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C-RAF Mutations Confer Resistance to RAF Inhibitors

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Abstract

Melanomas that contain B-RAF^{V600E} mutations respond transiently to RAF and MEK inhibitors; however, resistance to these agents remains a formidable challenge. Although B- or C-RAF dysregulation represents prominent resistance mechanisms, resistance-associated point mutations in RAF oncoproteins are surprisingly rare. To gain insights herein, we conducted random mutagenesis screens to identify B- or C-RAF mutations that confer resistance to RAF inhibitors. Whereas bona fide B-RAF^{V600E} resistance alleles were rarely observed, we identified multiple C-RAF mutations that produced biochemical and pharmacologic resistance. Potent C-RAF resistance alleles localized to a 14-3-3 consensus binding site or a separate site within the P loop. These mutations elicited paradoxical upregulation of RAF kinase activity in a dimerization-dependent manner following exposure to RAF inhibitors. Knowledge of resistance-associated C-RAF mutations may enhance biochemical understanding of RAF-dependent signaling, anticipate clinical resistance to novel RAF inhibitors, and guide the design of "next-generation" inhibitors for deployment in RAF- or RAS-driven malignancies. *Cancer Res; 73(15); 4840–51. ©2013 AACR.*

Introduction

Melanoma remains the deadliest form of skin cancer: its annual incidence has reached 76,250 cases, and metastatic melanoma accounts for 9,180 deaths in the United States each year (www.skincancer.org). The proliferation and survival of most melanomas is driven by genetic activation of the mitogen-activated protein kinase (MAPK) pathway, a signaling cascade whose core module consists of the RAS oncoprotein along with RAF, MEK, and ERK kinases (1). Approximately 50% of cutaneous melanomas harbor gain-of-function mutations in the B-RAF serine–threonine kinase—most commonly involving a valine-to-glutamic acid substitution at codon 600 (BRAF^{V600E}; ref. 2). An additional 15% to 20% of melanomas contain oncogenic N-RAS mutations (3, 4). Efforts to target this pathway in B-RAF–mutant melanoma have culminated in the emergence of small-molecule RAF and MEK inhibitors into clinical practice (5–8). Additional RAF inhibitors, including compounds with enhanced potency, are sure to follow. In the future, these agents will likely be used in combination with MEK inhibitors (9, 10).

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Despite these advances, enthusiasm for this new class of inhibitors has been tempered by the near-ubiquitous emergence of drug resistance after several months of treatment (6, 7, 11). Recent work has described 3 major categories of resistance to RAF inhibition in B-RAF^{V600E} melanoma. One avenue involves engagement of parallel (or "bypass") signaling modules, such as the kinase COT (12) or certain receptor tyrosine kinase–driven pathways (12–15). A second mechanism consists of activating somatic mutations in the downstream effector kinase MEK1 (11, 16, 17). The third category encompasses several distinct mechanisms that result in sustained RAF-dependent signaling, typically through ectopic C-RAF activation (18). Clinically validated examples include mutation of upstream C-RAF effectors (e.g., N-RAS; ref. 13) and expression of an alternative splice isoform of B-RAF (p61-B-RAF) that functions, in part, through enhanced dimerization (19). In principle, however, any mechanism resulting in dysregulated C-RAF activation could cause resistance to the selective RAF inhibitors in current clinical use (12). B-RAF amplification has also been reported in resistant specimens (20); conceivably, this mechanism may operate either through an excess of monomeric B-RAF^{V600E} or by potentiating B-RAF/C-RAF heterodimerization. All of the best supported resistance mechanisms produce sustained MEK/ERK activation, thus highlighting the continued importance of MAPK signaling in melanoma survival and progression.

The ability of dysregulated C-RAF to transduce a key resistance mechanism in BRAF^{V600E} melanoma accords well with recent observations that RAF inhibitors can paradoxically activate MEK/ERK signaling in the setting of upstream RAS activation (18, 21–25). Both RAS signaling and C-RAF activation are suppressed in B-RAF^{V600E} melanoma cells under steady-state conditions (12, 24), owing largely to a profound negative feedback regulation induced by oncogenic MAPK output in this setting. Presumably, pharmacologic interdiction

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of oncogenic B-RAF signaling may potentiate a cellular context more permissive for RAS-dependent C-RAF activation, which can occur by multiple mechanisms. C-RAF may become activated through either RAS-dependent recruitment to the plasma membrane (26) or RAS-independent mechanisms operant within the cytosol, where it undergoes conformational changes and binds to scaffold proteins such as 14-3-3 (27–29). 14-3-3 binding (especially the zeta isoform; ref. 28) and subsequent stabilization/activation of C-RAF is enabled by phosphorylation of activating residues such as serine 338 and tyrosine 341, situated in its negatively charged N-terminal regulatory region; or serine 621, located in the C-terminal region outside the kinase domain; and numerous other phosphorylation sites (22, 28–30). Moreover, homo- or heterodimerization appears crucial for C-RAF activation (12, 18, 21–25, 29).

Given the elaborate regulatory mechanisms that govern RAF-dependent signaling, it seems surprising that neither B- nor C-RAF point mutations have yet been observed in B-RAF^{V600E} melanomas that develop resistance to RAF inhibition. The paucity of RAF resistance mutations is particularly noteworthy given the overall prevalence of secondary point mutations in kinase oncoproteins in the setting of resistance to targeted agents (31). To gain additional insights into aspects of RAF regulation that might prove relevant for oncogenic signaling and the emergence of therapeutic resistance, we conducted random mutagenesis screens and biochemical studies to identify somatic B- or C-RAF variants capable of mediating resistance to RAF inhibitors.

Materials and Methods

Cell culture

Cell lines were obtained from the American Tissue Culture Collection or Allele Biotech and were cultured and maintained at 37°C in Dulbecco's Modified Eagle Medium supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2. Cells were passaged for less than 6 months after receipt and authenticated by short tandem repeat profiling.

B-RAF and C-RAF random mutagenesis screen

B-RAF and C-RAF cDNA were cloned into pWZL-Blast vector (gift from J. Boehm and W. C. Hahn) by recombinational cloning (Invitrogen). Specific mutations were introduced into B-RAF and C-RAF cDNA using QuickChange II Site Directed Mutagenesis (Stratagene). Random mutagenesis was conducted following a previously reported protocol (16). The mutagenized B-RAF and C-RAF plasmids were used to infect A375 melanoma cells. Following selection with blasticidin, cells were plated on 15 cm dishes and cultured in the presence of RAF inhibitor, PLX4720 (1.5 μmol/L) and RAF-265 (2 μmol/L) for 4 weeks until resistant clones emerged.

Sequencing of C-RAF and B-RAF DNA

PLX4720- and RAF-265–resistant cells emerging from the B-RAF random mutagenesis screens and PLX4720-resistant cells emerging from the C-RAF random mutagenesis screens were pooled and genomic DNA was prepared (Qiagen DNeasy). B-RAF and C-RAF cDNAs were amplified from genomic DNA using primers specific to flanking vector sequence at the 5′ and 3′ end and sequenced with Illumina 3G instrumentation according to manufacturer's instructions.

Analysis of massively parallel sequencing

Raw data from massively parallel sequencing lanes (Illumina; 2–3 million 36 base pair sequences per lane) were analyzed using a "next-generation" sequencing analysis pipeline. Output from data files representing the nucleotide sequence, per-base quality measure, variants detected, and alignment to cDNA reference sequence (as determined by alignment with the ELAND algorithm) were integrated and processed for each run.

Coverage (i.e., the number of fragments including each base of the cDNA reference) was determined for all bases, and variant alleles were mapped from individual DNA fragments onto the reference sequence. The frequency of variation for each non-wild-type allele was determined, and an average variant score (AVS) was calculated as the mean of all quality scores for the position and variant allele in question. All coding mutations were translated to determine the amino acid variation (if any) and data for high-frequency (>0.5%) and high-quality (AVS >7) mutations were loaded into the CCGD results database.

Retroviral infections

Phoenix cells (70% confluent) were transfected with pWZL-Blast-C-RAF or B-RAF$^{(V600E)}$-mutant libraries or the specific mutants using Fugene 6 (Roche). Supernatants containing virus were passed through a 0.45 μm syringe. A375 cells were infected for 16 hours with virus together with polybrene (4 μg/mL, Sigma). The selective marker blasticidin (3 μg/mL, Sigma) was introduced 48 hours postinfection.

Western blot analysis

Samples were extracted after washing twice with PBS and lysed with 150 mmol/L NaCl, 50 mmol/L Tris pH 7.5, 1 mmol/L EDTA, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, sodium orthovanadate, and protease inhibitor cocktail in the presence of 1% NP-40. The protein content was estimated with protein assay reagent (Bio-Rad) according to the manufacturer's instructions. Equal amounts of whole-cell lysates were loaded onto and separated by 8% to 16% SDS-PAGE readymade gels. Proteins were transferred to polyvinylidene difluoride membranes in a Trans-Blot apparatus. Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween 20 for 1 hour at room temperature or overnight at 4°C. Membranes were then incubated with monoclonal or polyclonal antibody raised against the protein of interest for 1 hour at room temperature or overnight at 4°C according to manufacturer guidelines and followed by 3 washes with TBS containing 0.1% Tween 20. The immunoreactivity of the primary antibodies total C-RAF, p-C-RAF(S338), p-ERK, total ERK, p-MEK, total MEK, 14-3-3ζ, Flag (Cell Signaling), B-RAF (Santa Cruz Biotechnology), V5 (Invitrogen), and actin (Sigma) was visualized with a secondary anti-rabbit (BD Transduction Laboratories) or anti-mouse (Santa Cruz Biotechnology) antibodies conjugated with horseradish peroxidase and subsequent development with ECL Plus (Amersham Biosciences) and autoradiography on X-OMAR TAR films.

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Immunoprecipitation

For immunoprecipitation with C-RAF antibody (BD Biosciences), protein G-Sepharose slurry (Thermo Scientific) was washed with 1× PBS and incubated with C-RAF antibody or normal mouse IgG (control) for 1 hour at 4°C. After three washes with lysis buffer, the beads were incubated with whole-cell lysates (0.5 mg of total protein) for 2 hours and then washed 3 times with lysis buffer. The proteins were then eluted by boiling in 1× SDS sample buffer. For immunoprecipitation of His/V5 or Flag-tagged plasmids, identical protocol was followed using Ni^{2+} (Qiagen) or Flag (Sigma) beads.

C-RAF kinase assay

293/T cells (70% confluent) were transfected with 6 μg pcDNA with Flag tag containing C-RAF-WT and C-RAF variant alleles. Forty-eight hours posttransfection, cells were treated with vemurafenib (Allele Biotech) for 1 hour. For C-RAF kinase assay with A375 cells, A375 cells infected with WT and mutant C-RAF alleles were cultured in the absence and presence of vemurafenib for 16 hours. Lysates were extracted by general protocol and immunoprecipitation using flag beads/C-RAF antibody was conducted overnight at 4°C, and bound beads were washed 3 times with lysis buffer, followed by kinase buffer (1×). The protein-bound Flag beads/bound C-RAF beads where incubated with 20 μL ATP/magnesium mixture, 20 μL of dilution buffer, and 0.5 μg of inactive MEK1 (Millipore) for 30 minutes at 30°C. The phosphorylated MEK product was detected by immunoblotting using a p-MEK antibody (Cell Signaling Technology).

Pharmacologic growth inhibition assays

Cultured cells were seeded into 96-well plates at a density of 3,000 cells per well for all melanoma short-term cultures including A375. After 16 hours, serial dilutions of the compound were conducted in dimethyl sulfoxide (DMSO) and transferred to cells to yield drug concentrations based on the potency of the drug, ensuring that the final volume of DMSO did not exceed 1%. The B-RAF inhibitor PLX4720 was purchased from Symansis, vemurafenib (Active Biochem), AZD6244 (Selleck Chemicals), and GSK1120212 (Active Biochem). Following addition of the drug, cell viability was measured using the Cell-Titer-96 aqueous nonradioactive proliferation assay (Promega) after 4 days. Viability was calculated as a percentage of the control (untreated cells) after background subtraction. A minimum of 6 replicates was made for each cell line and the entire experiment was repeated at least 3 times. The data from the pharmacologic growth inhibition assays were modeled using a nonlinear regression curve fit with a sigmoidal dose–response. These curves were displayed using GraphPad Prism 5 for Windows (GraphPad).

Results

Identification of resistance-associated RAF mutations by random mutagenesis

To identify somatic RAF mutations that confer resistance to RAF inhibitors, saturating random mutagenesis screens were conducted using the XL1-Red bacterial system and retroviral vectors expressing either B-RAF^{V600E} or C-RAF cDNAs (11, 16). The resulting mutagenized cDNA libraries were expressed in the A375 melanoma cell line, which harbors the B-RAF^{V600E} mutation and is highly sensitive to RAF inhibitors (16, 32–34). These cells were cultured in the presence of inhibitory concentrations of PLX4720 (1.5 μmol/L; B-RAF^{V600E} and C-RAF libraries) or RAF265 (2 μmol/L; BRAF^{V600E} library only). Drug-resistant colonies emerged after 28 days of drug exposure; however, the outgrowth of resistant colonies appeared qualitatively more robust following C-RAF mutagenesis compared with B-RAF^{V600E} mutagenesis screens (data not shown). Resistant colonies (∼1,000 total from PLX4720 and ∼600 total from RAF265 screens) were independently pooled and characterized by massive parallel sequencing as described previously (11, 16).

Characterization of putative resistance mutations in B-RAF^{V600E}

The spectrum of "secondary" B-RAF^{V600E} mutations that emerged following selection with RAF inhibitors is shown in Fig. 1A (PLX4720) and Supplementary Fig. S1A (RAF265). Mutations involving 3 residues (T521K, V528F, and P686Q) were observed in both mutagenesis screens and investigated further by mapping to the B-RAF^{V600E} crystal structure (PDB code: 3OG7, Fig. 1B; ref. 33). Additional alleles were independently identified in either the PLX4720 or RAF265 screen (Fig. 1A and Supplementary Fig. S1A). The threonine residue at position 521 (T521) maps to a linker region within the B-RAF catalytic domain (Supplementary Fig. S1B). The valine at position 528 (V528) resides immediately adjacent to the gatekeeper residue (threonine 529), which exerts significant interactions with several known RAF inhibitors (35). Although mutations of the gatekeeper residue were not observed in the mutagenesis screens, the V528F allele is predicted to perturb both the gatekeeper residue and the isoleucine at position 527, which may also generate important protein–drug interactions in the RAF inhibitor–bound protein (33). Interestingly, proline 686 localizes to a pocket in the C-terminus of the catalytic domain that is analogous to the myristic acid–binding site of the Abl kinase (36); however, myristylation of B-RAF^{V600E} has not been described.

To determine the functional effects of the putative B-RAF^{V600E} resistance alleles, mutations corresponding to each of the 3 amino acids were introduced into B-RAF^{V600E} cDNA and ectopically expressed in A375 cells. Surprisingly, neither ectopic B-RAF^{V600E} nor the putative B-RAF^{V600E} resistance mutants conferred pharmacologic resistance to PLX4720 when overexpressed in A375 cells (Fig. 1C). Furthermore, ectopic expression of B-RAF^{V600E} either by itself or harboring the candidate resistance mutations induced its own degradation without increasing MEK phosphorylation (p-MEK) compared with B-RAF^{V600E} (Supplementary Fig. S1C). Wild-type B-RAF (e.g., lacking the oncogenic V600E mutation) also had little effect on p-MEK in this context (Supplementary Fig. S1C and S1D). Indeed, the T521K and P686T variants within B-RAF^{V600E} resulted in modestly diminished p-MEK compared with "parental" B-RAF^{V600E} (Supplementary Fig. S1C), and expression of V528F within B-RAF^{V600E} markedly suppressed p-MEK levels (Supplementary Fig. S1C). Thus, forced expression of the putative B-RAF^{V600E} resistance alleles conferred either neutral

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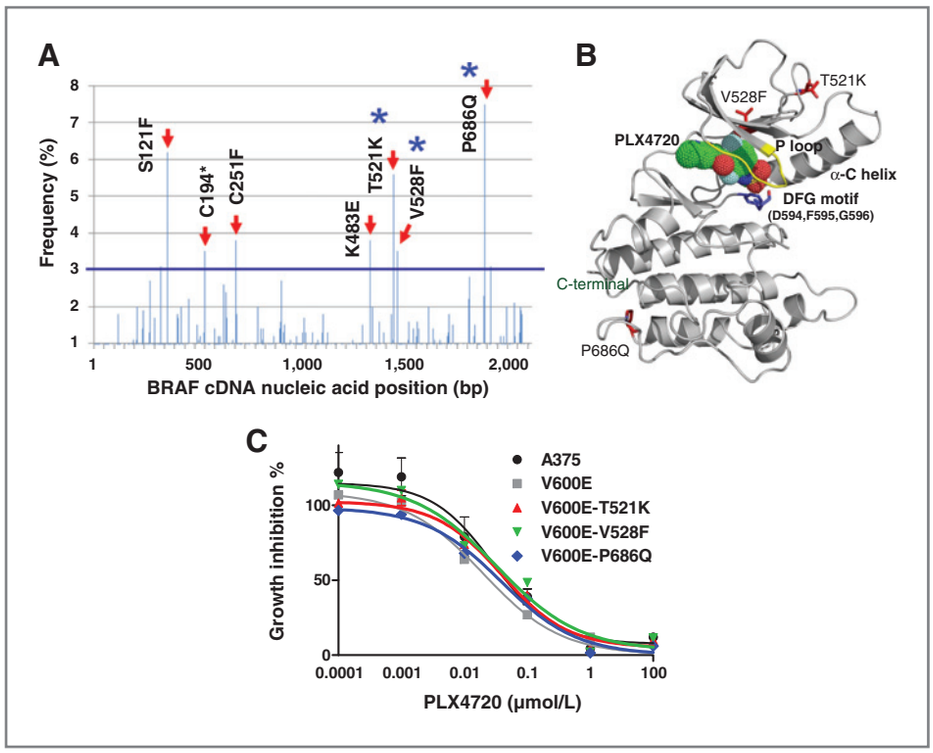

Figure 1. B-RAF^{V600E} mutations identified by random mutagenesis screens for resistance to RAF inhibition. A, the landscape of candidate mutations is shown from a random mutagenesis screen using the RAF inhibitor PLX4720 (*x*-axis, nucleic acid position across the B-RAF coding sequence; *y*-axis, frequency of high-confidence variants as a function of total variants detected). Corresponding amino acid substitutions for high-frequency mutations are indicated. Asterisks in blue denote B-RAF mutations also observed in mutagenesis screens using the RAF inhibitor RAF265. B, ribbon diagram of B-RAF^{V600E} (gray) bound by PLX4720 (space filling model; PDB code: 3OG7) depicting putative B-RAF resistance mutations (red sticks). The DFG motif (blue) and P-loop (yellow) are also shown (structures are rendered with PyMOL). C, growth inhibition curves of parental A375 cells engineered to express B-RAF^{V600E} and selected B-RAF^{V600E} or B-RAF mutants in response to PLX4720.

or deleterious effects on MEK activation. Moreover, despite the emergence of these B-RAF^{V600E} alleles from random mutagenesis screens using 2 independent RAF inhibitors, none conferred resistance to RAF inhibition when ectopically expressed in drug-sensitive melanoma cells (Fig. 1C). These mutations may conceivably provide a subtle advantage to melanoma cells during outgrowth under experimental drug selection; however, their biologic impact remains uncertain. Such ambiguities may also help explain why "secondary" B-RAF^{V600E} resistance mutations have not yet been observed clinically.

Identification and characterization of C-RAF resistance alleles

In striking contrast to the B-RAF^{V600E} results, C-RAF random mutagenesis screens produced robust PLX4720-resistant colonies in a relatively short time frame (3–4 weeks). The landscape of putative C-RAF resistance alleles included multiple recurrent variants (Fig. 2A) that localized to known functional motifs within the C-RAF kinase domain (Fig. 2B). Unlike the B-RAF^{V600E} variants described above, all C-RAF mutations examined (Fig. 2A, arrows) could be stably expressed in A375 cells (Supplementary Fig. S2A), without evidence of protein degradation. Furthermore, 8 of the 10 most prominent C-RAF mutations conferred biochemical resistance to the RAF inhibitor PLX4720 in A375 cells, as evidenced by p-MEK and p-ERK levels (Supplementary Fig. S2C). Thus, biologically consequential C-RAF mutations were readily identified by random mutagenesis.

Mapping of these C-RAF resistance alleles onto the primary structure (Fig. 2B) and the crystal structure of the C-RAF kinase domain (340–618; PDB code: 3OMV; Fig. 2C; ref. 25) showed that they aggregated within distinct functional motifs in either the N-terminal regulatory region or the C-terminal kinase domain (Fig. 2B; E104K, S257P, and P261T mutations could not be mapped, as the full-length C-RAF structure has not been solved to date.) The S257P and P261T mutations resemble 2 gain-of-function germline variants (S257L and P261S/L) observed in patients with Noonan syndrome, an autosomal dominant congenital disorder characterized by aberrant RAS/MAPK pathway activation (37). Both S257 and P261 occupy 1 of the 2 14-3-3 consensus binding sites present in C-RAF (located in the CR2 domain, Fig. 2B). As noted earlier, 14-3-3 proteins are known to bind RAF kinases and regulate their activity and stability (28). Conceivably, then, these variants may alter 14-3-3 binding to C-RAF, thereby enhancing its activity (37, 38).

The G356E and G361A variants mapped to the glycine-rich GxGxxG motif found in the ATP-binding P-loop. Somatic P-loop mutations have been found in several protein kinases (2) including BRAF (21, 22). The remaining mutations (S427T, D447N, M469I, E478K, and R554K) resided outside of the activation segment (Fig. 2B). Among these, S427 and D447 are reminiscent of certain activating variants (S427G and I448V) reported in patients with therapy-related acute myeloid leukemia (39). Moreover, E478K was previously characterized as an activating variant in a human colorectal carcinoma cell line and was also found to heterodimerize constitutively with B-RAF^{E586K} in a manner that increased kinase activity (25, 40). In the aggregate, these findings suggest that the C-RAF mutations may enact both predicted and novel biochemical mechanisms of resistance to RAF inhibition.

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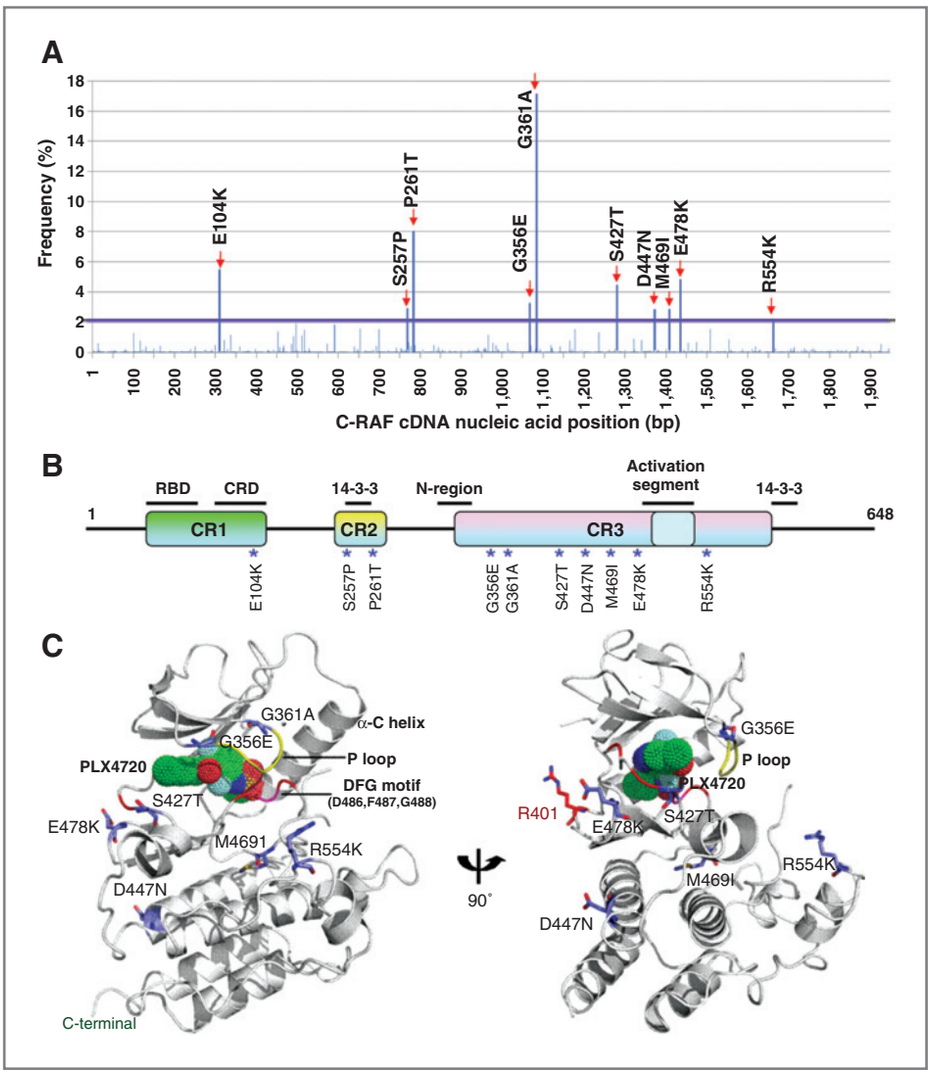

Figure 2. C-RAF resistance mutants identified by random mutagenesis screens. A, the spectrum of candidate mutations from a C-RAF random mutagenesis screen is shown for the RAF inhibitor PLX4720 (*x*-axis, nucleic acid position across the C-RAF coding sequence; *y*-axis, high-confidence variant frequency as a function of total variants detected). Corresponding amino acid substitutions for high-frequency mutations (>2%) are indicated. B, schematic of C-RAF primary structure, including key functional domains. CR, conserved region; CRD, cysteine-rich domain; RBD, Ras-binding domain. The location of recurrent resistance mutants (blue asterisks) is shown. C, left, crystal structure of C-RAF kinase domain (residues 340–618; gray) (PDB code: 3OMV) is shown, including representative C-RAF resistance mutants (blue sticks) along with a space-filling model of bound PLX4720. B-RAF^{V600E} bound to PLX4720 (PDB code: 3OG7) was structurally aligned with C-RAF (PDB code: 3OMV) to mimic a model of C-RAF in complex with PLX4720. The carbon$_{\alpha}$ root mean square deviation (RMSD) between B-RAF and C-RAF was <1 A°. The DFG motif (red-pink) and P-loop (yellow) are also indicated. Right, C-RAF structure rotated 90° to expose the dimer interface residue R401 (structures are rendered with PyMOL).

A subset of C-RAF resistance alleles confers pharmacologic resistance to RAF inhibitors

Next, the ability of biochemically validated C-RAF alleles to confer pharmacologic resistance to RAF inhibitors was examined by expressing the relevant cDNAs within A375 cells and conducting cell growth inhibition assays. In contrast to the B-RAF^{V600E} secondary mutations described above, 3 C-RAF alleles conferred profound resistance to both the tool compound PLX4720 and the structurally homologous clinical RAF inhibitor, vemurafenib (Fig. 3A and B). The S257P and P261T variants in the CR2 domain 14-3-3 binding region produced the most dramatic resistance effects (IC_{50} values were not reached at the 10 μmol/L drug concentration; Fig. 3A and B). The G361A variant (ATP-binding domain) also conferred >100-fold resistance to these RAF inhibitors compared with the wild-type C-RAF ($IC_{50} = 0.1–0.4$ μmol/L). The remaining variants exhibited modest (if any) effects on the A375 IC_{50} values (as exemplified by G356E and D447N), although E478K induced a discernible elevation of the "tail" of the PLX4720 IC_{50} curve (Fig. 3A). Moreover, only one allele (G361A) conferred any evidence of pharmacologic resistance to MEK inhibition (Supplementary Fig. S3A and S3B). Thus, some but not all of the biochemically validated C-RAF resistance alleles conferred substantial pharmacologic resistance to RAF inhibitors in BRAF^{V600E} melanoma cells.

C-RAF resistance alleles enable paradoxical C-RAF activation

To explore the mechanisms by which C-RAF mutations conferred resistance to small-molecule RAF inhibitors, biochemical studies were conducted to assess relative RAF/MEK/ERK activation across a representative panel of mutations at baseline and following exposure to 2 μmol/L RAF inhibitor (in this case, PLX4720). This concentration of RAF inhibitor was sufficient to achieve suppression of MEK/ERK phosphorylation in parental A375 cells and in those expressing wild-type C-RAF (Fig. 3C and Supplementary Fig. S2B). At baseline, C-RAF was inactive in both the presence and absence of PLX4720

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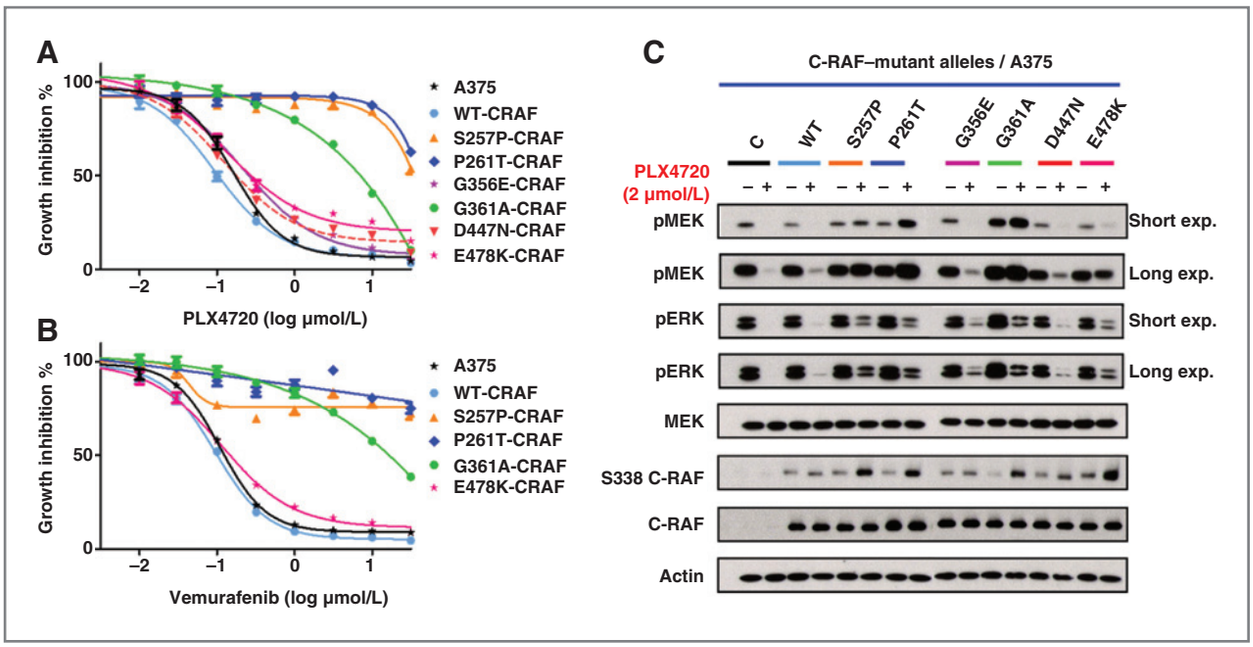

Figure 3. Functional characterization of C-RAF resistance mutants. A and B, growth inhibition curves of parental A375 cells (B-RAF^{V600E}) and cells engineered to express selected C-RAF resistance alleles in response to PLX4720 (A) and vemurafenib (B). C, A375 cells expressing representative C-RAF resistance mutants were treated with 2 μmol/L of PLX4720 for 16 hours. Immunoblotting studies were conducted using antibodies recognizing p-MEK1/2, p-ERK1/2, total MEK1/2, p-C-RAF (S338), total C-RAF, and actin (loading control).

based on serine 338 (S338) phosphorylation (Fig. 3C), as reported previously in the B-RAF^{V600E} context (12). Ectopic expression of C-RAF resistance alleles enabled sustained p-MEK and p-ERK (albeit moderately reduced) in the presence of 2 μmol/L PLX4720 (Fig. 3C). Moreover, the pharmacologically validated alleles (S257P, P261T, and G361A) showed evidence of C-RAF activation in the presence of PLX4720, as evidenced by increased p-C-RAF (S338) and a slight enhancement of p-MEK levels (Fig. 3C). This C-RAF activation of MEK/ERK signaling was suppressed by pharmacologic MEK inhibition (Supplementary Fig. S3A and S3B). Paradoxical activation of MEK/ERK signaling by selective RAF inhibitors has been described in the setting of activated RAS (24, 25) and ectopic C-RAF overexpression (12); however, somatic C-RAF point mutations that promote paradoxical, inhibitor-induced signaling have not previously been reported. Thus, C-RAF mutations that enable drug-induced kinase activation may promote resistance to RAF inhibitors.

Paradoxical MEK/ERK activation induced by RAF inhibitors involves dimerization of RAF proteins (24, 25). Moreover, a truncated form of B-RAF^{V600E} that shows enhanced dimerization confers resistance to RAF inhibitors (19). To determine whether the C-RAF resistance mutations might mediate resistance through increased dimerization, cotransfections were conducted in 293/T cells using expression constructs in which several representative C-RAF resistance alleles were differentially tagged with 2 distinct epitopes (His/V5 or Flag). Immunoprecipitation reactions were carried out using Ni^{2+} beads (to capture the His-tagged protein) followed by immunoblotting using anti-Flag antibody. In these experiments, all C-RAF mutations that conferred pharmacologic resistance to RAF inhibitors also exhibited robust homodimerization compared with wild-type C-RAF (S257P, P261T, G361A, and E478K; Fig. 4A). For these mutants, dimerization generally correlated with both increased total C-RAF expression and increased p-MEK levels (Fig. 4A, input lysate). Similar results were observed when His/V5-tagged C-RAF mutants were cotransfected with Flag-tagged wild-type C-RAF (Supplementary Fig. S4A), although the magnitude of MEK/ERK activation seemed qualitatively reduced (Supplementary Fig. S4A, input lysate). The 3 C-RAF mutants that conferred the most profound pharmacologic resistance to PLX4720 and vemurafenib (S257P, P261T, and G361A; Fig. 3B and C) also showed evidence of increased total protein accumulation (Fig. 4A, input lysate). Thus, the resistance phenotype linked to C-RAF mutations correlated with RAF expression and homodimerization. At present, we cannot determine whether the increased C-RAF expression associated with the resistance alleles is a cause or consequence of enhanced homodimerization.

In 293/T cells (which lack oncogenic B-RAF mutations), the C-RAF dimerization engendered by the presence of resistance mutations was sustained but not further enhanced upon exposure of transfected cells to RAF inhibitor (vemurafenib; Fig. 4B). However, both C-RAF activation (evidenced by S338 phosphorylation) and downstream MEK/ERK signaling were robustly induced by the RAF inhibitor (Fig. 4B, input lysate). To determine the effects of these resistance mutations on intrinsic C-RAF kinase activity, *in vitro* kinase reactions were conducted from 293/T cells cultured in the presence or absence of RAF inhibitor. In the absence of drug, steady-state C-RAF kinase activity (p-MEK) was not measurably increased by the resistance mutations in most cases (Fig. 4C). C-RAF^{G361A} was the

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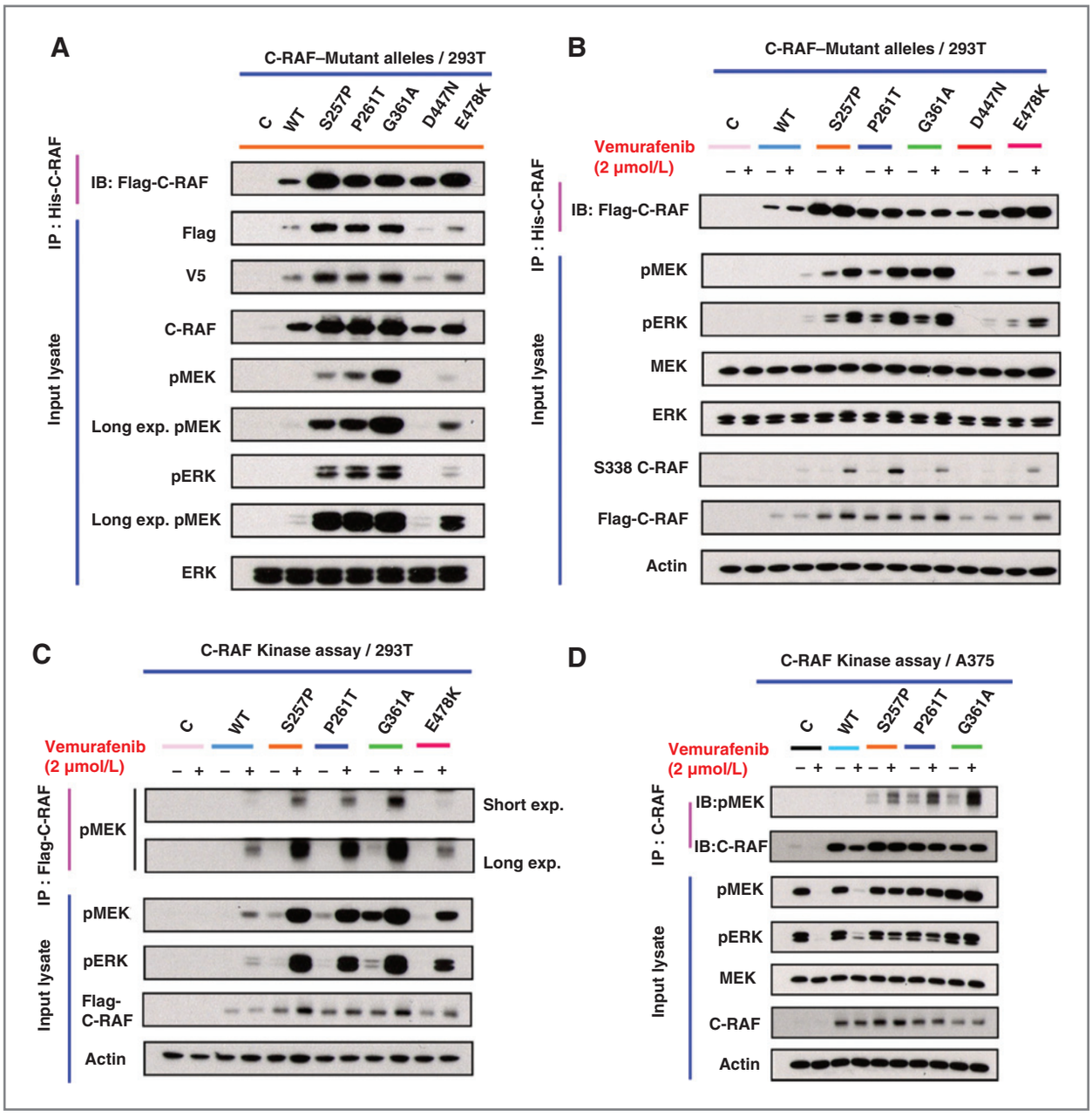

Figure 4. Homodimerization and kinase activity of C-RAF resistance mutants. A, 293/T cells coexpressing His/V5-tagged and Flag-tagged C-RAF resistance mutants for 48 hours were immunoprecipitated with nickel (see Materials and Methods) to pull down His-tagged C-RAF, and Flag-tagged C-RAF was assessed by immunoblotting. Input lysate was also assessed using antibodies that detected Flag-C-RAF, V5-C-RAF, total C-RAF, p-MEK, p-ERK, and total ERK. B, 293/T cells coexpressing His/V5-tagged and Flag-tagged C-RAF resistance mutants were treated with either vehicle (DMSO) or 2 μmol/L of vemurafenib for 1 hour, and His/V5-tagged C-RAF was immunoprecipitated as in A above. Input lysates were immunoblotted using antibodies recognizing p-C-RAF (S338), p-MEK, p-ERK, and actin (loading control). C, *in vitro* C-RAF kinase activity was measured in cell extracts derived from 293T cells transiently expressing Flag-tagged empty vector (C), wild type C-RAF (WT), and C-RAF harboring the resistance mutants S257P, P261T, G361A, and E478K. Assays were conducted in the presence or absence of 2 μmol/L vemurafenib (see Materials and Methods). Input lysate was also immunoblotted using antibodies that detect p-MEK1/2, p-ERK1/2, total MEK, total ERK, and actin. D, *in vitro* C-RAF kinase activity was measured in cell extracts derived from A375 cells (B-RAF^{V600E}; C) and A375 stably expressing wild-type C-RAF (WT), and C-RAF harboring the resistance mutants S257P, P261T, and G361A in the presence and absence of 2 μmol/L vemurafenib. Immunoblotting studies were conducted on input lysate using antibodies recognizing p-MEK1/2, p-ERK1/2, total MEK, total ERK, total C-RAF, and actin. All results are representative of 3 independent experiments.

one exception to this; here, modest steady-state kinase activity was detected that correlated with robust intrinsic p-MEK levels in the corresponding whole-cell lysates (Fig. 4C). In contrast, treatment of 293/T cells with 2 μmol/L vemurafenib before the *in vitro* kinase assays resulted in a marked upregulation of C-RAF kinase activity in all resistance alleles examined (Fig. 4C).

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Similar experiments in B-RAF^{V600E} melanoma cells (A375) revealed an increase in intrinsic kinase activity in the 3 most potent C-RAF resistance mutants examined (S257P, P261T, and G361A; Fig. 4D). This kinase activity was further augmented upon exposure of these cells to vemurafenib (Fig. 4D), as observed in 293/T cells. As expected, A375 cells showed elevated basal MEK phosphorylation that was inhibited by vemurafenib (Fig. 4D). Together, these results suggested that potent C-RAF resistance mutants enhanced RAF inhibitor–mediated C-RAF kinase activity.

C-RAF resistance mutants exhibit reduced 14-3-3 binding and increased B-RAF heterodimerization

The cumulative data above suggested that C-RAF mutations encompassing its 14-3-3 consensus binding site (S257P and P261T) and the ATP-binding region of the P loop (G361A) conferred pharmacologic and biochemical resistance to RAF inhibition, enhanced RAF dimerization, and increased C-RAF kinase activation upon treatment with RAF inhibitors. To investigate the role of 14-3-3 protein binding in relation to RAF dimerization, immunoprecipitation experiments were carried out from cells engineered to ectopically express C-RAF resistance alleles. To examine the effects of pharmacologic RAF inhibition on 14-3-3 binding and B-RAF heterodimerization, these experiments were carried out in both the absence and presence of RAF inhibition (in this case, vemurafenib). In the absence of RAF inhibitor, the C-RAF resistance alleles S257P, P261T, and G361A tended to show reduced interactions with 14-3-3ζ and increased interactions with B-RAF in 293/T cells (Fig. 5A) and, in particular, A375 (B-RAF^{V600E}) melanoma cells (Fig. 5B). In 293/T cells, the enhanced C-RAF/B-RAF heterodimerization triggered by C-RAF mutations correlated with C-RAF protein stabilization and robust MEK/ERK phosphorylation (Fig. 5A). On the other hand, the robust MEK/ERK activation observed in B-RAF^{V600E} melanoma cells was only marginally enhanced by the C-RAF resistance mutants (Fig. 5B); this result was expected given the constitutive oncogenic B-RAF signaling in these cells. Interestingly, one of the C-RAF mutants (G356E) exhibited very low 14-3-3ζ binding in both cellular contexts (Fig. 5A and B)—the reason for this is unknown. However, C-RAF^{G356E} showed no enrichment in B-RAF heterodimerization and no increase in MEK/ERK signaling under steady-state conditions. These results suggest that while reduced 14-3-3 binding may promote enhanced mutant C-RAF dimerization, some degree of 14-3-3 binding (perhaps within the C-terminal domain) is needed to promote maximal RAF-dependent signaling.

As expected, the RAF inhibitor vemurafenib induced B-RAF/C-RAF heterodimerization in 293/T cells ectopically expressing wild-type C-RAF (Fig. 5A) but abrogated this heterodimerization in A375 melanoma cells (Fig. 5B). In contrast, ectopic expression of the most robust C-RAF resistance mutants enabled sustained B-RAF heterodimerization even in the presence of drug in A375 cells (Fig. 5B). These results suggest that B-RAF^{V600E} may assume a dominant conformation that favors heterodimerization with resistance-associated C-RAF variants. Pharmacologic RAF inhibition had variable effects on the 14-3-3/C-RAF interaction depending on the cellular genetic context. In A375 cells (BRAF^{V600E}), vemurafenib modestly decreased the 14-3-3 zeta isoform (14-3-3 ζ) binding to wild-type C-RAF but

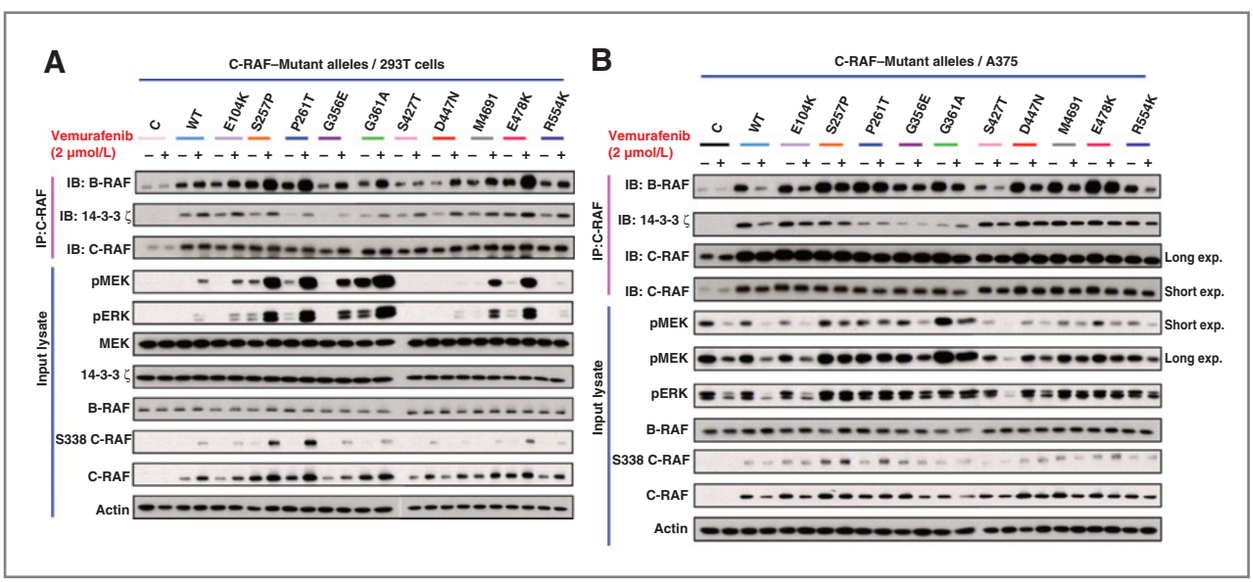

Figure 5. Heterodimerization and 14-3-3 binding properties of C-RAF resistance mutants. A, 293/T cells transiently expressing the indicated C-RAF resistance mutants in the absence (–) or presence (+) of 2 μmol/L vemurafenib for 1 hour were immunoprecipitated with C-RAF and levels of bound protein (B-RAF and 14-3-3 ζ; top) was assessed by immunoblotting. Immunoprecipitated C-RAF is also shown. Input lysate (bottom) shows 14-3-3 ζ, B-RAF, C-RAF, p-MEK1/2, p-ERK1/2, total MEK, p-C-RAF(S338), and actin. Results are representative of more than 2 independent experiments. B, A375 cells stably expressing the indicated C-RAF resistance mutants in the presence and absence of 2 μmol/L vemurafenib for 16 hours were immunoprecipitated with C-RAF and levels of bound protein (B-RAF and 14-3-3 ζ; top) were assessed by immunoblotting. Immunoprecipitated C-RAF is also shown. Input lysate was blotted for the same as in A. Results are representative of more than 2 independent experiments.

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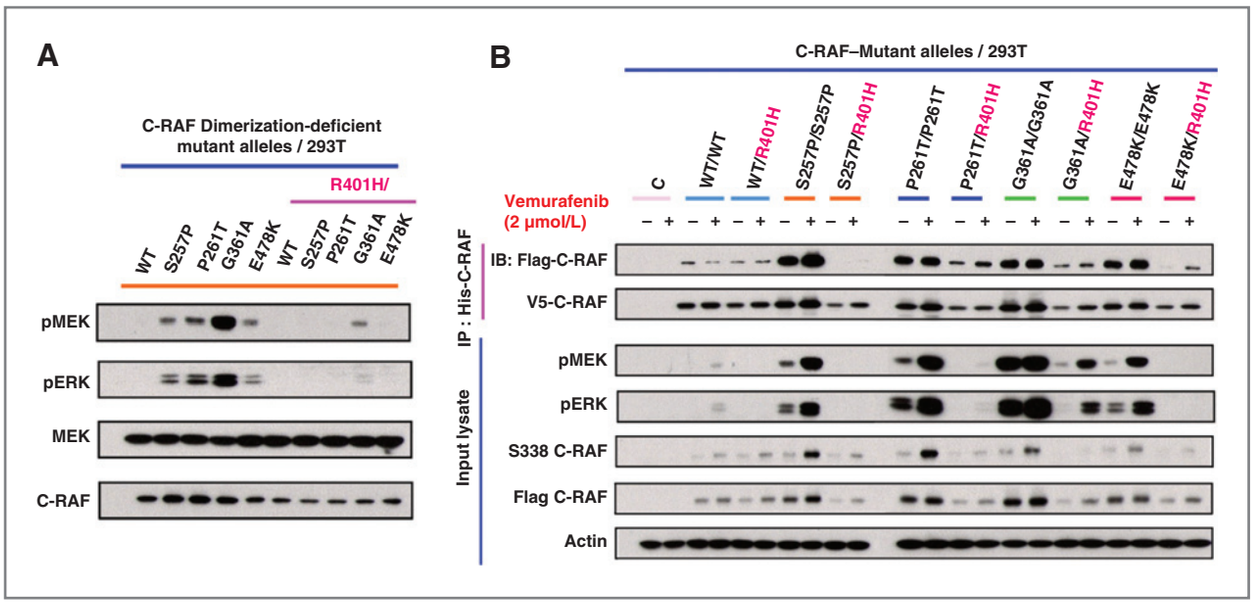

Figure 6. C-RAF resistance mutants require dimerization for MEK/ERK signaling. A, constructs expressing either Flag-tagged, wild-type C-RAF, or the indicated C-RAF resistance mutants in the absence (left) or presence (right) of the dimerization-deficient mutant R401H (pink) were expressed in 293T cells. Lysates were blotted using antibodies recognizing p-MEK, p-ERK, total MEK, or total C-RAF. B, 293/T cells coexpressing His-tagged C-RAF resistance mutants by themselves and in the dimerization deficient (pink) context were cultured in the absence or presence of vemurafenib (2 μmol/L, 1 hour). Immunoprecipitations were conducted using nickel beads and levels of Flag-tagged C-RAF were assessed by immunoblotting. Input lysates blotted with antibodies recognizing Flag-C-RAF, V5-C-RAF, total C-RAF, p-MEK, p-ERK, p-C-RAF(S338), and actin are also shown.

had no effect in the setting of the C-RAF^{S257P}, C-RAF^{P261T}, and C-RAF^{G361A} mutants (Fig. 5B). On the other hand, vemurafenib enhanced these 14-3-3/C-RAF interactions in 293/T cells (Fig. 5A). These findings lent further support to the premise that the resistance phenotype conferred by these C-RAF mutants in the B-RAF^{V600E} context involved enhanced RAF dimerization, which correlated with diminished 14-3-3/C-RAF interactions.

Enhanced MEK/ERK signaling by C-RAF resistance mutants requires dimerization

To test whether C-RAF dimerization is necessary for the enhanced MEK/ERK signaling conferred by C-RAF resistance mutants, an arginine–histidine mutation was introduced at residue R401 (Fig. 1C; C-RAF^{R401H}; Supplementary Fig. S4B). This mutant has previously been shown to disrupt C-RAF homodimerization (24, 25). We introduced the R401H dimerization deficient mutation into the respective C-RAF resistance alleles. As expected, C-RAF double mutants were rendered largely incapable of enhanced MEK/ERK signaling (Fig. 6A). Next, cotransfections were conducted using differentially epitope-tagged C-RAF resistance/R401H double mutants. As described earlier, the C-RAF resistance alleles augmented C-RAF homodimerization in a manner unaffected by RAF inhibitor (Fig. 6B). In contrast, introduction of the R401H allele suppressed C-RAF homodimerization and abrogated MEK/ERK signaling in most C-RAF mutant contexts examined. The exception to this was the C-RAF^{G361A} allele, which exhibited constitutive (albeit markedly reduced) MEK/ERK activation that was further induced by vemurafenib exposure, even when coexpressed with the dimerization-deficient double mutant. Together with the *in vitro* kinase activity results above, these data suggest that the C-RAF$^{G361A/R401H}$ allele may also contain increased intrinsic kinase activity. Overall, these results provide direct evidence that the enhanced MEK/ERK signaling conferred by C-RAF resistance mutants requires RAF dimerization. They may also provide a rationale for the future development of allosteric RAF inhibitors that disrupt the RAF dimerization interface.

Discussion

In this study, we present the results of random mutagenesis screens for mutations in B-RAF^{V600E} and C-RAF that confer resistance to small-molecule RAF inhibitors. Secondary mutations (or gene amplifications) affecting the target oncoprotein comprise a common mechanism of resistance to several targeted anticancer agents. In this regard, the power of *in vitro* mutagenesis approaches to identify clinically relevant, target-based mechanisms of resistance to kinase oncoprotein inhibitors has long been recognized. Studies of Abl kinase mutations that confer imatinib resistance were among the first to reveal the use of this approach. These efforts were followed by similar mutagenesis-based queries of the Flt3 receptor tyrosine kinase (41). More recent studies by our group in B-RAF^{V600E} melanoma leveraged this mutagenesis approach (expanded in scope through massively parallel sequencing of resistant clones) to identify mutations in the MEK1 kinase that confer resistance to MEK or RAF inhibitors. Several MEK1 mutations have since been found in melanomas that progressed following treatment with these agents (11, 17). This

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work therefore provided an early example of mutations situated downstream of the target oncoprotein that re-activate the salient pathway to produce resistance.

B-RAF^{V600E} melanoma has thus far proved unusual in that secondary B-RAF point mutations have yet to be described as a clinical means of resistance to RAF inhibitors (although B-RAF amplification has been observed; ref. 20). The results of our B-RAF^{V600E} mutagenesis screen suggest that secondary B-RAF^{V600E} mutations are plausible in principle; however, the ability of such mutations to confer profound resistance remains obscure. Indeed, only 1 of the 3 putative B-RAF^{V600E} resistance alleles identified by random mutagenesis produced sustained MEK/ERK signaling upon re-expression and challenge with a selective RAF inhibitor, and none engendered pharmacologic resistance to drug-sensitive B-RAF^{V600E} melanoma cells based on growth inhibition assays. While these results certainly do not exclude the possibility that secondary B-RAF^{V600E} resistance mutations might eventually be observed in relapsing melanoma specimens, they also support the premise that this specific mechanism may be less favored clinically. For example, it is conceivable that further elevations in intrinsic B-RAF kinase activity (already rendered several 100-fold more active that wild-type B-RAF by the V600E/K mutation) may be detrimental to the growth of melanoma cells. Alternatively, some point mutations that disrupt drug binding may prove antagonistic to overall oncogenic B-RAF signaling (e.g., the secondary V528F mutation identified herein). Additional mutagenesis studies that include both oncogenic and wild-type B-RAF may provide additional insights into target-based alterations that affect resistance to RAF inhibitors.

In light of these observations, the discovery of C-RAF resistance mutations is noteworthy as the first demonstration of any RAF family point mutation capable of conferring robust pharmacologic resistance to small-molecule RAF inhibitors in a B-RAF^{V600E} melanoma cell context. A prior study focusing on B-RAF gatekeeper alleles showed that such mutations could modify sensitivity to PLX4720; however, these experiments queried interleukin (IL)-3–independent growth in a Ba/F3 cell system (35). C-RAF is typically not required for melanoma cell viability in the setting of B-RAF^{V600E} (26, 42, 43), although it may become engaged in melanoma cells that contain non-V600E B-RAF mutations (22). Several C-RAF resistance mutants described here potentiate C-RAF kinase activity, downstream MEK/ERK signaling, and paradoxical augmentation by RAF inhibitors in the absence of upstream RAS activation (Supplementary Fig. S5). Despite their distinct locations within the C-RAF protein, they may converge onto a common resistance mechanism that favors the adoption of a dimerization-competent protein conformation. Given that the G361A mutation resides immediately adjacent to the P loop—next to the drug-binding region, this mutation may perturb the conformation of the DFG motif in a manner that potentiates an active kinase conformation. Although the C-RAF structure spanning the S257P and P261T mutations (including the CR2 14-3-3 binding region) has not yet been solved, our results strongly suggest that these mutations also promote a conformation that favors dimerization. Indeed, our experiments that incorporated a dimerization-deficient C-RAF mutant confirmed that these resistance alleles required dimerization for enhanced MEK/ERK signaling.

Multiple published studies have shown that 14-3-3 protein binding plays an important role in the regulation of RAF kinase activity (22, 29, 44). The 3 potent C-RAF resistance mutants identified in this study exhibit reduced (but not absent) 14-3-3 binding, which, in turn, is associated with increased RAF dimerization. In all cases, the end result is enhanced MEK/ERK signaling, consistent with prior studies that endorse the importance of C-RAF dimerization in MAPK pathway activation (24, 25). Overall, these results support a biphasic model for 14-3-3/C-RAF interactions wherein "full" C-RAF/14-3-3 interactions (involving both the CR-2 and C-terminal domains) may constrain C-RAF activity, but disruption of CR2-dependent 14-3-3 binding favors a C-RAF conformation that is permissive for enhanced RAF dimerization and (RAF inhibitor–inducible) kinase activity.

Of course, the gold standard for any study of anticancer drug resistance involves a demonstration that mechanisms identified experimentally are also operant in relapsing tumors. To date, C-RAF point mutations have not been linked to either *de novo* or acquired clinical resistance to RAF inhibitors (although it remains unclear to what extent C-RAF sequencing has been conducted in this setting). However, it has become clear that somatic C-RAF mutations may contribute to melanoma biology in some settings. In particular, the C-RAF^{E478K} mutation described here was previously reported in a colorectal cancer cell line that exhibited hypersensitivity to oncogenic RAS activation *in vitro* (40). This mutation, which is analogous to BRAF^{E586K} (3, 40), has also been found in gastric, lung, and breast cancer cell lines (45). More recently, we also identified the C-RAF^{E478K} mutation in a melanoma tumor that is wild type for both B-RAF and N-RAS (Supplementary Fig. S6; ref. 46), which is consistent with increased heterodimerization of this mutant with B-RAF leading to increased kinase activity under steady state (Figs. 4A and 5A). In the aggregate, our findings raise the tantalizing possibility that resistance-associated C-RAF mutations might yet be discovered—especially as the activity of such mutants becomes enhanced in the presence of RAF inhibitors. However, such speculation must be tempered by the fact that the 3 most potent resistance alleles described in this work cannot arise by the most common UV-associated base mutations (e.g., C-to-T transitions cannot generate the relevant amino acid substitutions at codons 257, 261, and 361). Undoubtedly, whole-exome sequencing of large numbers of treatment-refractory melanomas will prove highly informative in this regard, particularly as more potent RAF inhibitors progress through clinical development.

In conclusion, we have used random mutagenesis and biochemical studies to identify mutations in C-RAF that confer resistance to RAF inhibitors. These results have provided new insights into biochemical mechanisms that promote C-RAF dimerization and paradoxical MAPK pathway activation by RAF inhibitors (even in the absence of an upstream oncogenic module). Such knowledge may anticipate new therapeutic resistance mechanisms and speed the design of "next-generation" ATP-competitive or allosteric RAF inhibitors that

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circumvent plausible resistance mechanisms. In doing so, these studies may enable additional improvements in the clinical management of patients with RAF-dependent tumors.

Disclosure of Potential Conflicts of Interest

L.A. Garraway has a commercial research grant from Novartis, has ownership interest (including patents) from Foundation Medicine, and is a consultant/advisory board member of Novartis, Foundation Medicine, Millennium/Takeda, and Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: R. Antony, C.M. Emery, L.A. Garraway
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Antony, C.M. Emery, L.A. Garraway
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Antony, C.M. Emery, L.A. Garraway
Writing, review, and/or revision of the manuscript: R. Antony, C.M. Emery, L.A. Garraway
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Antony, A.M. Sawyer, L.A. Garraway

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C-RAF Mutations Confer Resistance to RAF Inhibitors

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