In Vivo MAPK Reporting Reveals the Heterogeneity in Tumoral Selection of Resistance to RAF Inhibitors

Kevin J. Basile, Ethan V. Abel, Neda Dadpey, Edward J. Hartsough, Paolo Fortina, and Andrew E. Aplin

Abstract
Activation of the ERK1/2 mitogen-activated protein kinases (MAPK) confers resistance to the RAF inhibitors vemurafenib and dabrafenib in mutant BRAF-driven melanomas. Methods to understand how resistance develops are important to optimize the clinical use of RAF inhibitors in patients. Here, we report the development of a novel ERK1/2 reporter system that provides a noninvasive, quantitative, and temporal analysis of RAF inhibitor efficacy in vivo. Use of this system revealed heterogeneity in the level of ERK1/2 reactivation associated with acquired resistance to RAF inhibition. We identified several distinct novel and known molecular changes in resistant tumors emerging from treatment-naïve cell populations including BRAF V600E variants and HRAS mutation, both of which were required and sufficient for ERK1/2 reactivation and drug resistance. Our work offers an advance in understanding RAF inhibitor resistance and the heterogeneity in resistance mechanisms, which emerge from a malignant cell population. Cancer Res; 73(23): 7101–10. ©2013 AACR.

Introduction
The MAPK/ERK1/2 signaling pathway is aberrantly regulated in multiple tumor types. Mutations in the serine/threonine kinase BRAF (mutant v-raf murine sarcoma viral oncogene homolog B1) that activate ERK1/2 signaling are found in 7% of human cancers with a high frequency (45%–50%) in cutaneous melanoma. Clinical inhibitors targeting steps in the ERK1/2 pathway are being actively pursued. Recent successes are the U.S. Food and Drug Administration (FDA) approval of the RAF inhibitor vemurafenib (PLX4032) and dabrafenib in late-stage mutant V600E BRAF melanomas. Vemurafenib, the RAF inhibitor vemurafenib (PLX4032) and dabrafenib in are the U.S. Food and Drug Administration (FDA) approval of the development of a novel ERK1/2 reporter system that provides a noninvasive, quantitative, and temporal analysis of RAF inhibitor efficacy in vivo. Use of this system revealed heterogeneity in the level of ERK1/2 reactivation associated with acquired resistance to RAF inhibition. We identified several distinct novel and known molecular changes in resistant tumors emerging from treatment-naïve cell populations including BRAF V600E variants and HRAS mutation, both of which were required and sufficient for ERK1/2 reactivation and drug resistance. Our work offers an advance in understanding RAF inhibitor resistance and the heterogeneity in resistance mechanisms, which emerge from a malignant cell population. Cancer Res; 73(23): 7101–10. ©2013 AACR.

Materials and Methods

Cell culture
Parental 1205Lu cells were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Parental A375 cells were purchased from the American Type Culture Collection. 1205LuTr is a subline with high Tet repressor (TR) expression (19). Lines were verified by DNA sequencing of multiple independent loci. 1205Lu cells were cultured in MCDB153 medium containing 20% Leibovitz L-15 medium, 2% FBS, 0.2% sodium bicarbonate, and 5 μg/ml insulin. A375 cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM)
with 10% FBS. PLX4720 and vemurafenib were provided by Dr. Gideon Bollag and Plexxikon Inc. AZD6244 (selumetinib) and GSK1120212 (trametinib) were purchased from Selleck Chemicals. Cells lines were authenticated by DNA sequencing at multiple loci.

**Lentiviral cloning**

pLenti4.3/V5-DEST, pLentino3/V5-DEST, pLentihygro3/V5-DEST and pLentipuro/V5-DEST vectors are modifications of pLentiv6/V5-DEST (Invitrogen). Renilla luciferase and GAL4-ELK1 were cloned into pENTR/D-TOPO (Invitrogen) from pRL-TK (Promega Corp.) and pFA2-ELK1 (Agilent Tech.), respectively. The 5’ upstream activation sequences (UAS) and minimal promoter of pFR-Luc (Agilent Tech.) was cloned into pENTR/D-TOPO upstream of an EGFP-firefly luciferase fusion gene. An additional 5 copies of the tandem UAS were added upstream (10 copies of UAS in total) to enhance transcription of the transgene. Stop codons were omitted from both Renilla luciferase and EGFP-firefly luciferase to allow for in-frame fusion with C-terminal V5 epitope found in all of the afore-mentioned lentiviral vectors. Wild-type HRAS, full-length BRAF V600E, BRAF full length without a stop codon, and a previously described (20) antibody were purchased from the following: ERK2, BRAF, HRAS, and cyclin A (Santa Cruz Biotech.); and phospho-ERK1/2 (Invitrogen); and V5 (Invitrogen); actin (Sigma-Aldrich); ERK2, BRAF, HRAS, and cyclin A (Santa Cruz Biotech.); and phospho-ERK1/2, BRAF V600E, BRAF V600E Mutation Detector Software v 2.0 (Life Technologies) using Mutation percentage of each sample was determined by curve experiment and compared to internal positive controls. Mutation percentage of each sample was determined by Mutation Detection Software v 2.0 (Life Technologies) using the Ct values of the mutant and wild-type assays.

**Generation of reporter cells**

1205LuTR cells were transfected for 72 hours with UAS/EGFP-firefly luciferase and Ubc/Renilla luciferase lentiviruses. Cells were selected simultaneously with 500 μg/mL Geneticin (Invitrogen) and 200 μg/mL Zeocin (Invitrogen). Resistant cells were subsequently transduced with Ubc/GAL4-ELK1 virus for 72 hours, followed by selection with 200 μg/mL HygroGold (Invitrogen). 1205LuTR reporter cells expressing high basal EGFP following transduction of GAL4-ELK1 virus were enriched by cell sorting for in vivo experiments.

**Dual luciferase assay**

Cells were lysed and firefly and Renilla luciferase activities measured using the Dual-Luciferase Assay System Kit (Promega) on a Glomax luminometer (Promega). Cells lysates were analyzed by Western blotting, as previously described (20). Antibodies were purchased from the following: GFP and V5 (Invitrogen); actin (Sigma-Aldrich); ERK2, BRAF, HRAS, and cyclin A (Santa Cruz Biotech.); and phospho-ERK1/2 and phospho-Rb Ser780 (Cell Signaling Technology).

**Competitive allele-specific TaqMan PCR**

CAST (competitive allele-specific TaqMan) PCR reactions were used to determine percentage of HRAS Q61 allele present in each sample. Reactions were conducted on the ABI 7500 FAST system (Life Technologies) as a quantitation/standard curve experiment and compared to internal positive controls. Mutation percentage of each sample was determined by Mutation Detector Software v 2.0 (Life Technologies) using the Ct values of the mutant and wild-type assays.

**PCR**

To detect Braf splice variants, cDNA libraries were generated using an oligo(dT) primer and used as templates for further PCR amplification using the following primers: forward, 5’-TTATAAGGACACCCAAGCAGTGTACACATCCC-3’. Reverse, 5’-TCAGTGGACAGAAAAGCACCACATATCC-3’. Cells were transfected with siRNAs at a concentration of 25 nmol/L using Lipofectamine RNAiMAX (Invitrogen). Nontargeting control (5’-UGUUUUACAGUGCACUA-3’), ERK1 (5’-CGCAGACUGUUGAGAAAUU-3’), ERK2 (5’-CAAGCCUGACCCUGAUUU-3’), BRAF full length (5’-CGAGACACUCUAAAGAAGU-3’), BRAF ΔEx 3–10 (5’-AUCUGAGAGAAAACUUCU-3’), BRAF ΔEx 2–8 (5’-CCAGAGAGAGUGUAGUU-3’), BCR ΔEx 2–8 (5’-CAGAGGACAGACCCGAA-3’, 5’-GAAAACCCCUAGAGAGUGAG-3’, 5’-GGAGACAGGAGGUGAAUGA-3’, 5’-GAGUGAGGAGGACGUACUA-3’, and HRAS #8 (5’-AGACUGGCGUGUGUCA-3’) siRNAs were purchased from Dharmacon.

**Cell viability assays**

Cells were plated at a confluence of 4 × 10⁶ cells per well of a 6-well plate and treated as indicated. Media and drugs were replenished once. After 5 days, 1 x AlamarBlue (Invitrogen) was added to each well and allowed to reduce for approximately 30 minutes. Medium was collected in triplicate from each
condition and the absorbances of oxidized and reduced AlamarBlue were measured at wavelengths 600 and 570 nm, respectively, in a Multiskan Spectrum spectrophotometer (Thermo Scientific). The change in viability was calculated from the resulting absorbances using the manufacturer’s guidelines. All conditions were normalized to dimethyl sulfoxide (DMSO) control.

Colony formation assays

Cells were plated at a confluency of 1.5 × 10^5 cells per 10-cm dish and treated as indicated. Media and drugs were replenished twice. After 7 days, cells were stained with crystal violet in formalin for 30 minutes. After excess stain was washed away, colonies were imaged on a Nikon Eclipse Ti inverted microscope (Nikon) with NIS-Elements AR 3.00 software (Nikon). The percent plate coverage is indicated as determined from 5 independent areas per plate using ImageJ software.

Statistical analyses

Where noted, the data were analyzed using 2-tailed, unpaired Student t test, assuming unequal variances. For experiments assessing in vivo tumor growth, data were analyzed using mixed effects model and Tukey multiple comparisons correction. Analyses were conducted using SAS software.

Study approval

All animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care accredited facility at Thomas Jefferson University (Philadelphia, PA).

Results

Generation of an ERK1/2 melanoma cell reporter system

Mutant BRAF melanoma cells, 1205LuTR, were transduced to express an EGFP-firefly luciferase fusion gene under the control of a minimal promoter with 10 tandem copies of the GAL4 upstream activation sequence (UAS; Supplementary Fig. S1). For an internal control, cells were also transduced with Renilla luciferase driven from the constitutive human ubiquitin C (Ubc) promoter. TR versions of 1205Lu cells were used to permit future molecular-based approaches. The resulting reporter cells (1205LuTR Ubc/Rl UAS/EGFP-Luc) exhibited virtually no firefly luciferase activity relative to Renilla activity (Supplementary Fig. S2A). To produce high firefly luciferase expression that was ERK1/2 responsive, cells were transduced with Ubc-driven GAL4-ELK1, a fusion protein with transcriptional activity that is dependent upon phosphorylation by ERK1/2 (refs. 21, 22; Supplementary Fig. S2A).

To determine responsiveness to RAF/MEK1/2 inhibition, we used 2 RAF inhibitors (PLX4720 and vemurafenib) and 2 MEK1/2 inhibitors (AZD6244/selumetinib and GSK1120212/trametinib). 1205LuTR reporter cells showed a strong reduction in the firefly luciferase activity relative to Renilla luciferase following 24 hours of treatment with these compounds (Fig. 1A). Depletion of ERK1 and ERK2 via siRNA transfection also dramatically lowered firefly luciferase activity (Fig. 1B and Supplementary Fig. S2B). Levels of V5-tagged EFGP firefly luciferase were reduced in response to RAF and MEK inhibitors, whereas Renilla luciferase levels remained unaffected as measured by Western blotting (Supplementary Fig. S2C) and flow cytometry (Supplementary Fig. S2D). These in vitro data show the generation of an ERK1/2 reporter melanoma model.

Efficient ERK1/2 inhibition in vivo precedes effects on tumor xenograft shrinkage by days

To test the application of this system in vivo, xenograft tumors were established by injecting 1205LuTR reporter cells intradermally into athymic nude mice. Tumors were allowed to form for 11 days at which time tumor volume and Renilla luciferase expression were comparable between mouse cohorts (Fig. 1C and Supplementary Fig. S3). Cohorts were then treated with either PLX4720 chow or control chow. After 2 days of treatment, firefly luciferase expression was significantly lower in PLX4720-treated mice compared to vehicle, an effect that was observed in all mice with a 77% to 98% range of inhibition (Fig. 1C and D). Firefly luciferase expression was normalized to tumor volume as determined by digital caliper measurements as opposed to Renilla luciferase intensity measurements due to less variability in the reading caused by the rapid turnover of the Renilla signal in vivo. Noticeable inhibition of xenograft growth by PLX4720 lagged behind reduction of ERK1/2 reporter activity and was only apparent in between days 4 and 7 (Fig. 1E). These data model temporal inhibition of ERK1/2 in vivo and show that pathway inhibition precedes inhibition of tumor growth.

ERK1/2 reactivation is associated with rapid tumor regrowth in vivo

We continued PLX4720 treatment of the 1205LuTR reporter xenografts. ERK1/2 activity was inhibited by PLX4720 through treatment day 28; however, xenografts displayed enhanced ERK1/2 activity between 28 and 35 days, which often increased to levels above those of pretreatment xenografts by day 42 (Fig. 2A). Reactivation of ERK1/2 was closely associated with enhanced tumor growth (Fig. 2B). Analysis of individual tumors showed a dramatic variation in the level of ERK1/2 reactivation. Half of the tumors (4/8) displayed ERK1/2 reactivation to a level above the pretreatment starting point (Fig. 2C and D). The remainder (4 of 8) xenografts showed partial reactivation of ERK1/2 (Fig. 2C and D). These data show that ERK1/2 pathway reactivation to different levels is associated with resistance to PLX4720 in vivo.

Heterogeneous molecular changes are found in PLX4720-resistant xenografts

To characterize molecular events that lead to ERK1/2 pathway reactivation in vivo, tumors were harvested at day 49 and hereafter referred to as PLX4720-resistant tumors (PRT). We were able to establish 4 of 8 PRTs as cell lines in the presence of PLX4720. Consistent with in vivo reporter activity, Western blotting showed heightened but varied
levels of phospho-ERK1/2 in all 4 PRT cell lines (#2, #3, #4, #6) compared with cell lines derived from vehicle-treated tumors and parental cells (Fig. 3A and Supplementary Fig. S4A). No differences in PDGFRβ, IGF1Rβ, ARAF, BRAF, CRAF, or phospho-AKT levels were detected between parental cells and PRT cells derived from 1205LuTR reporter cells (Supplementary Fig. S4A). Variation in phospho-ERK1/2 was also seen in cell lines derived from A375 xenograft tumors that had acquired resistance to PLX4720 in vivo (Supplementary Fig. S4D and S4E). A375-derived PRT #3 showed strong reactivation of ERK1/2 signaling and weak phosphorylation of AKT (Supplementary Fig. S4E). In contrast, A375-derived PRT #4 showed very low phosphorylation of ERK1/2 in the presence of PLX4720 but heightened phosphorylation of AKT (Supplementary Fig. S4E). These data show differences in ERK1/2 reactivation during in vivo acquired resistance to PLX4720 in a second melanoma cell line.

In the 1205Lu reporter system, sequencing of gDNA obtained from tumor tissue showed that all 8 PRTs maintained BRAF V600E positivity. PRT #8, however, showed enhancement of the BRAF V600E allele compared to vehicle-treated tumors indicating BRAF V600E homozygosity (Fig. 3B). This finding was confirmed by quantitation of IonTorrent sequencing and CAST PCR (Supplementary Fig. S4B and S4C). Although no PRTs showed acquisition of NRAS mutations (Fig. 3B), sequencing revealed an HRAS Q61K mutation in PRT #6 (Fig. 3B and Supplementary Fig. S4B). CAST PCR, which considers a sample positive if the level of HRAS Q61K present in the DNA sample is above 0.1%, did not detect mutant HRAS in the parental cells (Fig. 3C).
PCR analysis revealed that 2 of the 4 1205Lu reporter PRT cell lines (PRT #3 and PRT #4) expressed detectable levels of distinct BRAF variants (Fig. 3D). PRT #3 expressed a previously unreported variant that splices exon 2 with exon 11 of BRAF V600E (Fig. 3E). PRT #4 expressed a variant previously reported from a patient sample that splices exon 1 with BRAF V600E.

**Figure 2.** ERK1/2 reactivation in GAL4-ELK1 reporter cells xenografts that have acquired resistance to PLX4720 *in vivo*. A, 1205LuTR reporter cell xenografts were fed PLX4720 chow for 49 days. Xenografts were imaged for firefly luciferase using a 1-minute exposure time. Readings were normalized to tumor volume, as determined by digital caliper measurements. Columns represent mean firefly luciferase intensity, $n=8$. Error bars, SEM. B, columns represent mean fold change in tumor volume of PLX4720-treated group normalized to day 0 measurements. $n=8$. Error bars, SEM. C, images from individual mice with tumor progression associated with low ERK1/2 reactivation (mouse #5) and high ERK1/2 reactivation (mouse #6). D, analysis of GAL4-ELK1 reporter activity normalized to tumor volume in individual xenografts treated with PLX4720.
exon 9 of BRAF V600E (ref. 5; Fig. 3E). Both variants maintained the V600E mutation, lacked the RAS-binding domain and were detected by Western blotting using a C-terminal BRAF antibody (Fig. 3F). These data show heterogeneity in mechanisms of acquired resistance to RAF inhibitors from multiple cell lines.
Identified BRAF V600E variants and mutant HRAS promote ERK1/2 reactivation

Next, we tested whether the identified molecular changes in PRTs were necessary for resistance to PLX4720. We focused on 1205Lu reporter PRTs #3 (BRAF V600E ΔEx 3-10), #4 (BRAF V600E ΔEx 2-8), and #6 (HRAS Q61K). These cell lines showed strong resistance to phospho-ERK1/2 inhibition by PLX4720 in vitro; in contrast, PRT #2 was partially sensitive to PLX4720 (Fig. 3A). In PRT #3 and PRT #4, co-knockdown of full-length and variant forms of BRAF V600E effectively reduced levels of phospho-ERK1/2 (Supplementary Fig. S5A). To differentiate which form of BRAF V600E was important for signaling in PRTs #3 and #4, we used siRNAs designed to specifically target either full-length or variant BRAF. In the absence of PLX4720, knockdown of full-length BRAF had no effect on phospho-ERK1/2, whereas knockdown of BRAF V600E variants in their respective cell line reduced basal phospho-ERK1/2 levels (Fig. 4A and B). In PLX4720-treated conditions, knockdown of full-length BRAF V600E reduced phospho-ERK1/2 levels by 50% to 80%, whereas variant knockdown caused a near complete reduction (Fig. 4A and B). The viability of variant knockdown cells was also significantly reduced by PLX4720 treatment (Fig. 4C). BRAF variant–specific siRNAs did not inhibit phospho-ERK1/2 signaling in parental cells (Supplementary Fig. S5B).

In PRT #6, knockdown of HRAS in combination with PLX4720 treatment efficiently ablated phospho-ERK1/2 (Fig. 4D). This effect was specific to HRAS and was not observed with either NRAS or KRAS knockdowns (Supplementary Fig. S5C). In addition, viability was significantly decreased in HRAS depleted cells treated with PLX4720 (Fig. 4E). These data show

Figure 4. BRAF V600E variants and HRAS Q61K are necessary for resistance to PLX4720. A, PRT #3 was transfected with control (Cnt), full-length BRAF (Full), or BRAF ΔEx 3-10 variant siRNA. After 72 hours, cells were treated with DMSO (−) or PLX4720 (1 μmol/L) for 24 hours. Cells were then lysed and analyzed by Western blotting, as indicated. B, same as (A), except PRT #4 and BRAF ΔEx 2-8 siRNA were used. C, PRT #3 and #4 cells were transfected as described in (A) and (B), respectively. After 72 hours, cells were plated at clonal density and treated with DMSO or PLX4720 (1 μmol/L). Cells were processed for AlamarBlue staining. Columns represent mean, n = 3. Error bars, SEM. **P < 0.01 by unpaired, Student t test. D, PRT #6 cells were transfected with control (Cnt), HRAS smartpool, or HRAS #8 siRNA for 72 hours. Cells were then treated with DMSO (−) or PLX4720 (1 μmol/L) for 24 hours, lysed, and analyzed by Western blotting, as indicated. E, same as (D), except cells were processed for AlamarBlue staining. Columns represent mean, n = 3. Error bars, SEM. **P < 0.01 by unpaired, Student t test.
that the molecular changes found in PRT cell lines are required for ERK1/2 activation and viability in the presence of PLX4720.

**BRAF V600E variants and mutant HRAS are sufficient for resistance to PLX4720 treatment**

To determine sufficiency, we inducibly expressed either BRAF V600E ΔEx 3-10, BRAF V600E ΔEx 2-8, or HRAS Q61K in drug-naïve cell lines. Doxycycline-induced BRAF V600E variants and mutant HRAS expression prevented phospho-ERK1/2 inhibition by PLX4720 (Fig. 5A and B), whereas wild-type HRAS had no effect (Supplementary Fig. S6A). In growth assays, PLX4720 treatment significantly reduced colony formation and viability of parental cells, as expected (Fig. 5C and D and Supplementary Fig. S6B). However, expression of either BRAF V600E ΔEx 3-10, BRAF V600E ΔEx 2-8, or HRAS Q61K protected against PLX4720 inhibition of colony formation and viability (Fig. 5C and D and Supplementary Fig. S6B). These results were confirmed by introducing the transgenes into a second line, A375TR cells (Supplementary Fig. S6C–S6E). Ectopic expression of BRAF V600E ΔEx 3-10, BRAF V600E ΔEx 2-8, or HRAS Q61K in *in vivo* tumors mitigated inhibition of ERK1/2 reporter activity and tumor growth by PLX4720 compared to parental tumors (Fig. 5E–G). These results confirm that molecular changes detected in the PRT cell lines are sufficient to provide resistance to RAF inhibition.

**Discussion**

It is essential to quantitatively and temporally monitor the effect of kinase inhibitors on their target pathways *in vivo*. Here, we describe a luciferase-based reporter system to quantify changes in ERK1/2 signaling in mutant BRAF melanoma cells.

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**Figure 5. BRAF V600E variants and HRAS Q61K are sufficient to provide resistance to PLX4720.** A, 1205LuTR reporter cells harboring doxycycline-inducible BRAF V600E variants were induced with 100 ng/mL doxycycline for 24 hours, treated with DMSO (−) or PLX4720 (1 μmol/L) for a further 24 hours, lysed, and analyzed by Western blotting, as indicated. B, same as (A), except 1205LuTR reporter cells harboring doxycycline-inducible HRAS Q61K were used. C, cells used in (A) and (B) were plated at clonal density and treated with DMSO or PLX4720 (1 μmol/L). Cells were processed for crystal violet staining. Mean and SD are shown. n = 2–3. Same as (C), except cells were processed for AlamarBlue staining. Columns represent mean. n = 3. Error bars, SEM. **P < 0.01** by unpaired, Student *t* test. Ectopic expression of either BRAF V600E ΔEx 3-10, BRAF V600E ΔEx 2-8, or HRAS Q61K protected against PLX4720 inhibition of colony formation and viability (Fig. 5C and D and Supplementary Fig. S6B). These results were confirmed by introducing the transgenes into a second line, A375TR cells (Supplementary Fig. S6C–S6E). Ectopic expression of BRAF V600E ΔEx 3-10, BRAF V600E ΔEx 2-8, or HRAS Q61K in *in vivo* tumors mitigated inhibition of ERK1/2 reporter activity and tumor growth by PLX4720 compared to parental tumors (Fig. 5E–G). These results confirm that molecular changes detected in the PRT cell lines are sufficient to provide resistance to RAF inhibition.

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**Discussion**

It is essential to quantitatively and temporally monitor the effect of kinase inhibitors on their target pathways *in vivo*. Here, we describe a luciferase-based reporter system to quantify changes in ERK1/2 signaling in mutant BRAF melanoma cells.
The effects of mutant BRAF inhibition could be visualized \textit{in vivo} with PLX4720 rapidly and efficiently reducing firefly luciferase activity in xenografts. The importance of effective ERK1/2 inhibition is underscored by evidence that more than 80\% loss of phospho-ERK1/2 staining is correlated with a clinical response (2). Of note, this system selectively measures activity within the tumor as opposed to fine-needle aspirate samples, which may contain stromal components. Because of the quantitative readout, a use of this system is to conduct \textit{in vivo} side-by-side comparisons of modified compounds to select for those with the best efficacy and pharmacokinetic properties.

Reactivation of the ERK1/2 pathway is one of the main causes of acquired resistance in patients with melanoma who initially respond to RAF inhibitors (5, 7, 8). Using this reporter system, we show reactivation of the ERK1/2 pathway in tumors progressing while on PLX4720, providing an improved understanding of ERK1/2 reactivation kinetics \textit{in vivo}. Interestingly, there is heterogeneity in the response with some of the tumors showing only a partial reactivation of ERK1/2. This suggests that only a threshold of ERK1/2 reactivation may be necessary for relapse and/or variations in growth kinetics may be attributable to other signaling pathways. The use of this system to determine the extent and timing of ERK1/2 reactivation may be important in studying acquired resistance to RAF inhibitor–based combinations such as dabrafenib and trametinib, which is being tested in ongoing clinical trials (23).

Molecular changes associated with these PLX4720-resistant tumors also showed heterogeneity and included BRAF V600E homozygosity, HRAS mutation, and expression of BRAF V600E variants. Recently, another group generated a vemurafenib-resistant tumor from a patient-derived xenograft model and subsequent sublines (24). One of 9 sublines showed BRAF copy number gain, consistent with genomic amplification at this locus (24) and underscores our observation that multiple molecular changes can occur within a single cell line. Although there have been several reports of NRAS mutations associated with vemurafenib resistance (5, 8, 25), our data suggest that analysis of HRAS mutations is also warranted. In addition, we identified 2 distinct BRAF V600E variants in PRTs. We were unable to detect the presence of these molecular changes in the parental cells; however, we do not rule out that they are present in a very small percentage of cells and selected for during the \textit{in vivo} drug treatment. The heterogeneity of resistant tumors indicates that biopsies should be taken from multiple sites in a patient and that a wide range of RAS mutations and BRAF V600E variants should be included in molecular tests designed to detect the onset of resistance.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** K.J. Basile, E.V. Abel, A.E. Aplin  
**Development of methodology:** E.V. Abel, A.E. Aplin  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K.J. Basile, E.V. Abel, N. Dadpey, E.J. Hartsough, P. Fortina  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K.J. Basile, P. Fortina  
**Writing, review, and/or revision of the manuscript:** K.J. Basile, E.V. Abel, P. Fortina, A.E. Aplin  

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A.E. Aplin

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