techniques**
Human cell lines were cultured in DMEM (A375, WM852, HCT116, SW620, and PMWK) or RPMI (D04, MM485, MM415, and MM1791c) supplemented with 10% fetal bovine serum. For protein depletion, 3 × 105 D04 cells were transfected with 5nM CRAF (5′-AAGCACGGCTTAGATTGGAATA-3′) or NRAS (5′-CATGGACACGTACTCTTC TCG-3′) specific, or scrambled control (5′-AAACCGTGATTTCACCCGGGG-3′) siRNA using INTERFERin as recommended by the manufacturer (Polyplus Transfection SA). For transient expression studies, D04 cells were transfected using the Amaxa Nucleofector System as recommended by the manufacturer (Lonza). COS-7 cells were propagated, transfected, and extracted as described (Wan et al., 2004). For generation of stable lines, D04 cells were transfected with pMCEF-FLAG-CRAF or pMCEF-FLAG,T421NCRAF using Effectene as recommended by the manufacturer (Invitrogen) and selected in G418 (1 mg/ml).

Cell lysates were prepared with NP40 buffer as described (Wan et al., 2004). For immunoprecipitation, lysates were incubated with 2 μg BRAF F-7, 5 μg CRAF C-20 or 2 μg rabbit anti-myc antibodies, captured on Protein G sepharose 4B beads (Sigma) and analyzed by western blotting using standard protocols. Specific bands were detected using fluorescent-labeled secondary antibodies (Invitrogen; Li-COR Biosciences) and analyzed using an Odyssey Infrared Scanner (Li-COR Biosciences). For CIP treatment, immunoprecipitates were washed twice with NP40 lysis buffer, once in CIP buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, and 1 mM EDTA), and incubated with CIP with or without 0.2 mM Na3VO4 and 7 mM EDTA. The immunoprecipitates were washed in CIP buffer and western blotted. Coupled RAF kinase assays were performed with immunoprecipitated CRAF or BRAF as described (Wan et al., 2004). Membrane fractionation was as described (Garnett et al., 2005).

**Transgenic Mice**
Experiments were performed under Home Office license authority in accordance with United Kingdom Coordinating Committee on Cancer Research Guidelines (Workman et al., 1988) and with local Ethics Committee approval. To activate CreERT2, mice were treated with four doses (10 mg each) of topically applied tamoxifen as described (Dhomen et al., 2009). Genotyping was performed by PCR. BrafLSL-V600E and BrafLox-D594A were analyzed as described for BrafLSL-VE600E and BrafLox-D594A respectively and Tyr::CreERT2 was analyzed as described (Dhomen et al., 2009). KrasLSL-SETD2 was analyzed using primers

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Figure 7. A Model of Paradoxical CRAF Activation by BRAF

(A) In the presence of oncogenic RAS, BRAF is cytosolic, where it maintains itself in an inactive conformation in a manner that depends on its own kinase activity. CRAF is recruited to the plasma membrane by RAS and activates the pathway.

(B) When BRAF is inhibited by genetic or chemical means, it is no longer autoinhibited and is recruited to the plasma membrane by RAS, where it binds to CRAF. Although BRAF does not itself signal, it can act as a scaffold to enhance CRAF activity and consequently enhance signaling through the pathway.

(C) Pan-RAF inhibitors hyperactivate CRAF because they inhibit BRAF, but they simultaneously inhibit CRAF, leading to paradoxical activation of CRAF without pathway activation.

(D) T421NCRAF (T421N) escapes the paradoxical activation by the pan-RAF inhibitors, because it no longer allows them to bind, so is freely activated due to BRAF inhibition.
5′-CGCA
gCGCGCGCCG-3′ and 5′-CCAT
GCTCTTGGACTGCG-3′. For expression analysis, RNA was prepared (QIAgEN RNAeasy, QIAgEN) and first-strand cDNA synthesis was performed with 500ng total RNA and random hexanucleotides (Random Primers, Invitrogen). Specific genes were amplified under linear conditions for analysis as described (Dhomen et al., 2009). For Kras cDNA sequencing, a 238 bp fragment of Kras cDNA was PCR amplified using primers 5′-GGGCGACGCCTGTAGCAGCG-3′ and 5′-CTGAAGGGTC ATACCTGACCC-3′ and sequenced using automated dyeideoxy sequencing.

For immunohistochemistry (IHC), tissues were fixed and analyzed as described (Dhomen et al., 2009). Positive (a well characterized sample of mouse melanoma) and negative (omission of the primary antibody and substitution with preimmune serum) controls were included in each slide run. Immunohistochemical staining was analyzed by two of the authors on a multi-headed microscope. Tumor cell lines were established by mechanically dissociating tumors in DMEM/20%FCS/Primocin (0.1mg/ml - InvivoGen) and clonal lines were selected by limiting dilution.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and three tables and can be found with this article online at doi: 10.1016/j.cell.2009.12.040.

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