In vivo MAPK reporting reveals the heterogeneity in tumoral selection of resistance to RAF inhibitors

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Abstract

Activation of the ERK1/2 mitogen-activated protein kinases (MAPKs) 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 utility of RAF inhibitors in patients. Here we report the development of a novel ERK1/2 reporter system that provides a non-invasive, 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.

Keywords

vemurafenib; RAF; ERK1/2; luciferase; resistance

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 FDA-approval of the RAF inhibitor vemurafenib (PLX4032) and dabrafenib in late-stage mutant V600E
BRAF melanomas. Vemurafenib, PLX4720 (the tool compound of vemurafenib) and dabrafenib are selective inhibitors of the mitogen-activated protein kinase kinase (MEK)-ERK1/2 pathway in mutant V600E BRAF cells (1-3). Patients with effective responses showed >80% inhibition of phosphorylated ERK1/2, as judged by immunohistochemical staining of biopsy samples (2).

RAF inhibitors such as vemurafenib are burdened by two issues. First, nearly all patients who initially respond to vemurafenib, acquire resistance and develop progressive disease. Acquired resistance is associated with reactivation of the ERK1/2 pathway by mechanisms involving mutations in neuroblastoma RAS viral oncogene homolog (NRAS), expression of COT1/MAP3K8, acquisition of MEK1 mutations, amplification of mutant BRAF and expression of BRAF V600E splice variants (4-8). Additionally, resistance mechanisms that are MEK-ERK1/2 independent have been described (8-11). MEK1/2 inhibitors elicit minimal clinical effects in patients progressing on vemurafenib (12) and there is a critical need to develop second-line treatments for vemurafenib-resistant disease. A second issue is that vemurafenib-induced paradoxical activation of the ERK1/2 pathway in wild-type BRAF cells has been linked to the formation of cutaneous squamous cell carcinoma/keratoacanthoma (cuSCC/KA), adenomas and leukemia (13-18). This paradoxical signaling effect is driving the design of next-generation of RAF inhibitors.

In this study, we developed an in vivo ERK1/2 activity reporter system in mutant BRAF melanoma cells. We utilized this system to temporally quantitate the effect of the RAF inhibitor, PLX4720, on ERK1/2 activity across the whole tumor in vivo and to analyze ERK1/2 pathway reactivation and associated genetic changes during acquired resistance to PLX4720.

Materials and Methods

Cell culture

Parental 1205Lu cells were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia). Parental A375 cells were purchased form the American Type Culture Collection (Manassas, VA). 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% fetal bovine serum, 0.2% sodium bicarbonate, and 5 μg/ml insulin. A375 cells were cultured in DMEM with 10% FBS. PLX4720 and vemurafenib were provided by Dr. Gideon Bollag and Plexxikon Inc. (Berkeley, CA). AZD6244 (selumetinib) and GSK1120212 (trametinib) were purchased from Selleck Chemicals (Houston, TX). Cells lines were authenticated by DNA sequencing at multiple loci.

Lentiviral cloning

pLenti4.3/V5-DEST, pLentinoe3/V5-DEST, pLentihygro3/V5-DEST and pLentipuro/V5-DEST vectors are modifications of pLenti6/V5-DEST (Invitrogen, Carlsbad, CA). Renilla luciferase and GAL4-ELK1 were cloned into pENTR/D-TOPO (Invitrogen) from pRL-TK (Promega Corp., Madison, WI) and pFA2-Elk1 (Agilent Tech., Santa Clara, CA), 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 epitopes found in all of the aforementioned lentiviral vectors. Wild-type HRAS, full length BRAF V600E, BRAF V600E ΔEx 3-10 and BRAF V600E ΔEx 2-8 were
amplified from cDNA libraries and cloned into pENTR/D-TOPO. HRAS Q61K was generated via site directed mutagenesis of pENTR/D-TOPO/HRAS-WT. Transgene cassettes were transferred to their respective lentiviral vectors by LR Clonase II (Invitrogen) and lentiviruses were packaged in 293FT cells as previously described (19).

**Generation of reporter cells**

1205LuTR cells were transduced 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™ (Invivogen, San Diego, CA). 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).

**Western blotting**

Cell lysates were analyzed by Western blotting, as previously described (20). Antibodies were purchased from the following: GFP and V5 (Invitrogen); actin (Sigma-Aldrich, St Louis, MO); ERK2, BRAF, HRAS and Cyclin A (Santa Cruz Biotech., Santa Cruz, CA); and phospho-ERK1/2 and phospho-Rb Ser780 (Cell Signaling Technology, Beverley, MA).

**In vivo experiments**

1205LuTR reporter cells (1×10⁶) were injected intradermally into female athymic mice (NCr-nu/nu:NCI-Frederick, MD) and allowed 11 days to reach appropriate volume (~100mm³).

Mice were then fed either AIN-76A (Vehicle) chow or AIN-76A with 417 ppm PLX4720 chow (Plexxikon Inc.). Digital caliper measurements of tumor size were taken in order to calculate tumor volume using the following formula: volume = (length × width²) × 0.52. *In vivo* bioluminescence was performed using the Caliper IVIS Lumina-XR System (Caliper Life Sciences, Hopkinton, MA) and the data-acquisition LivingImage Version 4.0 software (Caliper Life Sciences). For renilla luciferase, mice were imaged after tail vein injection of Rediject coelenterazine (100 μL of 150 μg/mL stock, Caliper Life Sciences). For firefly luciferase, mice were imaged after intraperitoneal injection of D-luciferin (100 μL of 15 mg/mL stock, Caliper Life Sciences). Firefly luciferase was measured at least one hour after renilla luciferase measurement, and diminished signal intensity was confirmed before each firefly luciferase image was acquired. Signal intensity was quantified within a region of interest, as defined by the LivingImage software and was appropriately adjusted using tumor volume measurements. Due to the damage inflicted on the tail veins following multiple injections, we chose to normalize tumor volume by the more consistently determined digital caliper measurements for the extended experiments.

**Competitive Allele-Specific TaqMan® (CAST) PCR**

CAST (competitive allele-specific TaqMan) PCR reactions were used to determine percentage of HRAS Q61 allele present in each sample. Reactions were performed 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.

**Polymerase chain reaction (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′-TTATAAGATGCGCGCTGAGCG-3′; reverse, 5′-TCAGTGAAGGAAACGCACCATTCCC-3′.

**siRNA transfections**

Cells were transfected with siRNAs at a final concentration of 25 nmol/L using Lipofectamine RNAiMAX (Invitrogen). Non-targeting control (5′-UGGUUUACAGUGCAUUAA-3′), ERK1 (5′-CCGCAAGUGCUUAAGAAAAAUU-3′), ERK2 (5′-CCAAAGCUCUGGACUUAAUU-3′), BRAF full length (5′-CCGAGACAGUCUAAAGAAAUU-3′), BRAF ΔEx 3-10 (5′-AUAUCUGGAAACACUACUUU-3′), BRAF ΔEx 2-8 (5′-UCGGGAGGACUUAUUU-3′), HRAS smartpool (5′-CCAUCACGUAUCCGAA-3′, 5′-GAACCCUCCUGAUGAGAAGU-3′, 5′-GGAAGCAGUUGCUUUGAAUAGU-3′, 5′-GAGUUGGAGGAUGGCCUCU-3′) and HRAS #8 (5′-AGACUUGGCCUGUUGACAU-3′) siRNAs were purchased from Dharmacon (Lafayette, CO).

**Cell viability assays**

Cells were plated at a confluency of 4 × 10^4 cells per well of a 6-well plate and treated as indicated. Media and drugs were replenished once. After 5 days, 1× 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 nM 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 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, Tokyo, Japan) 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 two-tailed, unpaired, student t-test, assuming unequal variances. For experiments assessing in vivo tumor growth, data were analyzed using mixed effects model and Tukey’s multiple comparisons correction. Analyses were performed using SAS software.

**Study approval**

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in an Association for the Assessment and Accreditation of
Laboratory Animal Care (AAALAC) 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 utilized 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 (21, 22) (Supplementary Fig. S2A).

To determine responsiveness to RAF/MEK1/2 inhibition, we used two RAF inhibitors (PLX4720 and vemurafenib) and two 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 while renilla luciferase levels remained unaffected as measured by Western blotting (Supplementary Fig. S2C) and flow cytometry (Supplementary Fig. S2D). These *in vitro* data demonstrate 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 vehicles, an effect that was observed in all mice with a 77-98% 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 pre-treatment 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 pre-treatment starting point (Fig. 2C and D). The remainder (4/8) 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 are referred to as PLX4720-resistant tumors (PRTs). We were able to establish four of eight 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 four 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 E). A375-derived PRT #3 showed strong reactivation of ERK1/2 signaling and weak phosphorylation of AKT (Supplementary Fig. S4E). By 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 genomic DNA 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 Competitive Allele-Specific TaqMan® (CAST) PCR (Supplementary Fig. S4B and C). Although no PRTs showed acquisition of NRAS mutations (Fig. 3B), sequencing revealed an HRAS Q61K mutation in PRT #6 (Fig. 3B and Supplementary Figure 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 two of the four 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 exon 9 of BRAF V600E (Fig. 3E). Both variants maintained the V600E mutation, lacked the RAS binding domain and were detected by Western blot using a C-terminal BRAF antibody (Fig. 3F). These data demonstrate 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; by 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 50-80%, 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). Additionally, viability was significantly decreased in HRAS depleted cells treated with PLX4720 (Fig. 4E). These data show 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-6E). 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. The effects of mutant BRAF inhibition could be visualized 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 >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. Due to the quantitative read-out, a utility of this system is to perform 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 melanoma patients 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 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 utility 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 on-going 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 out of nine 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. Additionally, we identified two 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Generation and validation of ERK1/2 reporter cells

(A) 1205LuTR reporter cells were treated with DMSO, PLX4720 (1 μM), vemurafenib (1 μM), AZD6244 (3.3 μM) or GSK1120212 (65 nM) for 24 hours. Dual-luciferase assays were performed. Columns represent mean. n=3. Error bars, s.e.m. **p-value<0.001 by unpaired, student t-test. (B) 1205LuTR reporter cells were transfected with control (Cnt) or ERK1 + ERK2 siRNAs. After 72 hours, dual-luciferase assays were performed. Columns represent mean. n=3. Error bars, s.e.m. **p-value<0.001 by unpaired, student t-test. (C) Xenograft models of 1205LuTR reporter cells in mice that were fed either AIN-76A (Vehicle) chow or AIN-76A with 417 ppm PLX4720 chow were imaged for renilla luciferase activity.
luciferase and firefly luciferase using a one minute exposure time. Representative images are shown from mice at day 3. \( n=8 \). (D) Quantification of firefly luminescence. Columns represent fold change in firefly luciferase signal intensity adjusted for tumor volume (digital caliper measurement) compared to mean firefly luciferase signal intensity in vehicles. \( n=8 \). Error bars, s.e.m. *\( p\)-value<0.05 by unpaired, student t-test. (E) Columns represent mean fold change in tumor volume in 1205LuTR reporter xenografts fed vehicle or PLX4720 chow. \( n=8 \). Error bars, s.e.m. *\( p\)-value<0.05 or **\( p\)-value<0.0001 by mixed effects model and Tukey’s multiple comparisons test.
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 one 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, s.e.m. (B) Columns represent mean fold change in tumor volume of PLX4720-treated group normalized to day 0 measurements. n=8. Error bars, s.e.m. (C) Images from individual mice with tumor progression associated with low ERK1/2 reactivation (mouse #5) and high ERK1/2 reactivation (mouse #6).

Cancer Res. Author manuscript; available in PMC 2014 December 01.
reactivation (mouse #6). (D) Analysis of GAL4-ELK1 reporter activity normalized to tumor volume in individual xenografts treated with PLX4720.
Figure 3. Characterization of molecular changes in PLX4720-resistant tumors
(A) Cell lines were derived from either vehicle-treated (Veh) or PLX4720-resistant (PRT) 1205LuTR reporter xenograft tumors. All cell lines were cultured in the absence of drug for four days followed by treatment with DMSO or PLX4720 (1 μM) for 24 hours. Cells were then lysed and analyzed by Western blotting, as indicated. (B) Sequencing of genomic DNA from Veh #2, PRT #6 and PRT #8 tumor tissue for BRAF exon 15, NRAS exon 2 and HRAS exon 2. (C) Table of results from CAST PCR for HRAS Q61 allele. (D) PCR amplification of BRAF from cDNA derived from either parental cells or PRT cell lines. Fragments ~2.3 kb correspond to full length BRAF transcript. Fragments ~1.2 kb correspond to BRAF V600E ΔEx variants. (E) Exon arrangement of BRAF variants found in

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PRTs #3 and #4. (F) Parental 1205LuTR reporter cells or PRTs #2, #3, #4, or #6 cell lysates were analyzed by Western blotting, as indicated.
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 μM) for 24 hours. Cells were then lysed and analyzed by Western blotting, as indicated. (B) Same as (A), except PRT #4 cells 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 μM). Cells were processed for AlamarBlue® staining. Columns represent mean. n=3. Error bars, s.e.m. **p-value<0.01 by unpaired, student t-test. (D) PRT #6 cells were transfected with control (Cnt), HRAS

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smartpool or HRAS #8 siRNA for 72 hours. Cells were then treated with DMSO (−) or PLX4720 (1 μM) 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, s.e.m. *p-value<0.05 and **p-value<0.01 by unpaired, student t-test.
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 hrs, treated with DMSO (−) or PLX4720 (1 μM) for a further 24 hrs, 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 μM). Cells were processed for crystal violet staining. Mean and standard deviation are shown. n=2. (D) Same as (C), except cells were processed for AlamarBlue® staining. Columns represent mean. n=3. Error bars, s.e.m. **p-value<0.01 by unpaired,
student t-test. (E) Xenograft models using cells from (A) and (B) were fed vehicle or PLX4720 chow. Representative images are shown from mice at day 3. \( n = 5 \) for parental groups, \( n = 8 \) for other groups. (F) Quantification of firefly luminescence of each PLX4720-treated group in (E). Columns represent fold change in firefly luciferase compared to mean vehicle firefly luciferase. \( n = 5 \) for parental groups, \( n = 8 \) for other groups. Error bars, s.e.m. \(*p\text{-value}<0.05\) and \(**p\text{-value}<0.01\) by unpaired, student t-test. (G) Columns represent mean fold change in tumor volume of each PLX4720-treated group in (E) normalized to mean fold change in tumor volume of each vehicle-treated group. \( n = 5 \) for parental groups, \( n = 8 \) for other groups. Error bars, s.e.m. \(*p\text{-value}<0.05\) and \(**p\text{-value}<0.01\) by mixed effects model and Tukey’s multiple comparisons test.