SUPPLEMENTARY DATA

MATERIALS AND METHODS

Cells, antibodies and reagents. Vemurafenib-trametinib-resistant A375 cells (A375-VR/TR) were derived from A375-VR cells after culture for several weeks in the presence of both VMF (1.3 μM) and trametinib (40 nM). SK-Mel-239 and SK-Mel-239-VR cells were a kind gift from Drs. Poulikos Poulikakos and Emily Bernstein (1). Foreskin melanocytes were provided by Dr. Marisol Soengas (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). Antibodies to BRAF and p90RSK were from BD Biosciences (San Diego, CA) and Abcam (Cambridge, UK), anti-β-actin and vinculin antibodies were purchased from Sigma Aldrich (St Louis, MO), and Erk1/2, phospho-Erk1/2, AKT, phospho-AKT (Ser473), phospho-p90RSK (Thr359/Ser363) and phospho-S6 Ribosomal Protein (Ser235/236) antibodies from Cell Signaling (Danvers, MA). Anti-MEK1, CHOP, IGFBP5 and RhoGDIα antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA), and phospho-MEK1/2 (S218/S222) and pan-Ras antibodies from Millipore (Billerica, MA). Antibodies to ephrin B2, NUAK1/ARK5 and Slug were from Abcam. Antibodies to Rac1 were obtained from BD Biosciences (San Diego, CA), anti-RhoA from Santa Cruz Biotech (Santa Cruz, CA). SCH772984 (ERK1/2 inhibitor), Triciribine (Akt inhibitor) and the NSC 23766 Rac GTPase inhibitor were from Selleckchem. Thapsigargin was a gift from Dr. Natalia Rodríguez-Muela (Centro de Investigaciones Biológicas, Madrid).

Rho GTPase assays. GTPase assays to detect the active forms of Rac and RhoA were performed as described (2), using GST-PAK-CD to detect active Rac, and GST-C21 for active RhoA. After elution from glutathione-agarose beads, proteins were immunoblotted with anti-Rac or anti-RhoA antibodies.

DNA sequencing. RNA was isolated with TriReagent (Sigma Aldrich) and reverse-transcribed to cDNA by standard methods using M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen), using specific oligonucleotides for BRAF V600 (CCATATCATTGAGACCAAATTTGAGATG and GGCACTCTGCCATTAATCTCTTCATGG) and for N-RASQ61 (GAGTACAAACTGGTGGTGGT and TTCGTGGGCTTGTTTTGTAT). PCR profiles for BRAFV600 were 94°C for 2 min, followed by 40 cycles at 94°C for 1 min, 60°C for 2 min and 72°C for 2 min, with a final incubation at 72°C for 7 min. For N-RASQ61 were 95°C for 1 min, followed by 35 cycles at 95ºC for 30 sec, 50ºC for 30 sec, 72ºC for 45 sec, with a final incubation at 72º for 10 min. The amplified cDNA was purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen), and sequenced with specific oligonucleotides (BRAFV600: TGCACAGGGCATGGATTACT; N-RASQ61: GACAATCCAGCTAATCCAGA) by Sanger DNA sequencing at Secugen (Centro de Investigaciones Biológicas, Madrid, Spain).

Invasion assays. Invasions were performed as earlier reported (3). Briefly, cells were loaded on the upper compartments of invasion chambers (Corning Costar, Corning, NY) coated with Matrigel (BD Biosciences), whereas the lower compartments were filled with invasion medium with or without 10% FBS. After 48h invasive cells were fixed, stained and counted under a microscope.

FIGURE LEGENDS

Figure S1. Characterization of vemurafenib-resistant melanoma cells. Cells were incubated without (Ctrl) or with the indicated concentrations of vemurafenib (VMF) or trametinib (TMT), and tested in MTT assays (A, n=3), or by immunoblotting (B). (C)

VMF-resistant A375-VR cells were incubated for 2 weeks without (-) or with (+) VMF (1.3 μM), and subsequently subjected to cell proliferation assays as in (A). Parental A375 are shown as controls. (n=2). (D) Tumor cells from metastases of mice inoculated with A375 or A375-VR cells were extracted, cultured and subsequently tested for 48 h in MTT assays in the presence of VMF.

Figure S2. Sensitivity of A375-VR cells to other inhibitors of the MAP kinase pathway. Cells were cultured for 48 h in medium (Ctrl) or with the indicated concentrations of trametinib (TMT) (A), SCH772984 (B) or triciribine (C), and tested in MTT assays (n=3). (D) *SPRED* and *COT* mRNA expression from the indicated cells was analyzed by qPCR. (E, F) Cells were incubated in the absence or presence of the indicated inhibitors, and subsequently subjected to western blotting with antibodies to the shown proteins.

Figure S3. Characterization of invasive properties ofA375-VR cells and sensitivity to a Rac inhibitor . (A) Cells were subjected to invasion assays across matrigel (n=4, $^{\Delta\Delta\Delta}p<0.001$). (B) Cells were analyzed in GTPase assays for Rac and Rho activation (n=4-5; $^{**}p<0.01$, $^{*}p<0.05$). (C-E) Cells were cultured for 48 h in medium without (Ctrl), or with the indicated concentrations of NSC23766 in the absence (C) or presence of different amounts of vemurafenib (D), and tested in MTT assays (n=2-3), or by immunoblotting (E).

Figure S4. Characterization of miRNA expression in melanoma cells resistant to BRAF and MEK inhibitors, and in foreskin melanocytes. (A) Six different VMF-resistant clones of SK-Mel 239 cells were analyzed by qPCR for expression of the indicated miRNAs. (B) Parental and resistant cells were incubated for 48 h in medium without (Ctrl) or with the indicated concentrations of VMF, TMT or the combination of both inhibitors, and tested in MTT assays (n=3). (C) miRNA expression from parental

or the indicated resistant A375 cells was tested by qPCR. (D) Cultured normal foreskin melanocytes were exposed for 24 h to VMF alone (1 μM), or to combined VMF (100 nM) and TMT (4 nM), and subsequently subjected to qPCR analyses for miRNA expression. Expression of miRNAs was significantly upregulated $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, or significantly reduced, $^{\Delta\Delta}p<0.01$ or $^{\Delta}p<0.05$ (n=2-3).

Figure S5. Overexpression or silencing of miRNAs in A375, SK-Mel 28 and A375-VR cells. (A, B) A375 cells were subjected to lentiviral-mediated gene transfer for overexpression (H-miR vectors) or silencing (Zip vectors), respectively, and subsequently tested by qPCR for the expression of the indicated miRNAs. (C) The indicated control A375 transductants were tested for 48 h in MTT assays in the absence (Ctrl) or presence of the indicated concentrations of VMF. Proliferation was significantly augmented, $^{*}p<0.05$ (n=3). (D, E) Analysis by qPCR of miRNA-204-5p and miRNA-211-5p expression in single and double-miRNA SK-Mel-28 miRNA transductants. ***Expression was significantly increased, $p<0.001$, or reduced $^{\Delta\Delta}p<0.01$ (n=2-3).

Figure S6. Role of miR-204-5p, miR-211-5p and miR-140-3p in melanoma resistance to MAP kinase inhibitors and to AKT blockers. The indicated control (H-Scr) and miR A375 and SK-Mel-28 transductants were tested for 48 h in MTT assays in the absence (Ctrl) or presence of the indicated concentrations of TMT or VMF and TMT combinations (A, B), SCH772984 (50 nM) and VMF (100 nM) (C), or to triciribine (5 μM) (D). Proliferation was significantly augmented, $^{**}p<0.01$, $^{*}p<0.05$ (n=3-4). (E) A375-VR cells were transfected with a combination of miRNA-204-5p and miRNA-211-5p miRIDIAN microRNA hairpin inhibitors or with a negative control, and miRNA levels determined by qPCR (n=2). ***Expression was significantly reduced, $^{\Delta\Delta\Delta}p<0.001$.

Figure S7. miRNA role in VMF resistance in BRAF wild type cells. (A) SK-Mel 103 cells were exposed for 48 h to VMF alone (1 μM), or to combined VMF (100 nM) and TMT (4 nM), and subsequently subjected to qPCR analyses for miRNA expression (n=3). Expression was significantly increased $^{**}p<0.01$, $^{*}p<0.05$, or reduced ($^{\Delta\Delta\Delta}p<0.001$). (B) Cells were transiently transfected with miRNA-204-5p miRIDIAN microRNA hairpin inhibitor or with a negative control, and transfectants tested by quadriplicate in cell proliferation assays (n=3; $^{\Delta\Delta\Delta}p<0.001$).

Figure S8. Analyses of miRNA target expression in cells overexpressing miR-204-5p and miR-211-5p and in A375-VR cells. The mRNA levels of the indicated miRNA targets were determined by qPCR. $^{\Delta\Delta\Delta}$Expression was significantly reduced, $p<0.001$ or $^{\Delta}p<0.05$, or **stimulated, $^{*}p<0.05$ (n=3-6).

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