Supporting Information

Emery et al. 10.1073/pnas.0905833106

Cell Lines and Primary Melanoma Cultures. A375 melanoma cells and 293T retroviral producer cells (ATCC) were cultured in DMEM (MediaTech) with 10% FBS (Gemini). A primary melanoma culture (M307) was derived from an axillary lymph node metastasis that progressed following AZD6244 treatment. This culture was generated by treating fresh biopsy material with dispase for 4 h and collagenase for 3 h at 37 °C. Cells were washed (PBS) and seeded into RPMI medium (MediaTech) supplemented with 10% FCS (36). Treatment-naïve BRAF(V600E) melanoma primary cultures (WM3482 and WM3457) were kindly provided by Dr. M. Herlyn (Wistar Institute) and cultured in RPMI, 10% FBS and 1% penicillin plus streptomycin.

MEK1 Expression Constructs. *MEK1* cDNA was cloned into the pWZL-Blast vector (kindly provided by J. Boehm and W. C. Hahn) by recombinational cloning (Invitrogen) to generate pWZL-Blast-MEK1. Specific mutations were introduced into *MEK1* cDNA using QuikChange II Site Directed Mutagenesis (Stratagene).

MEK1 Random Mutagenesis Screen. MEK1 cDNA was cloned in pWZL(Blast) via Gateway cloning technology (Invitrogen). To generate random mutant libraries, the plasmid was propagated in *Escherichia coli* deficient for the DNA repair genes *MutS*, *MutD5*$^{-}$, and *MutT*$^{-}$ (XL1-Red, Stratagene), thus introducing mutations randomly within the plasmid. DNA extracted from these bacteria was subsequently transformed into XL1-Blue (Stratagene) to amplify the library. To determine the approximate coverage of mutations within the library, a parallel experiment was performed using the pDNR-1r-SacB vector. Expression of *SacB* is lethal in sucrose-containing media. The vector was replicated in XL1-red according to the manufacturer's protocol. Following transformation, bacteria were plated in triplicate in the presence or absence of sucrose at different dilutions. Colonies were then quantified to infer a frequency of mutation (number of colonies – sucrose/number of colonies + sucrose). The frequency determined by this method is an underestimate of the actual mutation frequency: stop codons are most often scored, but silent and nondeleterious mutations cannot be detected. We estimate that the actual mutation rate might approach a value four times greater than that scored by this assay. The number of *MEK1*-containing *E. coli* was quantified on each 15-cm plate, and the cDNA library was collected by pooling bacterial colonies to generate coverage of ≈60-fold (e.g., each base had the potential to be mutated 60 individual occasions within the library as a whole). This was achieved by harvesting bacteria from 40 × 15-cm plates (≈54,000 colonies per plate). The mutagenized MEK1 plasmid or nonmutagenized control was used to infect A375 melanoma cells. After selection with blasticidin, cells were plated on 15-cm dishes and cultured in the presence of MEK inhibitors (AZD6244 or CI-1040; 1.5 μM or 2 μM, respectively) for 4 weeks until resistant clones emerged.

***MEK1* PCR.** *MEK1* exon 3 was amplified using forward primer 5′-CTTTCATCCCTTCCTCCCTC-3′ and reverse primer 5′-CACCTCCCAGACCAAAGATTAG-3′. MEK1 exon 6 was amplified using forward primer 5′-CTTCTCTTCCCCAATCTACCTGTG-3′ and reverse primer 5′-CCTACCCAGCACAAGACTCTG-3. 20 ng of genomic DNA was added to each PCR. Thermocycling reactions were as follows; 95 °C/5 min, followed by 30 cycles of (95 °C/15 seconds, 52 °C/20 seconds, 68 °C/30 seconds), followed by 68 °C/10 min. *MEK1* cDNA was amplified using forward primer 5′-CGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGG-3′ and reverse primer 5′-GAGGCCAGCATCGGTTGGTGTG-3′. Thermocyling reactions were 95 °C/5 min, 35 × (95 °C/15 seconds, 66 °C/60 seconds, 68 °C/90 seconds), 68 °C/10 min. All reactions were performed using *Pfx* polymerase (Invitrogen).

Sequencing of *MEK1* DNA. AZD6244- or CI-1040-resistant cells emerging from the random mutagenesis screens were pooled and genomic DNA was prepared (Qiagen DNeasy). MEK1 cDNA was amplified from genomic DNA using primers specific to flanking vector sequence at the 5′ and 3′ end. In separate experiments, genomic DNA was prepared from melanoma tumor samples, and PCR was performed using intronic primers that amplified exons 3 and 6 from *MEK1*. PCR products were gel purified, pooled, and subjected to single molecule sequencing using an Illumina 3G instrument according to the manufacturer's instructions. Sequence reads were filtered on the basis of quality metrics and assigned a variant score according to the manufacturer's instructions. For ≈100 clones, MEK1 cDNA was PCR amplified (as above) and sequenced by the Sanger method using established protocols.

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 (developed by C.H. and L.N.). 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 *a*verage *v*ariant *s*core (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. For the AZD6244 screen, an AVS below 10 was considered as background, and an AVS above 15 was considered as an outlier.

Pharmacologic Growth Inhibition Assays. Cultured cells were seeded into 96-well plates at a density of 5,000 cells per well for all melanoma short-term cultures; 3,000 cells were seeded for the A375 cell line. Following adherence of the cells (16 h or overnight), serial dilutions of the compound were performed in DMSO and transferred to cells to yield final drug concentrations ranging from 100 μM to 1 × 10^{-6} μM, ensuring that the final volume of DMSO did not exceed 1%. Compounds used included the MEK inhibitors CI-1040 (purchased from Shanghai Lechen International Trading Co.), AZD6244 (purchased from Selleck Chemicals) and the B-RAF inhibitor PLX4720 (purchased from Symansis). Following addition of a drug, cells were incubated for 96 h. Subsequently, cell viability was measured using the CellTiter-Glo viability assay (Promega). Viability was calculated as a percentage of the control (untreated cells) after background subtraction. A minimum of three replicates was made for each

cell line and drug combination, and the entire experiment was repeated at least three 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). GI_{50} values were calculated by determining the slope of the line connecting the data points that flanked the 50% point. The GI_{50} of each MEK allele was compared to A375 using a two-sample Student's *t*-test; the M307 line was compared to treatment-naïve short-term cultures. H_A (alternative hypothesis) was used: the mean GI_{50} of the query cell line was compared to the mean GI_{50} of A375 or naïve short-term culture at $\alpha = 0.05$ (one-sided test). All *P*-value calculations were performed using MATLAB Statistics Toolbox (MATLAB Version 7.1, MathWorks).

Western Blot Analysis. Immunoblot studies were performed using standard procedures. Briefly, melanoma cells were lysed with TNN buffer containing protease inhibitor (Roche), NaF, and $NaVO_3$ (1 mM each). Lysates were quantified (Bradford assay), denatured (95 °C), and resolved by SDS gel electrophoresis. Protein was transferred to nitrocellulose membranes and probed with primary antibodies recognizing *p*-ERK1/2, *p*-MEK1/2 (Ser-217/221), MEK1/2, and α-tubulin (Cell Signaling Technology; 1:1,000 dilution). After incubation with the appropriate secondary antibody (anti-rabbit or anti-mouse IgG, HRP-linked; 1:1,000 dilution) (Cell Signaling Technology), proteins were detected using chemiluminescence (Pierce). Biochemical effects of MEK or B-RAF inhibition were assessed by Western blot analysis of cell lysates collected following 16-h drug exposure at varying concentrations.

MEK1 Kinase Assays. The 293T cells (≈70% confluent) were transfected with 15 μg pc-DNA-DEST40 containing MEK-WT, MEK(DD), kinase-dead MEK1 (contains the K97M mutation), or one of several MEK1 variant alleles. At 48 h postinfection, lysates were generated by standard methods. Pull-down using cobalt beads was performed for 30 min at 4 °C on 1 mg of whole cell extract. The protein-bound cobalt beads where incubated with 20 μL ATP/magnesium mixture (20 mM Mops pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na3VO4, 1 mM DTT, 75 mM MgCl2, and 0.5 mM ATP), 20 μL of dilution buffer (20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT), and 1 μg of inactive ERK1 (obtained from Millipore) for 30 min at 30 °C. The phosphorylated ERK1 product was detected by immunostaining using a *p*-ERK1/2 antibody (Cell Signaling Technology), and relative *p*-ERK signals were quantified using densitometry, normalized to the amount of input MEK1, and compared to MEK1-WT as a reference.

Colony Formation Assays. For each cell line, 40,000 cells where seeded into 15-cm dishes in triplicate. Media (± drug) was changed every 7 days. When dense colonies formed (e.g., foci with high cell concentration), cells where stained using 0.5% crystal violet solution. Vehicle-treated cells formed colonies at 7 days; MEK(P124L) in 0.5 μM or 0.25 μM AZD6244; MEK(P124L) and MEK(DD) in 1.5 μM, 1 μM, 0.5 μM or 0.25 μM PLX4720 formed colonies at 14 days. All other cells formed colonies by 28 days. Expression of MEK1-WT in A375 cells resulted in low-level breakthrough growth but minimal dense colony formation. Stained plates were imaged and dense colonies were quantified using ImageQuant TL software.

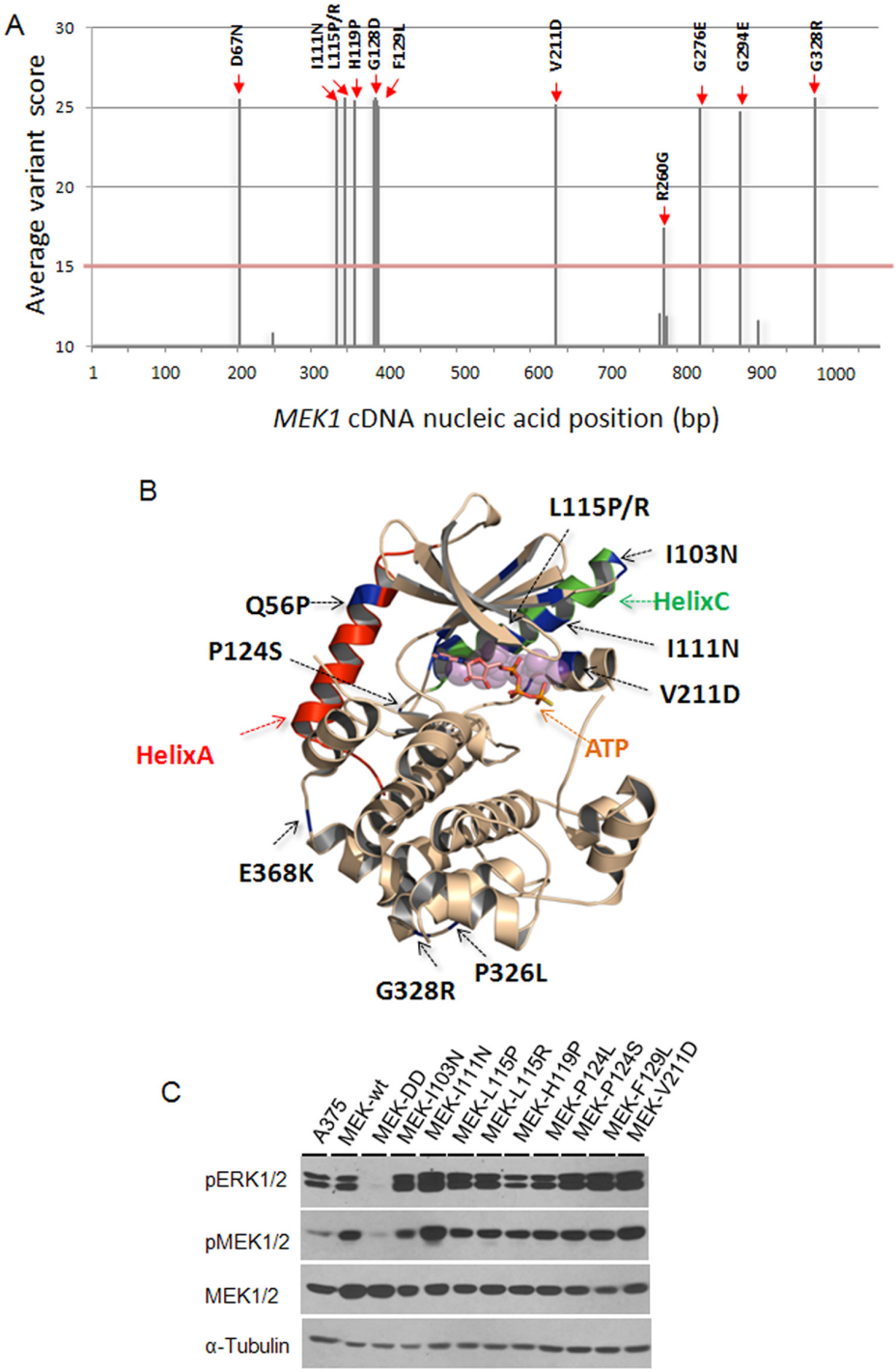

Fig. S1. CI-1040 mutagenesis screen and relative expression of MEK resistance alleles. (*A*) The average variant scores (AVS) of mutations across the *MEK1* coding sequence is shown for a random mutagenesis screen using CI-1040. The corresponding amino acid changes are shown for high frequency alleles. (*B*) An enlarged view of the MEK1 structure from Fig. 1*B* (main text) is shown. Helix A (red) and helix C (green) are indicated, as are several resistance alleles. The locations of ATP (orange) and an arylamine MEK inhibitor (purple) bound to MEK1 are shown. (*C*) Relative levels of pERK1/2, pMEK1/2, MEK1/2, and α-tubulin (control) are shown for A375 cells expressing resistant *MEK1* alleles.

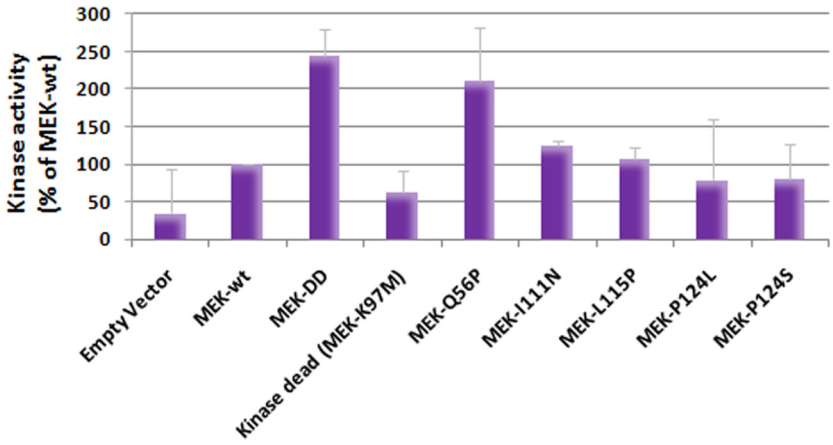

Fig. S2. In vitro kinase assays of selected MEK1 resistance alleles. Relative pERK1 induction in vitro is shown in graphical format following addition of cobalt bead pull-down material (see *SI Text*) for empty vector, MEK-WT, MEK(DD), a kinase-dead MEK allele (K97M), MEK1(Q56P), MEK1(I111N), MEK1(L115P), MEK1(P124L), and MEK1(P124S).

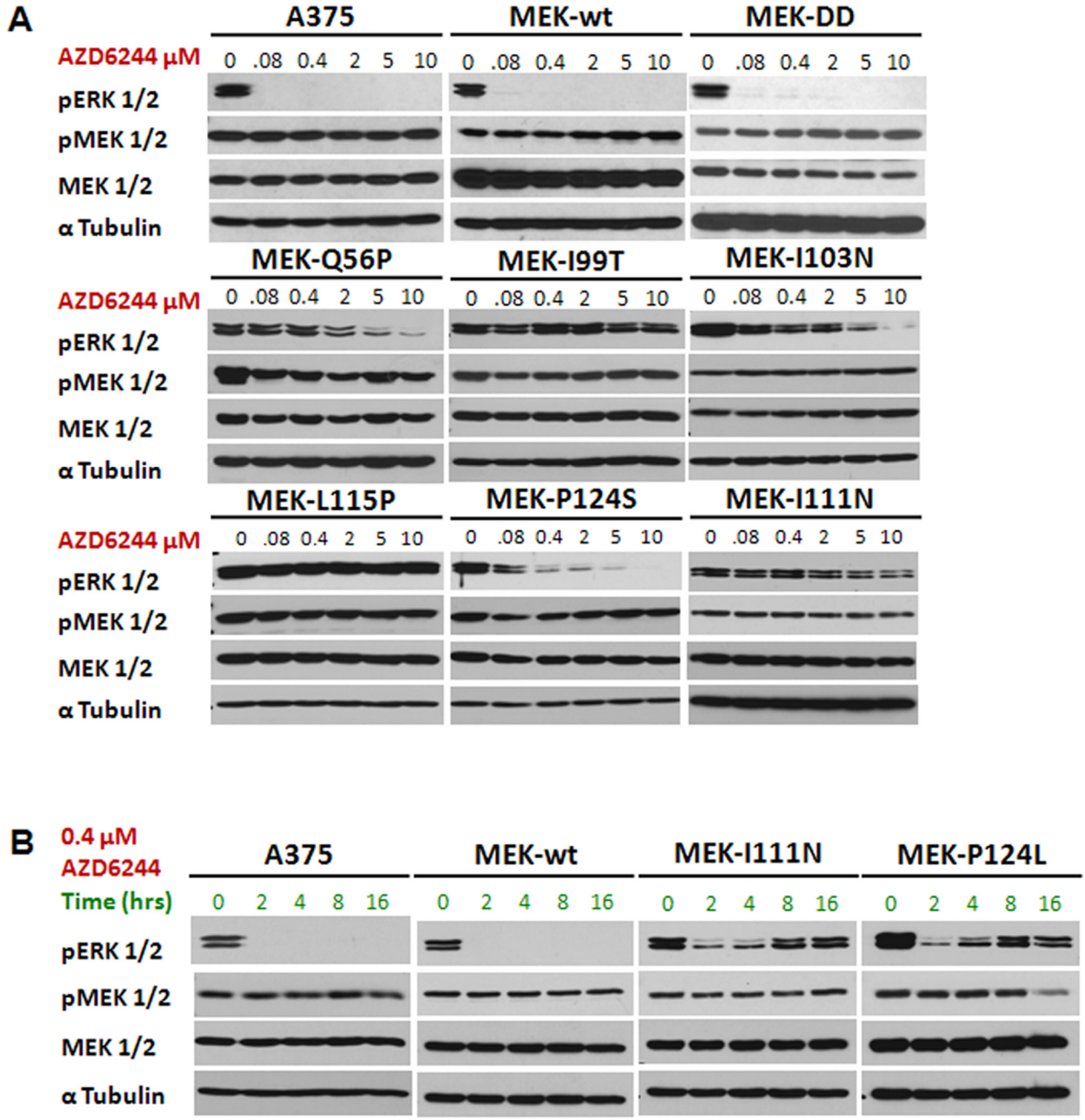

Fig. S3. Biochemical characterization of MEK1 resistance alleles using AZD6244. (*A*) The levels of pERK1/2, pMEK1/2, MEK1/2, and α-tubulin are shown for A375 cells expressing MEK1 mutations following 16-hour incubation with AZD6244 at 10 μM, 5 μM, 2 μM, 0.4 μM, 0.08 μM, and 0 μM. (*B*) A375 cells expressing MEK(WT), MEK(I111N), or MEK(P124) were treated with 0.4 μM AZD6244 and incubated for 0, 2, 4, 8, and 16 h. The levels of pERK1/2, pMEK1/2, MEK1/2, and α-tubulin are shown.

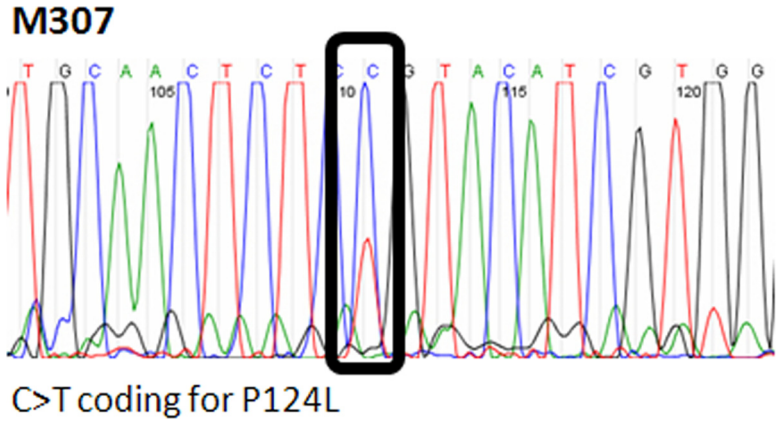

Fig. S4. The *MEK1(P124L)* mutation in M307 melanoma cells cultured ex vivo. The Sanger sequencing chromatogram from *MEK1* exon 3 is shown for the M307 line. A C → T transition, indicative of MEK^{P124L}, is evident.

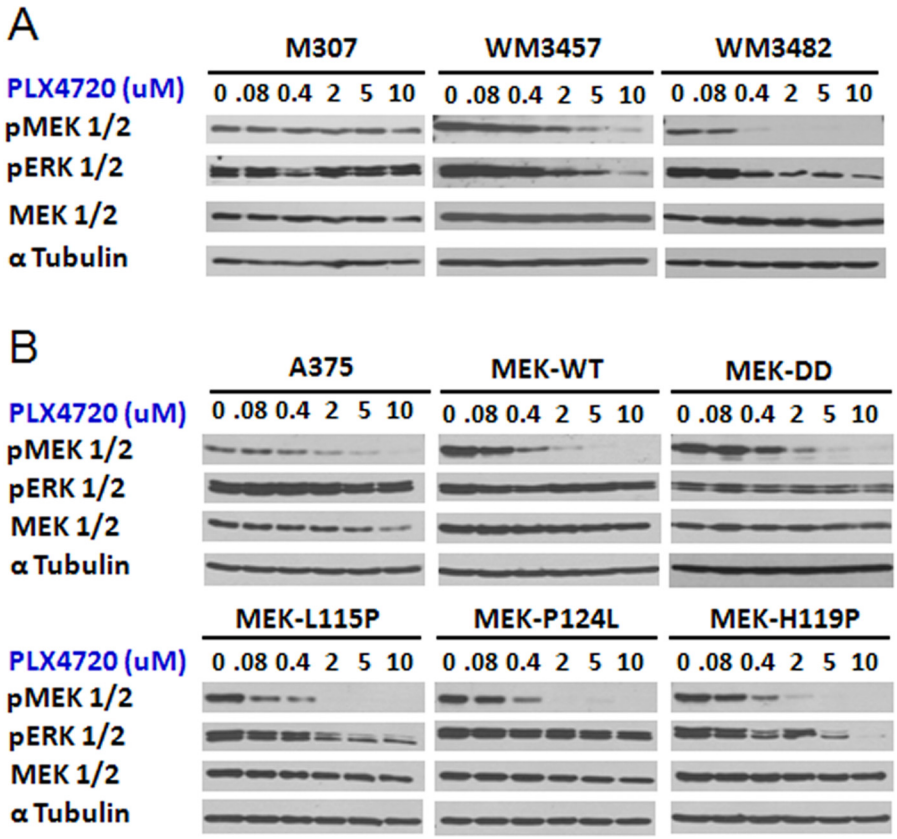

Fig. S5. Biochemical characterization of MEK1 resistance alleles using PLX4720. (*A*) Immunoblot studies of *p*-ERK, *p*-MEK1/2, and MEK1/2 are shown following treatment with increasing concentrations of PLX4720 in treatment-naïve or AZD6244-resistant melanoma cells. (*B*) Immunoblot studies of pERK1/2, pMEK1/2, and MEK1/2 are shown for A375 cells expressing resistant *MEK1* alleles following 16-h incubation with PLX4720 at 10 μM, 5 μM, 2 μM, 0.4 μM, 0.08 μM, and 0 μM.

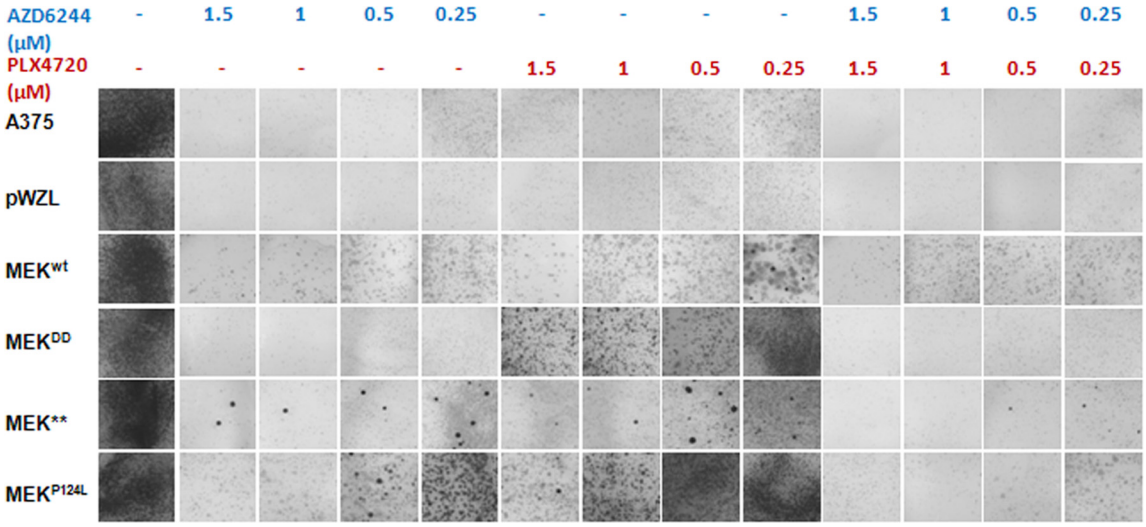

Fig. S6. Colony formation assays following AZD6244 and/or PLX4720 exposure. Representative colony images are shown for parental A375 and cells expressing empty vector (pWZL-BLAST), MEK(DD), or MEK (P124L). Cells were treated with vehicle (for 7 days), AZD6244 alone, PLX4720 alone, or AZD6244 + PLX4720 at the indicated concentrations. All drug treatments were for 4 weeks.

Table S1. Summary of inhibitor-resistant *MEK1* alleles

MEK1 allele	Method of identification	AZD* AVS†	CI‡ AVS†	Sanger freq. (%)	Experimental validation
Q56P	Massively parallel	17.7	—	—	Validated
D67N	Massively parallel and Sanger	—	25.5	1	Not done
L74R	Massively parallel and Sanger	—	8.7	25	Failed
I99T	Massively parallel	21.1	—	—	Validated
I103N	Sanger	—	—	1	Validated
K104N	Massively parallel	19.5	—	—	Not done
A106T	Sanger	—	—	1	Failed
I107M	Sanger	—	—	1	Failed
I111N	Massively parallel and Sanger	21.4	25.4	73	Validated
L115P	Massively parallel	21.3	25.6	—	Validated
L115R	Massively parallel and Sanger	—	24.7	1	Validated
H119P	Massively parallel	20.8	25.4	—	Validated
E120D	Massively parallel	20.1	—	—	Not done
P124Q	Massively parallel	9.1	—	—	Not done
P124S	Sanger	—	—	—	Validated
G128D	Massively parallel	21.3	25.4	—	Not done
F129L	Massively parallel and Sanger	—	25.6	1	Validated
F133L	Massively parallel	21.9	—	—	Not done
G167R	Sanger	—	—	1	Failed
V211D	Massively parallel and Sanger	18.2	25.2	1	Validated
L215P	Massively parallel	21.3	—	—	Not done
R260G	Massively parallel	8.3	17.4	—	Failed
G276E	Massively parallel	10.7	25	—	Not done
G297E	Massively parallel	—	24.7	—	Not done
P326L	Massively parallel	21.9	—	—	Not done
G328R	Massively parallel	22.8	25.6	—	Not done
E368K	Massively parallel	21.1	25.6	—	Not done

*AZD6244.
†Average variant score.
‡CI 1040.

Table S2. GI_{50} values for cell lines and MEK1 alleles

Cell culture	AZD6244 (μM)	PLX4720 (μM)	CI-1040 (μM)
A375	0.04	0.91	0.07
A375-MEK-WT	0.04*	1.29*	0.11**
A375-MEK-DD	0.05*	16.80*	0.11*
A375-MEK-Q56P	8.27***	7.72***	3.88***
A375-MEK-I99T	3.69***	0.617*	0.72**
A375-MEK-I103N	1.89**	2.94***	6.14***
A375-MEK-I111N	5.96***	2.94***	7.59***
A375-MEK-L115P	55.05**	1.97**	11.34***
A375-MEK-L115R	43.65***	1.17***	6.80**
A375-MEK-H119P	9.08***	1.42***	6.91**
A375-MEK-P124S	0.17***	3.44***	0.62***
A375-MEK-P124L	0.19**	2.84***	0.56***
A375-MEK-F129L	7.32***	2.79***	6.05**
A375-MEK-V211D	113.90**	2.73**	8.47***
M307	7.18***	20.28***	—
WM3457	0.05	0.07	—
WM3482	0.01	0.05	—

*, $p < 0.05$; **, $p < 0.01$; ***, $p > 0.001$.

Table S3. Patient characteristics

Patient	Age (years)	Gender	Breslow (mm)	Staging at trial entry	Pretreatment biopsy location	Posttreatment biopsy location	Durations of stable disease (days)	*BRAF* status
1	48	Female	6	T4b Nx Mx	Back, left	Skin, thoracal, left	77	V600E
2	66	Male	3.1	T3b N3 M1c	Skin, axillary, thoracal	Skin, shoulder, left	48	WT
3	72	Female	5.5	T4b N2b M1b	Lung, upper lobe	N/A	40	—
4	48	Female	0.3	T1a N1b M1c	Axillary lymph node, left	Skin, retro-auricular, right	39	V600E
5	63	Male	Unknown	Tx Nx M1c	Skin, axillary, left	N/A	78	WT
6	49	Female	0.8	T1a N3 M1c	N/A	Skin, neck, left	51	WT
7	58	Male	7	T4 N1 M0	Axillary lymph node, left	Axillary lymph node, left	Ongoing	V600E