DATA SUPPLEMENT

Detailed Case Studies

Patient 1

Patient 1 was a 72-year-old man who was originally diagnosed with Stage IIIC melanoma of the left ear with parotid and left neck nodal involvement. He was initially treated with wide excision, left neck dissection, and adjuvant radiation therapy to the left neck. One year later, routine imaging showed recurrent left neck disease. He underwent another resection (***pre-treatment biopsy)*** and intensity modulated radiation therapy (IMRT) to the resection basin. Clinical mutational analysis of the tumor revealed both BRAF V600E and V600K mutations. Over the subsequent six months, he developed multiple areas of disease in the neck, chest, and paraesophageal regions. He was enrolled in a phase I/II study of dabrafenib and trametinib with initial, marked regression of his subcutaneous and nodal based lesions in his head and neck. Approximately three months later, he developed subcutaneous and nodal progression in the neck region only, and was removed from study (***resistant biopsy***). Following discontinuation, he was treated with an anti-PDL1 antibody for four months until progression and then with 4 cycles of ipilimumab. He died of his disease approximately nine months after discontinuing RAF and MEK inhibitor therapy.

Patient 2

Patient 2 was a 48-year-old man who developed widely metastatic melanoma with pleural, pericardial, pulmonary, renal, and bladder metastases 5 years after initial

diagnosis of Stage IB melanoma of the chest wall. A pleural biopsy (***pre-treatment biopsy)*** confirmed the diagnosis of metastatic melanoma, and clinical mutational analysis identified a BRAF V600E mutation. He was enrolled on a phase I/II study of dabrafenib and trametinib and had initial regression of his disease and improvement of his disease-related symptoms. Three months after study enrollment, however, routine scans demonstrated marked disease progression (***resistant biopsy****)*. He died approximately six months after his initial presentation of metastatic disease.

Patient 3

Patient 3 was a 42-year-old man who rapidly developed unresectable soft tissue disease after wide excision and lymph node dissection of a stage IIIC melanoma of the left thigh (***pre-treatment biopsy*****)**. Clinical mutational analysis revealed a BRAF V600E mutation, and he began therapy on a phase I/II study of dabrafenib and trametinib approximately six months after surgery. After nearly one year on trial, he developed progressive disease (***resistant biopsy*****)**. He was treated briefly with ipilimumab, but died of disease progression after four cycles, approximately three months after discontinuing the BRAF and MEK inhibitor therapy.

Patient 4

Patient 4 is a 56-year-old man originally diagnosed with stage IIIB melanoma of the lower back who completed surgical management and one year of adjuvant interferon alpha-2B. Approximately one year later, he developed in-transit

metastases and underwent resection followed by GM-CSF. Six months later, routine imaging revealed new chest wall and axillary metastases; GM-CSF was discontinued and clinical mutation analysis revealed a BRAF V600E mutation (***pre-treatment biopsy)***. He was enrolled on a phase I/II study of dabrafenib and trametinib and had complete regression of his disease. After nine months on study, he developed a new nodule at a previous resection site that was removed and found to be melanoma. Since there was no other evidence of disease, he remained on study for another nine months until he developed a lateral abdominal wall lesion. This lesion was also resected (***resistant biopsy*)**, and to date he has been on therapy for more than 30 months.

Patient 5

Patient 5 was a 49-year-old man who presented with metastatic melanoma involving his right inguinal and pelvic nodes, liver, lungs, and bone (AJCC Stage IV M1c); mutational analysis of right groin nodal biopsy revealed a BRAF V600E mutation (***pre-treatment biopsy*)**. A subsequent dermatologic evaluation revealed a primary lesion on his right thigh that was excised. He enrolled on a phase I/II of dabrafenib and trametinib and remained on treatment for eight months. After a brief partial response, he subsequently developed disease progression in his inguinal and pelvic nodal basins (***resistant biopsy*)**. Following discontinuation of therapy, he was treated with several different therapies without significant response, and died due to rapid disease progression approximately 21 months after his initial diagnosis.

Supplementary Tables

TABLE 1: Annotated SNVs and Indels in All Samples (spreadsheet) – Annotated SNVs and indels identified by WES in all samples

TABLE 2: Changes in Cancer Cell Fraction for All SNVs and Indels in All Samples (spreadsheet) – Comparison of cancer cell fractions (CCF) for SNVs and indels identified by WES in all samples

TABLE 3: Alterations with Significantly Enriched CCF from Pretreatment to Resistant (spreadsheet) – List of alterations detected by WES in each patient that changed from subclonal/undetectable in the pretreatment sample to clonal in the resistant sample

TABLE 4: Candidate Resistance Genes from RNAi and ORF Screens (spreadsheet) – Genes identified as candidate resistance effectors from ORF or RNAi functional screens (1, 2)

TABLE 5: Alterations in Each Patient in Genes That Scored in Functional Screens (spreadsheet) – List of alterations detected by WES in each patient for which the gene was identified as a candidate resistance effector in ORF or RNAi functional screens (1, 2)

TABLE 6: **Coverage and Metrics** (spreadsheet) – Sequencing metrics for all samples

Supplementary Figures

FIGURE 1. Copy number profiles from pre-treatment and resistant tumors

Copy number analysis of whole exome data from pre-treatment and resistant tumors from the 4 patients not shown in the main figures (Patients 1, 2, 4 and 5).

FIGURE 2. MEK1^{P162S} does not confer resistance to dabrafenib/trametinib *in vitro*.

Growth inhibition curves are shown for dabrafenib (A), trametinib (B), VX-11e (C) and combined dabrafenib/trametinib (D) for A375 (BRAF^{V600E}) melanoma cells (grey) and A375 cells expressing wild type MEK1 (MEK WT; blue) or MEK1^{P162S} (red).

FIGURE 3. Additional MEK1 and MEK2 mutations found in patients with acquired resistance to RAF inhibitor monotherapy confer resistance to dabrafenib/trametinib *in vitro.*

Growth inhibition curves are shown for combined dabrafenib/trametinib treatment for A375 (BRAF^{V600E}) melanoma cells and A375 cells expressing various mutations in MEK1 (A) or MEK2 (B). MEK1^{C121S} and MEK1^{G128V} were identified in patients with acquired resistance to vemurafenib (3, 4). MEK2^{L46F} and MEK2^{C125S} were also identified in patients with acquired resistance to vemurafenib(4). MEK2^{K101M} is a kinase-dead mutant (5), and MEK2-DD is a constitutively active mutant (5).

Supplementary Methods

Patients and Tumor Samples

All patients provided written informed consent for research biopsies and genomic profiling of tumor and normal DNA/RNA, as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-181). Surgical biopsies were obtained prior to treatment with dabrafenib/trametinib and again after the development of resistance. All tumor tissue was stored at -80°C until DNA and RNA extraction was performed. A blood sample was obtained during the course of treatment, and whole blood or peripheral blood nuclear cells were stored at -80°C until DNA extraction was performed.

Whole Exome Sequencing

DNA extraction: DNA extraction was performed as previously described (6).

Library Construction: DNA libraries for massively parallel sequencing were generated as previously described (6) with the following modifications: the initial genomic DNA input into the shearing step was reduced from 3μg to 10-100ng in 50μL of solution. For adapter ligation, Illumina paired-end adapters were replaced with palindromic forked adapters (purchased from Integrated DNA Technologies) with unique 8 base index molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, all reagents used for end repair, A-base addition, adapter ligation, and library enrichment PCR were purchased from KAPA Biosciences in 96-reaction

kits. In addition, during the post-enrichment solid phase reversible immobilization (SPRI) bead cleanup, elution volume was reduced to 20µL to maximize library concentration, and a vortexing step was added to maximize the amount of template eluted from the beads. Libraries with concentrations above 40ng/µl, as measured by a PicoGreen assay automated on an Agilent Bravo instrument, were considered acceptable for hybrid selection and sequencing.

Solution-phase hybrid selection: The exon capture procedure was performed as previously described (6) with the following modifications: prior to hybridization, any libraries with concentrations >60ng/µL (as determined by PicoGreen) were brought to 60ng/µL, and 8.3µL of library was combined with blocking agent, bait, and hybridization buffer. Libraries with concentrations between 50 and 60ng/µL were normalized to 50ng/µL, and 10.3µL of library was combined with blocking agent, bait, and hybridization buffer. Libraries with concentrations between 40 and 50ng/µL were normalized to 40ng/µL, and 12.3µL of library was combined with blocking agent, bait, and hybridization buffer. Finally, the hybridization reaction was reduced to 17 hours, with no changes to the subsequent capture protocol.

Preparation of libraries for cluster amplification and sequencing: After post-capture enrichment, libraries were quantified using PicoGreen, normalized to equal concentration using a Perkin Elmer MiniJanus instrument, and pooled by equal volume on the Agilent Bravo platform. Library pools were then quantified using quantitative PCR (KAPA Biosystems) with probes specific to the ends of the

adapters; this assay was automated using Agilent's Bravo liquid handling platform. Based on qPCR quantification, libraries were brought to 2nM and denatured using 0.2 N NaOH on the Perkin-Elmer MiniJanus. After denaturation, libraries were diluted to 20pM using hybridization buffer purchased from Illumina.

Cluster amplification and sequencing: Cluster amplification of denatured templates was performed according to the manufacturer's protocol (Illumina). HiSeq v3 cluster chemistry and flowcells, as well as Illumina's Multiplexing Sequencing Primer Kit. DNAs were added to flowcells and sequenced using the HiSeq 2000 v3 Sequencing-by-Synthesis method, then analyzed using RTA v.1.12.4.2 or later. Each pool of whole exome libraries was subjected to paired 76bp runs. An 8-base index sequencing read was performed to read molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

Sequence data processing: Exome sequence data processing was performed using established analytical pipelines at the Broad Institute. A BAM file was produced with the Picard pipeline (http://picard.sourceforge.net/), which aligns the tumor and normal sequences to the hg19 human genome build using Illumina sequencing reads. The BAM was uploaded into the Firehose pipeline (http://www.broadinstitute.org/cancer/cga/Firehose), which manages input and output files to be executed by GenePattern (7).

Sequencing quality control: Quality control modules within Firehose were applied to all sequencing data for comparison of the origin for tumor and normal genotypes and to assess fingerprinting concordance. Cross-contamination of samples was estimated using ContEst (8).

Somatic Alteration Assessment

MuTect (9) was applied to identify somatic single-nucleotide variants. Indelocator (http://www.broadinstitute.org/cancer/cga/indelocator) was applied to identify small insertions or deletions. Artifacts introduced by DNA oxidation during sequencing were computationally removed using a filter-based method (10). Annotation of identified variants was done using Oncotator (http://www.broadinstitute.org/cancer/cga/oncotator). Copy ratios were calculated for each captured target by dividing the tumor coverage by the median coverage obtained in a set of reference normal samples. The resulting copy ratios were segmented using the circular binary segmentation algorithm (11). Genes in copy ratio regions with segment means of greater than $\log_2(4)$ were evaluated for focal amplifications, and genes in regions with segment means of less than $\log_2(0.5)$ were evaluated for deletions.

Cancer Cell Fraction Analysis

Genome wide copy-ratios were estimated from whole-exome sequencing (WES) data by comparison of the observed depth of coverage at each exon to that achieved in normal samples. Allelic copy-ratios were then estimated by analysis of allelic

fractions for all heterozygous SNPs identified in the paired normal sample. These allelic copy-ratios were then analyzed using ABSOLUTE (12) to generate cancer cell fractions as previously described.

Whole Transcriptome Sequencing

RNA extraction: RNA was extracted from frozen tissue using the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions, including the optional on-column DNase digest. All samples were quantified using Nanodrop and quality was evaluated using Agilent's Bioanalyzer 2100.

cDNA Library Construction: Total RNA was quantified using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and normalized to 4ng/ul. An aliquot of 200ng for each sample was transferred into library preparation which was an automated variant of the Illumina Tru Seq™ RNA Sample Preparation protocol (Revision A, 2010). This method uses oligo dT beads to select mRNA from the total RNA sample followed by heat fragmentation and cDNA synthesis from the RNA template. The resultant cDNA then goes through library preparation (end repair, base 'A' addition, adapter ligation, and enrichment) using Broad designed indexed adapters substituted in for multiplexing. After enrichment the libraries were quantified with qPCR using the KAPA Library Quantification Kit for Illumina Sequencing Platforms and then pooled equimolarly. The entire process is in 96-well format and all pipetting is done by either Agilent Bravo or PerkinElmer JANUS Mini liquid handlers.

Illumina Sequencing: Pooled libraries were normalized to 2nM and denatured using 0.2 N NaOH prior to sequencing. Flowcell cluster amplification and sequencing were performed according to the manufacturer's protocols using either the HiSeq 2000 v3 or HiSeq 2500. Each run was a 76bp paired-end with an eight-base index barcode read. Data was analyzed using the Broad Picard Pipeline which includes de-multiplexing and data aggregation.

Analysis of Transcriptome Data

Fusion detection was performed as previously described (13). The fusion detection pipeline begins by identifying putative fusion events supported by at least two distinct chimeric read pairs (i.e. two ends of the read mapping inside two different genes located on two different chromosomes or at least 1Mb apart), which mapped to sufficiently unique/non-repetitive genomic locations. To avoid products that are non-functional and/or would be subject to nonsense-mediated decay, the orientation of the aligned first and second reads in a chimeric read pair were required to be consistent with the coding strand orientation of the two genes forming the putative fusion. An exon-exon junction reference set is generated from the list of putative fusion events by taking the 5' exon from the first gene and the 3' exon from the second gene. This exon-exon junction reference is then used to attempt to align the initially unmapped reads for which a paired mate was aligned inside one of the genes forming the putative fusion transcript. The evidence for a fusion transcript is a combination of the observations of chimeric read pairs and

"split reads" spanning an exon-exon junction between the two genes involved in a fusion.

To identify the presence of splice isoforms in BRAF, MAP2K1, MAP2K2, or NRAS, transcriptome data from each patient was manually reviewed in the Integrated Genome Viewer (IGV). For each gene, we generated Sashimi plots (http://arxiv.org/abs/1306.3466). Each plot was manually reviewed for changes in isoform expression between pre-treatment and matched post-resistance tumor sample.

Expression of candidate alleles was determined by manual review of the transcriptome sequencing data in the Integrated Genome Viewer (IGV).

Plasmids and Site-directed Mutagenesis

The tet-inducible construct, pCW57.1, was a generous gift of Dr. David Root and Dr. John Doench (The Broad Institute of Harvard and MIT). The V5-tagged tet-inducible construct, pLIX_403, was obtained from Addgene (41395). MEK1 cDNA was described previously (3) and MEK2 cDNA was obtained from Addgene (23555). Site-directed mutagenesis was performed in the pDONR (Invitrogen) construct using Quick-Change II (Stratagene) according to the manufacturer's instructions. The pDONR vector containing the MEK cDNA was then recombined into pCW57.1 or pLIX_403 vectors using LR Clonase II (Invitrogen).

Viral infections

293T cells (70% confluent) were transfected with expression vectors (pCW57.1 MEK1 (tet-inducible) or pLIX_403 MEK2 (V5 tagged, tet-inducible), together with packaging vectors Δ8.91 and VSVG, using X-tremeGene 9 (Roche). Viral supernatants were passed through a 0.45μm filter syringe and added to A375 cells plated the previous day. A375 cells were infected for 16 hours with virus in the presence of polybrene (4ug/ml, Sigma); puromycin was introduced 48 hours post infection to create stable cell lines.

Cell lines

A375 cells were purchased from ATCC and were maintained in DMEM with 10% heat inactivated FBS. A375 cells stable expressing tet-inducible MEK constructs (pCW57.1 MEK1or pLIX_403 MEK2-V5) were maintained in 10% tet-approved FBS (Clontech) and 2μg/mL puromycin.

In vitro pharmacologic growth inhibition assays

Dabrafenib (RAF inhibitor), trametinib (MEK inhibitor) and VX-11e (ERK inhibitor) were purchased from ChemieTek. For growth inhibition analysis, A375 cells expressing MEK1 or MEK2 constructs were seeded in 96-well plates at 2000 cells/well and allowed to adhere for 16 hours. Afterwards, media containing serial dilutions of inhibitor was added, ensuring that the final volume of DMSO did not exceed 0.1%. Doxycycline (1μg/mL) was added to the media at the time of drug treatment to induce the protein expression. Cells were incubated for 72 hours in the

presence of drug, and viability was measured by the CellTiter96 AQueous assay (Promega). Viability was calculated as a percentage of the untreated cells after background subtraction. Six replicates were performed in each cell line for the different drug treatments, and the entire experiment was also repeated three times. Data from the pharmacologic growth-inhibition assays were modeled using a nonlinear regression curve fit with a sigmoidal dose response using GraphPad Prism 5 (GraphPad Software).

Western blot analysis

Immunoblot studies were performed using standard procedures. Briefly, melanoma cells were lysed with RIPA buffer containing both protease and phosphatase inhibitor cocktails (Roche). Lysates were quantified (BioRad Protein Assay), denatured at 95°C, and resolved by SDS gel electrophoresis. Protein was transferred to nitrocellulose membranes and probed with primary antibodies recognizing p-ERK1/2 (Santa Cruz Biotechnology, 1:10,000), ERK 1/2, p-MEK1/2 (Ser217/221), MEK1/2, and vinculin (Cell Signaling Technology; 1:1000 dilution). After incubation with the appropriate secondary antibody (anti-rabbit or anti-mouse IgG, HRP-linked; 1:1000 dilution) (Cell Signaling Technology), proteins were detected using chemiluminescence (Pierce).

Statistical analysis

All statistical analyses were performed using the R statistical package.

References

1. Johannessen CM, Johnson LA, Piccioni F, Frederick DT, Donahue MK, Narayan R, et al. A cyclic AMP-regulated melanocyte lineage program confers resistance to MAP kinase pathway inhibition. Nature. 2013;Manuscript Accepted.

2. Whittaker SR, Theurillat JP, Van Allen E, Wagle N, Hsiao J, Cowley GS, et al. A genome-scale RNA interference screen implicates NF1 loss in resistance to RAF inhibition. Cancer discovery. 2013 Mar;3(3):350-62. PubMed PMID: 23288408. Pubmed Central PMCID: 3606893.

3. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011 Aug 1;29(22):3085-96. PubMed PMID: 21383288. Pubmed Central PMCID: 3157968.

4. Van Allen EM, Wagle N, Sucker A, Treacy D, Goetiz EM, Place CS, et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. Submitted. 2013.

5. Senawong T, Phuchareon J, Ohara O, McCormick F, Rauen KA, Tetsu O. Germline mutations of MEK in cardio-facio-cutaneous syndrome are sensitive to MEK and RAF inhibition: implications for therapeutic options. Human molecular genetics. 2008 Feb 1;17(3):419-30. PubMed PMID: 17981815.

6. Fisher S, Barry A, Abreu J, Minie B, Nolan J, Delorey TM, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted

capture libraries. Genome biology. 2011;12(1):R1. PubMed PMID: 21205303. Pubmed Central PMCID: 3091298.

7. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. Nature genetics. 2006 May;38(5):500-1. PubMed PMID: 16642009.

8. Cibulskis K, McKenna A, Fennell T, Banks E, DePristo M, Getz G. ContEst: estimating cross-contamination of human samples in next-generation sequencing data. Bioinformatics. 2011 Sep 15;27(18):2601-2. PubMed PMID: 21803805. Pubmed Central PMCID: 3167057.

9. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nature biotechnology. 2013 Mar;31(3):213-9. PubMed PMID: 23396013.

10. Costello M, Pugh TJ, Fennell TJ, Stewart C, Lichtenstein L, Meldrim JC, et al. Discovery and characterization of artifactual mutations in deep coverage targeted capture sequencing data due to oxidative DNA damage during sample preparation. Nucleic acids research. 2013 Apr 1;41(6):e67. PubMed PMID: 23303777. Pubmed Central PMCID: 3616734.

11. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics. 2004 Oct;5(4):557-72. PubMed PMID: 15475419.

12. Carter SL, Cibulskis K, Helman E, McKenna A, Shen H, Zack T, et al. Absolute quantification of somatic DNA alterations in human cancer. Nature biotechnology. 2012 May;30(5):413-21. PubMed PMID: 22544022.

13. Berger MF, Levin JZ, Vijayendran K, Sivachenko A, Adiconis X, Maguire J, et al. Integrative analysis of the melanoma transcriptome. Genome research. 2010 Apr;20(4):413-27. PubMed PMID: 20179022. Pubmed Central PMCID: 2847744.