Supplemental Information

Concurrent MEK2 mutations and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma.

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I. Supplemental Figures Legends

II. Supplemental Experimental Procedures

Figure S1, Resistance to BRAF and MEK inhibitors is coupled to MAPK reactivation Related to Figure 1.

(A) Immunohistochemical analysis of paired BRAF-mutant melanoma tumor biopsies at pre-treatment, day 15, and progression. The patient was administered a loading-dose regimen of 6/6/2 mg QD of trametinib. Progression biopsy was obtained a month after trametenib was discontinued.

(B-E) 451Lu parental and 451Lu-MR trametinib-resistant sublines were treated with the indicated concentrations of MEK (B, C) or BRAF (D, E) inhibitors for 72h. Cell viability was assessed by MTT assays and calculated relative to the DMSO (vehicle control)-treated cells. Data are represented as mean ± SEM (n=6).

(F) Mel1617 parental and Mel1617-MR trametinib resistant sublines were treated with the indicated concentrations of paclitaxel (Top) or carboplatin (Bottom) for 72h. Cell viability was determined by MTT assays. Data are represented as mean ± SEM (n=6).

(G) Mel1617 parental and Mel1617-MR trametinib resistant cells were treated with the indicated doses of AZD6244 or dabrafenib for 20h. Equal amounts of total cell protein lysates were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies.

(H-K) 451Lu parental and isogenic trametinib-resistant (MR) sublines were treated with increasing concentrations of the indicated MEK (H-I) or BRAF (J-K) inhibitors for 20h. Equal amounts of protein lysates were resolved by SDS-PAGE and analyzed by immunoblotting.

Figure S2. A de novo MEK2-Q60P mutation decreases sensitivity to MEK and BRAF inhibitors. Related to Figure 2.

(A) A375P BRAF-V600E melanoma cells were transduced with wild-type MEK2 (WT) or MEK2-Q60P. Transduced cells were treated with trametinib or dabrafenib for 72h. MEK2 wild-type transduced cells are more sensitive to trametinib (top) (IC50 = 4 ± 0.2 nM) and dabrafenib (bottom) (IC50 = 24 ± 4 nM) than cells expressing MEK2-Q60P (trametinib IC50 = 16 ± 2 nM; dabrafenib IC50 = 110 ± 17 nM).

(B-E) Mel1617-MR cells were infected with lentiviral vectors expressing a non-targeting control shRNA (shNT2) or MEK2 shRNA (shM2-1, shM2-2). Infected cells were sorted by GFP expression. (B, C) Cell lines were treated with DMSO, the indicated doses of AZD6244, or dabrafenib for 20h. Cell lysates were analyzed by immunoblotting. (D,E) Mel1617 parental and Mel1617-MR infected cell lines were treated with increasing doses of AZD6244 or dabrafenib for 72h. Cell viability was calculated relative to untreated cells. Representative results of three independent experiments are shown. Data are represented as mean ± SEM, n=5.

(F-M) Mel1617-MR cells were infected with lentiviral vectors expressing a non-targeting control shRNA (shNT2) or MEK1 shRNA (shM1-3, shM1-3/1). Infected cells were sorted by GFP expression. Cell lines were treated with DMSO or the indicated doses of trametinib, PLX4720, AZD6244, or dabrafenib for 20h. Cell lysates were analyzed by immunoblotting (F-I). For cell viability assays Mel1617 parental and Mel1617-MR infected cell lines were treated with increasing doses of the indicated compounds for 72h (J-M). Cell viability was calculated relative to untreated cells. Representative results of three independent experiments are shown. Data are represented as mean ± SEM, n=5.

(N-Q) Mel1617 cells were infected with lentiviral vectors expressing empty vector (pLKO.1), MEK2 shRNA (shM2-1, shM2-2) or MEK1 shRNA (shM1-1, shM1-3). Cells were analyzed by immunoblotting (N,O). Mel1617 parental cells were treated with increasing doses of trametenib (P, Q) for 72h. Cell viability was calculated relative to untreated cells. Data are represented as mean ± SEM, n=7. Data shown in panels P and Q are from one representative experiment; the same Mel1617-pLK0.1 control is depicted in both panels.

Table S1. Related to Figure 2. Western blots for Figure 2C were quantified using LiCor Odyssey system.

Figure S3. Analysis of potential mechanisms of resistance in trametenib-resistant cells, related to Figure 3.

(A) Mel-1617 parental and trametinib-resistant sublines were treated with DMSO, 0.1μM dabrafenib or 0.1 μM trametinib for 24 hr. RTK arrays were probed with whole cell lysates. Target proteins were visualized with fluorescent-based detection and fluorescent intensity was normalized to DMSO-treated parental cells. Data represent mean value ± SD (n=2).

(B) DNA isolated from parental and trametenib-resistant sublines was analyzed by aCGH. No difference in copy number of MAP3K8/COT (top) and NF1 (bottom) are seen in the parental and trametenib-resistant sublines

(C) Cell lysates from parental and trametinib-resistant cells were analyzed for immunoblotting with the indicated antibodies. All sublines expressed the tumor suppressor PTEN and express similar levels of MAP3K8/COT. Although additional bands (<70KD) were detected with a monoclonal BRAF antibody, these bands were present in cell lysates from parental (sensitive) and resistant sublines.

(D) Mel1617 ectopically expressing WT-BRAF, BRAF-V600E or MEK-Q60P, and trametinib resistant Mel1617 (MR) cells were treated with 1 μM PLX4720 (PLX). Cell lysates were analyzed by immunoblotting with the indicated antibodies.

Figure S4. Resistance to the combination of BRAF and MEK inhibitors is linked to sustained phosphorylation of S6K, related to Figure 4.

(A-B) Mel1617 parental cells ectopically expressing mutant MEK2-Q60P at low (A) or high (B) levels were grown as tumor xenografts in NSG mice. Once tumors were >200 mm^3 mice were randomized into two groups and treated orally with 3 mg/kg trametenib once a day. Tumor volume was measured with calipers.

(C-D) Tumor volume of 451Lu parental (C) and 451Lu-MR drug resistant (D) xenografts treated with vehicle or trametinib (3 mg/kg po qd) (mean ± SEM, n=5). A likelihood ratio testing nested model showed that the trends of relative tumor growth are significantly different between the two sublines (p=0.002). A mixed-effect model analysis indicated that tumor growth is significantly slower after treatment with trametinib in the parental cells (p=0.033) compared with the resistant cells. Wilcoxon rank sum test shows that the tumor weight at the end of experiment is significant lower in the parental cells treated with trametinib compared with vehicle control (p=0.008), whereas there is no significant difference in tumor weight at the end of experiment in resistant cells treated with trametinib compared with vehicle control (p=0.117).

(E) Total proteins were extracted from xenograft tumors derived from Mel1617 parental, Mel1617-MR (MR), or Mel1617 cells ectopically expressing low (Q60P-low) or high (Q60P-

high) levels of mutant MEK2 treated with vehicle (Veh) or trametinib (Tram; 3 mg/kg po qd). Mice were sacrificed 4h after receiving the last dose of trametinib. The effect of trametinib on the MAPK pathway was assessed by immunoblotting with anti-pERK antibodies. BRAF levels are also shown and histone H3 (H3) was used as protein loading control.

(F) Tumors derived from 451Lu or 451Lu-MR cells were extracted 4h after the last dose and total protein lysates analyzed by immunoblotting with the indicated antibodies.

(G-H) Mel1617-MR cells (G) or A375 cells expressing MEK2-Q60P (H) were treated with dabrafenib (Dabr), trametinib (Tram), or the combination of both drugs (Comb). Total cell lysates were analyzed by immunoblotting.

(I-K) Mel1617-MR (I), Mel1617 expressing MEK2-Q60P (J), and Mel1617-MR cells expressing MEK2 shRNA (shM2-2; K) were treated with single agent trametinib or dabrafenib or the combination of both drugs at the indicated doses. Cell viability was assessed by MTT assays after 72h of drug treatment and calculated relative to untreated controls. Data are represented as mean ± SEM, n=7.

(L-M) 451Lu parental and isogenic trametinib resistant (MR) sublines were treated with increasing concentrations of trametenib (L) or PLX4720 (M) for 20h. Equal amounts of protein lysates were resolved by SDS-PAGE and analyzed by immunoblotting.

(N) Mel1617-MR cells were treated with the indicated drugs (0.1 μM) for 24h. Cells were collected, lysed and analyzed by immunoblotting with the indicated antibodies.

(O) Sequenom iPLEX assay depicting MEK2 nucleotide determination. DNA was isolated

from a Xenograft tumor derived from a progression biopsy of a patient treated with the combination of dabrafenib and trametinib. Specific assay depicted is for MEK2-Q60P mutation. ‘A’=wild type nucleotide; ‘C’=mutant nucleotide

Supplemental Experimental Procedures

Reagents

Trametinib (GSK2011212) and dabrafenib (GSK2118436) were provided by GlaxoSmithKline, PLX4720 and PLX4032 (vemurafenib) were provided by Plexxikon, and selumetinib (AZD6244) was purchased from Selleckchem (Houston, TX). Alamar Blue® was purchased from Invitrogen. Propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, and all other chemicals and solutions were from Sigma (St. Louis, MO) unless otherwise indicated.

Immunohistochemistry

Immunohistochemistry was performed by Mosaic Laboratory (Lake Forest, CA). Tissues were stained with the following antibodies: pERK rabbit clone 20G11 (Cell Signaling, Danvers, MA; Catalog# 4376); Ki-67 mouse clone MiB-1 (Dako, Carpinteria, CA; Catalog# M7240). Tumor samples were fixed in 10% neutral buffered formalin for no more than 24 hours prior to dehydration and paraffin embedding. Melanoma samples were stained with a red chromogen to distinguish true staining from melanin.

BRAF amplification in tumor samples

BRAF amplification was determined in patient tumor samples using TaqMan® Copy Number Assay for BRAF and TaqMan® Copy Number Reference Assay RNaseP (Applied Biosystems). Normal female genomic DNA isolated from formalin fixed paraffin embedded (FFPE) tissue was used as control DNA for reference samples. In a 384-well plate, 10ng genomic DNA from indicated samples were combined with TaqMan® 20X BRAF Copy Number Assay, 20X RNaseP Reference Assay, and TaqMan® 2x Genotyping Master Mix according to protocol. Reactions were run using a real-time PCR instrument with the following PCR conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec then 60 °C for 60 sec. BRAF copy number was normalized to RNaseP copy number, resulting in a ΔCt from which the 2–ΔCt value was derived.

Cell lines and viability assays

BRAF-V600E melanoma cells (Mel1617, 451Lu, WM983B-BR, WM164, and WM88) were cultured in DMEM supplemented with 5% fetal bovine serum and harvested at 60%–80% confluence, unless otherwise noted. 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Each parental cell population (Mel1617, 451Lu, WM88, WM983B, and WM164) was independently treated with increasing concentrations of trametinib from 0.1 nM up to 1 μM in a stepwise manner to generate an isogenic resistant subline (-MR). Cells with the ability to grow in 1μM of trametinib were obtained ~4 months after the initial drug exposure. Cell viability was measured using MTT or Alamar Blue as previously described (Villanueva et al., 2010).

Immunoblotting

For Western blot analysis, cells were washed with cold PBS containing 100 μM Na_3VO_4, scraped, collected by centrifugation, and quick-frozen in dry ice before lysis. Cells were lysed and equal amounts of protein (25-50 μg) were subjected to SDS-PAGE and proteins transferred onto nitrocellulose membranes (Amersham). Following overnight incubation with primary antibodies, membranes were incubated with Alexa Fluor-labeled secondary antibodies (IRDye 680LT goat-anti mouse or IRDye 800CW goat-anti rabbit antibodies (LicorBiosciences, Lincoln, NE) for 1 hr. Fluorescent images were acquired and quantified by Li-COR Odyssey Imaging System.

RTK array analysis was performed using PathScan® RTK Signaling Antibody Array Kit (Cell Signaling) according to the manufacturer's instructions. Briefly, whole cell lysates (200 μg) were incubated on slide-based RTK antibody arrays, detected with biotinylated detection antibody cocktails and visualized with fluorophore (DyLight680)-linked Streptavidin. Fluorescent images were acquired and quantified by Li-COR Odyssey Imaging System.

PCR, Bidirectional Sequencing and Mutation Analysis

PCR primers were designed to amplify all coding exons and intronic flanking regions of *MAP2K1* (NM_002755.2) and *MAP2K2* (NM_030662.2). For sequencing, the PCR primers were modified on the 5' end to include M13 forward (GTAAAACGACGGCCAGT) and reverse (CAGGAAACAGCTATGACC) sequences. Primer sequences and PCR conditions are available on request. Bidirectional sequencing was conducted with AB BigDye Terminator v1.1 Cycle Sequencing Kit (Austin, TX) according to manufacturer's guidelines and run on an

ABI3130xl capillary sequencing instrument (Applied Biosystems, Foster City, CA). Mutation analysis was performed by Mutation Surveyor 5.00 (SoftGenetics LLC, State College, PA).

Sequenom (iPlex) genotyping

Genotyping was done for *AKT1* E17K, *AKT3* E17K, BRAF G466A/E/R/V, V600E/D/K/E/L/R, K601E, *CDK4* K22Q, R24C/H, *CTNNB1* D32A/E/G/V, S37F/Y/DEL, S45F/Y, *FBXO4* H364R, I377M, *GNA11* Q209L/P, *GNAQ* Q209H/L/P/R/X, *KIT* W557R, L576P, V599A/D, K642E, R634W, D816H/V, D820Y, N822I, Y823D, A829P, *MEK1* C121S, *MEK2* F57S, Q60P, K61E/T, L119P, *MET* Y1248H, NRAS G12A/C/D/R/S/V, G13A/C/D/R/S/V and Q61E/H/K/L/P/R. For genotyping using Sequenom's MassArray spectrometry platform, samples were plated and sent to PSOM Molecular Profiling Facility. An initial PCR amplification of the DNA was performed in a 5 µl reaction consisting of 0.8ul HPLC grade water, 0.5ul of 10X PCR buffer with 20mM MgCl2, 0.4 µl of 25mM MgCl2, 0.1 µl of 25mM dNTP mix, 1 µl of 0.5 µM primer mix, 0.2 µl Sequenom PCR enzyme and 2ul of genomic DNA (5ng/µl). PCR conditions were an initial cycle at 94°C for 2 min; 45 cycles at 95°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec; and a final step at 72°C for 5 min. This was followed by a shrimp alkaline phosphatase (SAP) treatment of the samples with 2 µl of the SAP mix consisting of 1.53 µl of HPLC grade water, 0.17 µl of SAP buffer, and 0.3 µl of SAP enzyme. The thermal cycling conditions were 37 °C for 40 min followed by 85 °C for 5 min. Following SAP treatment, a single base pair extension reaction was performed using Sequenom's iPLEX Gold chemistry, where 2 µl of the iPLEX reaction mix was added to the samples. The reaction mix consisted of 0.62 µl of HPLC grade water, 0.2 µl of iPlex buffer, 0.2 µl of iPlex terminator mix, 0.94 µl of primer mix, and 0.04 µl of iPlex enzyme. Thermal cycling conditions an initial cycle at 94°C for 30 sec; 40 cycles at 94°C for 5 sec, [52°C for 5 sec and 80°C for 5

sec (repeat 5 times per cycle)]; and a final step at 72°C for 3 min. The samples were then resin treated and spotted on a SpectroCHIP to be run on Sequenom's MassArray platform.

MEK2 constructs, shRNA, and lentivirus infection

Primers used to generate mutant MEK2-Q60P were:

MEK2mut forward

5'-CGGCTGGAAGCCTTTCTCACCCCGAAAGCCAAGGTCGGCGAACTC-3' and MEK2mut reverse

5'-GAGTTCGCCGACCTTGGCTTTCGGGGTGAGAAAGGCTTCCAGCCG-3'.

The mutant construct was subcloned into the lentiviral vector pLU-EF1a-PGK-GFP (courtesy of D. Schultz, The Wistar Institute). Both constructs were fully sequenced to confirm the engineered mutation and that no additional mutations had been randomly introduced. Wild-type MEK2 in pLentipuro/TO/GW was a generous gift of Dr. Andrew Aplin (Thomas Jefferson University, Philadelphia PA).

Lentiviral MEK1, MEK2, and BRAF shRNA in pLKO1 backbone were obtained from OpenBiosystems. Lentiviruses were produced by transfection of 293T cells with the packaging plasmids along with the lentiviral shRNA vector using Lipofectamine 2000 (Invitrogen) reagent following the manufacturer's instructions. Melanoma cells were exposed to virus in the presence of 8 µg/mL polybrene for 16-18h. Infected cell populations were selected with antibiotics or sorted for GFP using flow cytometry. Expression of mutant genes or shRNA knockdown efficiency was determined by western blot analysis for the respective proteins using specific antibodies.

Array-based Comparative Genomic Hybridization (aCGH)

aCGH was performed on DNA extracted from Mel1617 and 451Lu (parental and resistant cell lines). Control DNA used for comparison was pooled female genomic DNA from Promega. DNA labeling was performed using BioPrime Total for Agilent aCGH from Invitrogen following manufacturer's instructions. Briefly, genomic DNA was fragmented by restriction digest using Alu I and Rsa I restriction enzymes. Control and experimental DNA were labeled with Cy3 and Cy5 fluorescent dye, respectively. Once labeled, control and experimental samples were combined and hybridized to Agilent SurePrint G3 Human CGH 2x400K microarrays, following manufacturer's instructions. Arrays were scanned using Agilent's High-Resolution C Scanner. Extracted data was analyzed using BioDiscovery's Nexus 6 copy-number software. Copy number variation was assessed using the CBS-like Rank Segmentation algorithm provided with Nexus 6.

Quantitative real time PCR

Total RNA was reverse transcribed using Superscript First-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used with 100 ng cDNA template and 70 nM primers for the evaluation of target gene and GAPDH expression. Primers used were purchased from OriGene (Rockville, MD). A negative control without cDNA was run with each assay. Amplifications were performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All experiments were performed in triplicate. Baseline and threshold values for genes were set using the ABI 7000 Prism Software. Expression of mRNA was assessed using the relative standard curve method according to Applied Biosystems' Chemistry Guide. Expression ratios of controls were normalized to 1.

Tumor Xenografts

Mice were injected with $1x10^6$ melanoma cells per site (left and right flank) in a suspension of matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) / complete media at a ratio of 1:1. Tumor growth was assessed twice weekly by caliper measurement. Once tumors reached an average tumor volume of 200 - 300 mm^3 mice were randomized into two treatment groups. Tumor volume was estimated using the formula (length x width x width)/2. Trametinib, Dabrafenib and 458 were suspended in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 in dH2O (pH 8.0). Mice were dosed once daily by oral gavage (po qd). Animals were sacrificed 4 hours after last dosing.

Relative tumor weight was used for data analysis, and was calculated as the individual measured tumor weight divided by the average tumor weight in the vehicle group of the same cell line. Shapiro-Wilk test was used to examine data normality and variance ratio test was used to examine the equality of variances between two groups. ANOVA (or Kruskal-Wallis test for non-normal distribution data) and post-hoc test with Bonferroni procedure was performed to examine the cell line effect on relative tumor weight. The trends of mean tumor volume over time were compared between treatments groups within same cell line using a mixed-effect model with the random effect at mouse level. A mixed-effect model with linear or quadratic function with or without random slopes of follow-up time (days) was compared and determined to provide the best fit. A likelihood ratio testing nested models (with versus without the interaction term of cell lines and days) was used to examine if trends were significantly different among treatment groups. Similar mixed-effect models were used to examine the tumor growth trends among cell lines.

To generate CRPDX (Combination therapy resistant patient-derived xenografts), a fresh tumor biopsy was minced using cross blade technique, digested in collagenase IV for 30min with repeated trituration. Approximately 100mm^3 of digested tissue was implanted subcutaneously in the flank of NSG mice in matrigel. Serial transplantation was performed in a similar fashion. Tumor grafts reached ~1000 mm^3 approximately 8 weeks post implantation for every mouse passage.

Table S1. Related to Figure 2. Western blots for Figure 2C were quantified using Li-Cor Odyssey system.

	Vector	MEK2	MEK2-Q60P
MEK2/Vinculin	0.21	2.53	2.65
pMEK/MEK	0.53	0.68	1.96
pERK/ERK	0.11	0.44	10.39