Supplemental Information

Table S1. Annotated list of gene expression changes in primary human melanocytes expressing oncogenic BRAFa.

| Probe Name | Gene Title | Gene Symbol | Fold change | GO Biological Process |
|------------|------------|-------------|-------------|-----------------------|
| 204475_at  | matrix metallopeptidase 1 | MMP1         | 315.9       | proteolysis           |
| 204614_at  | serpin peptidase inhibitor, clade B, 2 | SERPINB2     | 284.2       | anti-apoptosis        |
| 205239_at  | amphiregulin | AREG         | 192.6       | cell-cell signaling /growth |
| 209278_s_at | tissue factor pathway inhibitor 2 | TFPI2         | 188.0       | blood coagulation     |
| 230746_s_at | Stanniocalcin 1 | STC1         | 103.7       | calcium ion homeostasis |
| 214974_x_at | chemokine (C-X-C motif) ligand 5 | CXCL5         | 103.7       | chemotaxis / inflammation |
| 1555339_at | RAP1A, member of RAS family | RAP1A        | 41.4        | regulation of cell cycle |
| 227140_at  | CDNA FLJ11041 fis | ---          | 38.8        | ---                   |
| 205767_at  | epiregulin | EREG         | 33.8        | EGFR signaling        |
| 206569_at  | interleukin 24 | IL24         | 30.9        | immune response       |
| 224480_s_at | lung cancer metastasis-associated | MAG1         | 26.1        | metabolic process     |
| 218322_at  | brain expressed, X-linked 1 | BEX1         | 21.8        | nervous system dev    |
| 242005_at  | ---          | ---          | 17.3        | ---                   |
| 206044_s_at | v-raf viral oncogene homolog B1 | BRAF         | 16.3        | MAPK signal transduction |
| 204602_at  | dickkopf homolog 1 (Xenopus laevis) | DKK1         | 16.0        | Wnt receptor signaling |
| 202859_x_at | interleukin 8 | IL8          | 15.0        | chemotaxis / inflammation |
| 203889_at  | secretogranin V (7B2 protein) | SCG5         | 14.9        | protein transport     |
| 206172_at  | interleukin 13 receptor, alpha 2 | IL13RA2      | 14.1        | ---                   |
| 39402_at   | interleukin 1, alpha | IL1A         | 14.1        | inflammation           |
| 213479_at  | neuronal pentraxin II | NPTX2        | 13.5        | synaptic transmission  |
| 224321_at  | transmembrane protein with EGF-like | TMEFF2       | 12.7        | ---                   |
| 201645_at  | tenascin C (hexabrachion) | TNC          | 11.6        | cell adhesion         |
| 212298_at  | neuropilin 1 | NRP1         | 10.4        | angiogenesis           |
| 210512_s_at | vascular endothelial growth factor A | VEGFA        | 9.8         | angiogenesis           |
| 206652_s_at | tachykinin, precursor 1 | TAC1         | 8.9         | tachykinin signaling  |
| 202437_at  | cytochrome P450, family1B | CYP1B1       | 8.4         | electron transport    |
| 242871_at  | progestin & adipoQ receptor family V | PAQR5        | 7.4         | ---                   |
| 206115_at  | early growth response 3 | EGR3         | 6.9         | regulation of transcription |
| 222853_at  | fibronectin leu rich transmembrane 3 | FLRT3        | 6.7         | cell adhesions        |
| 201920_at  | solute carrier family 20, member 1 | SLC20A1      | 6.5         | DNA repair / transcription |
| 210095_s_at | insulin-like growth factor binding 3 | IGFBP3       | 6.4         | regulation of cell growth |
| 208692_at  | dual specificity phosphatase 6 | DUSP6        | 6.4         | protein dephosphorylation |
| 203939_at  | 5'-nucleotidase, ecto (CD73) | NT5E         | 6.2         | DNA metabolic process  |
| 209921_at  | solute carrier family 7 member 11 | SLC7A11      | 5.8         | protein complex assembly |
| 209803_at  | pleckstrin homology-like domain,A2 | PHLD2A       | 5.8         | imprinting / apoptosis |
| 218451_at  | CUB domain containing protein 1 | CDCP1        | 5.7         | ---                   |
| 205249_at  | early growth response 2 | EGR2         | 5.4         | regulation of transcription |
| 205476_at  | chemokine (C-C motif) ligand 20 | CCL20        | 5.2         | chemotaxis / inflammation |
| 216598_s_at | chemokine (C-C motif) ligand 2 | CCL2         | -7.3        | chemotaxis / inflammation |
| 226587_at  | CDNA FLJ33569 fis | ---          | -5.4        | ---                   |
| 205961_s_at | PC4 and SFRS1 interacting protein 1 | PSIP1        | -5.2        | regulation of transcription |

a: Genes shown are differentially expressed more than 5 fold compared to control group.
b: Genes with multiple probe sets are shown with data from a single representative probe set.
Figure S1. Network target mapping of BRAF downstream effector genes in primary human melanocytes. Mapping of network targets induced by activated BRAF kinase identifies cell growth, cell proliferation, and apoptosis as cellular processes that are critically mediated by the BRAF gene signature. The ten most highly upregulated BRAF kinase target genes in primary human melanocytes were subjected to network target mapping using a data mining software (Pathway Architect, Stratagene, La Jolla, CA) to detect cellular processes. Eight BRAF effector genes (green oval) were recognized as nodal genes.
Figure S2

(a) Melanocytes

(b) Fibroblasts

Figure S2. Melan-A immunofluorescence staining of melanocytes isolated from neonatal foreskins and used for global gene expression profiling. (a) melanocytes were fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 respectively. Cells were
incubated with primary antibody for melan-A (monoclonal mouse anti-human, clone A103, DAKO) and FITC conjugated secondary antibody. (b) Melan-A immunofluorescence staining of short-term cultured fibroblasts isolated from newborn foreskins were included as a negative control.

Supplemental Materials and Methods

Cell culture
Primary human melanocytes were prepared from neonatal foreskins as previously outlined (Dunlap et al., 2004). In order to minimize genetic variability melanocytes from 4–5 individuals were pooled for each culture. Once epidermal cells were dispersed with trypsin and neutralized in serum-containing media, cells were plated in melanocyte growth medium (Cell Applications, Inc., San Diego, CA). A pure population of epidermal melanocytes was obtained by eliminating possibly contaminated fibroblasts with 100 μM geneticin (G418) treatment for 3 days. Cells were used at passage 2 for viral infection. Melanoma cell lines WM852 and WM793 were obtained from Meenhard Herlyn and cultured in DMEM with 10% FCS.

Lentivirus preparation and primary cell transduction
BRAFV600E and dead kinase mutant BRAFT595A/S602A constructs have been described previously (Karbowniczek et al., 2004) and were obtained from Gavin Robertson (Hershey, PA). BRAF mutants were amplified by PCR using the following primers: 5'-GGATCCCAGTGTGGTGGTA-3' and 5'-CCACTGTGCTGGCGAATTC-3'. PCR amplified products were blunt ended and inserted into Eco RV site of a bicistronic lentiviral vector (Yu et al., 2003). Virus was produced in HEK293T cells using previously described protocols (Yu et al., 2003). Viral transduction of primary human melanocytes was performed as described previously (Dunlap et al., 2004) with slight modification. Briefly, 1 x 10^{6} melanocytes were plated in a well of a 6-well cell culture plate with melanocyte growth medium for 24 hours prior to viral transduction. Cells were infected with a multiplicity of infection (MOI) of 2 and 10 in the presence of 6 ug/ml of polybrene (Sigma, St. Louis, MO) for 4 hours. Primary melanocytes transduced with an MOI of 10 were used for the comparative analysis of expression profiles to detect BRAF downstream effectors. Primary melanocytes transduced with an MOI of 2 used for the rest of the experiments. Transduced cells were maintained for 5 days in culture prior to the initiation of studies. At this point more than 99% of melanocytes expresses GFP (a marker for transduced melanocytes) indicating very
high transduction efficiency by mutant BRAF expressing lentivirus (Dunlap et al., 2004). The melanocytes were also stained with a melanocytic marker (Melan-A) in order to ruled out possible transdifferentiation into a mesenchymal cell (Figure S2).

**Antibodies for western blotting**

Cell lysates were prepared from melanocytes on day 4 after infection. Western blotting was performed using standard techniques and reactive proteins were visualized using ECL reagents. Antibodies used included: Raf-B (sc-5284, Santa Cruz), Phospho-MEK1/2 (#9121, Cell Signaling), MEK1/2 antibody (#9122, Cell Signaling). For Amphiregulin Western Blot Analysis, 500 μg of conditioned media collected from primary human melanocytes was subjected to immunoprecipitation using 2 μg of human amphiregulin monoclonal antibody (MAB262, R&D Systems) and Protein A/G PLUS-agarose beads (sc-2003, Santa Cruz). The resulting immunoprecipitated proteins were denatured and run on a 12% polyacrylamide gel. Western blotting was performed using standard techniques and was visualized using ECL reagents. The primary antibody used was biotinylated human amphiregulin antibody (BAF262, R&D Systems) and the secondary antibody was a 1:10,000 dilution of streptavidin-HRP (21126, Pierce).

**Expression profiling of BRAF^{V600E} melanocytes**

Primary human melanocytes (1.0 x 10^6/well of a 6-well plate) were transduced by BRAF^{V600E} expressing lentivirus and control virus with MOI of 2 respectively in duplicate (total 4 samples, two samples of primary human melanocytes withBRAF^{V600E} and two samples of melanocytes with control GFP). Cells were harvested 5 days following infection and total RNA was extracted and purified as described previously (Ryu et al., 2007). RNA quality check, double strand complementary DNA synthesis, hybridization with Human Genome U133 Plus 2.0 Array Chips (Affymetrix Inc., Santa Clara, CA), initial data extraction, normalization using RMAExpress software (Irizarry et al., 2003) and statistical analysis for the identification of differentially expressed genes were performed at the Johns Hopkins Medical Institute Microarray Core Facility. Briefly, RNA samples were analyzed with Affymetrix GeneChip human U133 Plus 2.0 Arrays (Affymetrix Inc., Santa Clara, CA). Quality of the microarray experiment was assessed with affyPLM and Affy, two Bioconductor packages for statistical analysis of microarray data. To estimate the gene expression signals, data analysis was conducted on the chips’ CEL file probe signal values at the Affymetrix probe pair (perfect match (PM) probe and mismatch (MM) probe) level, using the statistical algorithm Robust Multiarray Analysis (RMA) expression measure (Irizarry et al., 2003) with Affy. This probe level data processing includes a normalization.
procedure utilizing a quantile normalization method (Irizarry et al., 2003) to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization and/or scanning. Exploratory data analysis (EDA) was performed with the preprocessed data above. Between-treatment and between-replicate variations were examined with the pair-wise MvA plots, in which the base 2 log ratios (M) between two samples are plotted against their averaged base 2 log signals (A). With the signal estimates, Multidimensional Scaling (MDS) analysis was also performed to assess sample variability. The quality assessment and MDS analyses identified and disqualified a discordant sample chip from one sample of melanocytes with BRAFV600E. Comparative analysis of gene expression profiles for the identification of differently expressed genes and statistical analyses for p-value calculations were therefore performed with the expression data from one sample of melanocytes with BRAFV600E and two samples of melanocytes with GFP control. Gene Ontology analysis and network target mapping to detect cellular processes that may be regulated by the BRAF downstream effectors were performed using Spotfire® (Spotfire Inc., Cambridge, MA) and Pathway Architect software (Stratagene, La Jolla, CA) respectively. Upon excluding the discordant chip, the signal data were obtained with the remaining chips using the RMA algorithm above. With the signal intensities estimated above, an empirical Bayes method implemented in the bioconductor package EBarrays, was attempted with both the Gamma-Gamma and lognormal-normal modeling methods to estimate the posterior probabilities of the differential expression of genes between the sample conditions (Kendziorski et al., 2003). Though the Gamma-Gamma modeling fits better than the lognormal-normal modeling, both parametric modeling methods fit poorly. It has been shown in a simulation study on a yeast example that in spite of poor fit, the resulting inference methods from the parametric modeling have good operating characteristics (Newton et al., 2004). Thus, the posterior probability from the Gamma-Gamma modeling might provide a way to make inferences on differential gene expression in this study in spite of the poor fit. Specifically, the criterion of the posterior probability > 0.5, which means the posterior odds favoring change, was used to produce the differentially expressed gene list. All Bioconductor packages are available online and all computation was performed under R environment (Ihaka and Gentleman, 1996). The complete dataset is accessible as GSE13827.

MMP-1 gene silencing by siRNA
The MMP-1 siRNA (5'-gagcaagatgtggacttag-3') and scrambled siRNA (5'-gattcaggtgtagaacgag-3') sequences were selected from previously published paper (Yuan et al., 2005) and
synthesized from Dharmacon siDESIGN Center. Transfection of the siRNAs was performed using 2 µL of DharmaFECT 1 transfection reagent (Dharmacon). The same molar concentration of siRNA (100 nM) and negative control (scramble) as reported in the study performed by Dr. Yuan et al. were tested. It turned out that 100 nM siRNA worked well for the wild-type B-RAF WM852 cell line but with minimal effect on the B-RAF mutant WM793 cell line. Therefore, optimization for each cell line was performed in order to obtain comparable data for analysis. The optimum amount of siRNA for efficient knock-down of MMP-1 in WM852 and WM792 cells were 100 nM and 250 nM respectively. Cells were collected and pelleted 24 hours after transfection for RNA extraction using the RNeasy Mini Kit (Invitrogen), cDNA production by using SuperScript™ First-Strand Synthesis System for RT-PCR according to manufacturer’s instructions (Invitrogen), and semi-quantitative duplex PCR to confirm MMP-1 knockdown.

Quantitative Real Time Polymerase Chain Reaction Assay

The relative expression levels of MMP-1 in WM852 and WM793 were determined by quantitative real time polymerase chain reaction (qRT-PCR) analysis. The following primer oligonucleotides were used: MMP-1 forward (5'-TGCTTGACCCTCAGACAGCT-3'), MMP-1 reverse (5'-GATGGGAGGCAAGTTGAAAA -3'), GAPDH forward (5'-CATGAGAAGTATGACAACAGCCT-3'), GAPDH reverse (5'-AGTCCTTCCACGATACCAAGT-3'). RNA was extracted using the RNeasy Mini Kit (Invitrogen). 3 µg of RNA for each sample was used for cDNA synthesis using SuperScript First-Strand Synthesis System according to manufacturer’s instructions (Invitrogen). The qRT-PCR was carried out in a total volume of 20 µL per reaction, each containing 1 µL cDNA, 10 µL SYBR Green PCR master mix (Applied Biosystems), 1 µL of 10 µM forward primer, and 1 µL of 10 µM reverse primer. DNA was amplified using the following parameters: 50 ºC for 2 minutes, 95 ºC 10 minutes, followed by forty cycles of 95 ºC for 30 seconds, 55 ºC for 35 seconds, and 72 ºC for 30 seconds. MMP-1 gene expression was normalized using reference primers against GAPDH.

³H-thymidine Cell Proliferation Assay

WM852 and WM793 cell lines were seeded at 15,000 cells per well into a 96-well tissue culture plate (Becton Dickinson) 24 hours after siRNA transfection. Twenty-two hours later, 10 µL of 0.1 µCi/µL ³H-thymidine (Perkin Elmer) was added to each well. After 4 hours, the cells were washed with PBS and 50 µL of trypsin was added into each well for 10 minutes. The trypsinized cells were harvested to a filtermat using the Perkin Elmer FilterMate cell harvester (Perkin
Elmer). The dried filtermat was sealed in a sample bag with 4 mL of scintillation fluid and then read on a Perkin Elmer 1450 Microbeta Liquid Scintillator (Perkin Elmer).

**Conditioned Media Analysis of MMP-1 and amphiregulin**

Cell culture media with no serum was added to the cell lines. After 72 hours, the media was collected and the protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). The amount of human MMP-1 pro and active forms was determined using the RayBio® Human MMP-1 ELISA kit (RayBiotech, Inc.) following the manufacturer's instructions. MMP-1 activity in the collected media was measured using the SensoLyte Plus™ 520 MMP-1 Assay Kit (AnaSpec) following the manufacturer’s protocol including the step to add 4-aminophenylmecuric acetate (Weinberg *et al.*, 1995) to the samples in order to activate all the pro-MMP-1. Activated (cleaved) amphiregulin levels in conditioned media were determined using an ELISA (DY262, R&D Systems) following the manufacturer’s instructions.

**References**

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Yuan J, Dutton CM, Scully SP (2005) RNAi mediated MMP-1 silencing inhibits human chondrosarcoma invasion. J Orthop Res 23:1467-74.

Footnotes:
a. http://www.bioconductor.org
b. http://www.r-project.org
c. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13827