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Supplemental Information

Anaplastic Lymphoma Kinase Confers Resistance to BRAF Kinase Inhibitors in Melanoma

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SUPPLEMENTARY FIGURES AND LEGENDS

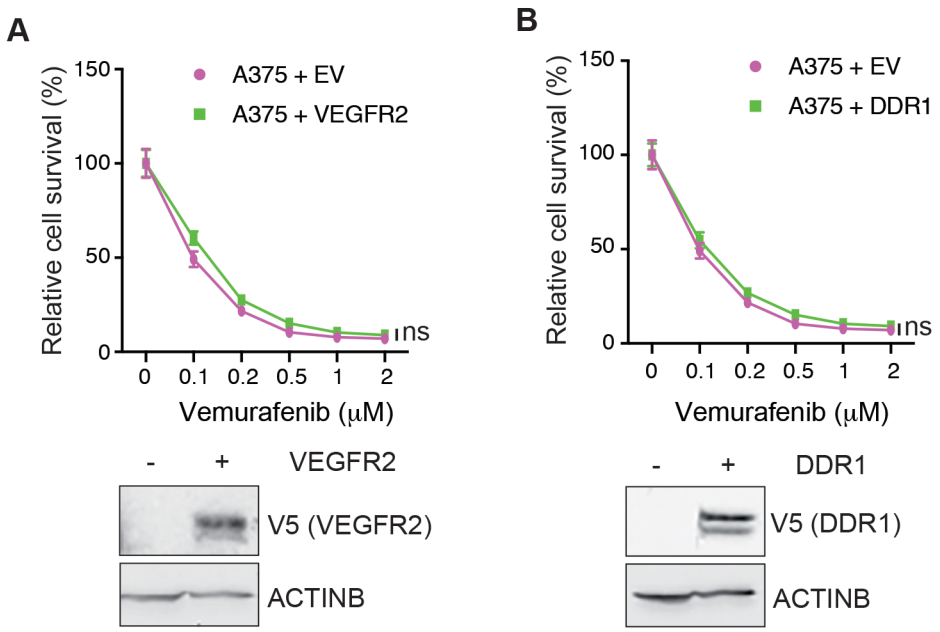

Figure S1. Ectopic expression of VEGFR2 or DDR1 did not cause BRAFi resistance, related to Figure 2. (A) (Upper panel) A375 cells ectopically expressing empty vector (EV) or VEGFR2 were treated with either DMSO or the indicated concentrations of vemurafenib for 3 days and analyzed for survival using the MTT assay. Relative survival (%) for each cell line in reference to DMSO treated cells is shown. (Lower panel) Immunoblot showing ectopic expression of V5-agged VEGFR2 and ACTINB. **(B)** (Upper panel) A375 cells ectopically expressing empty vector (EV) or DDR1 were treated with either DMSO or the indicated concentrations of vemurafenib for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) for each cell line in reference to DMSO treated cells is shown. (Lower panel) Immunoblot showing ectopic expression of V5-tagged DDR1 and ACTINB. Data is presented as mean ± SD. ns= not significant p-value.

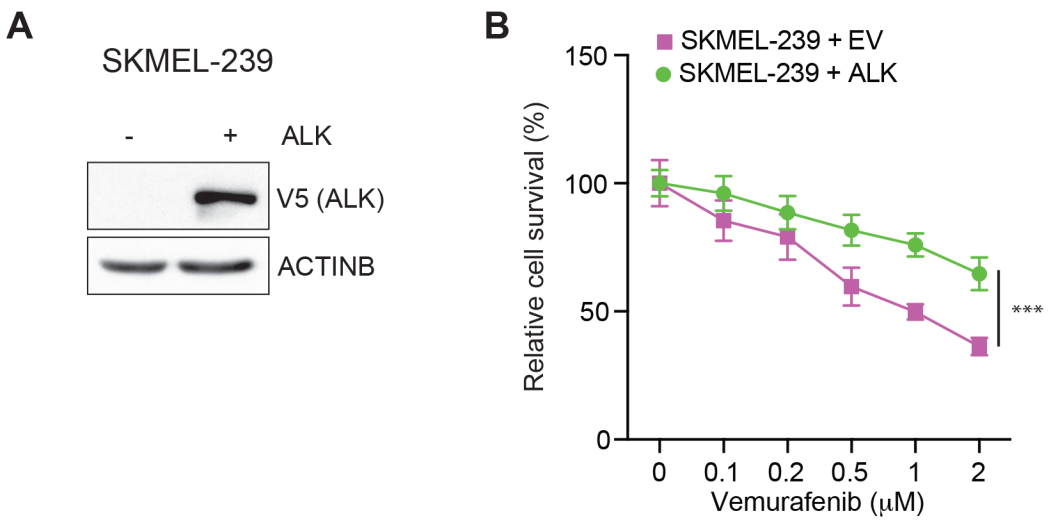

Figure S2. Ectopic expression of ALK causes BRAFi resistance in SKMEL-239 cells, related to Figure 3. (A) ALK-V5 was overexpressed in SKMEL-239 parental cells and expression was confirmed by immunoblot with V5 antibody. ACTINB is used as a loading control. **(B)** SKMEL-239 cells ectopically expressing empty vector (EV) or ALK were treated with either DMSO or the indicated concentrations of vemurafenib for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) for each cell line in reference to DMSO treated cells is shown. Data is presented as mean ± SD. p-value. *** represents $p<0.001$.

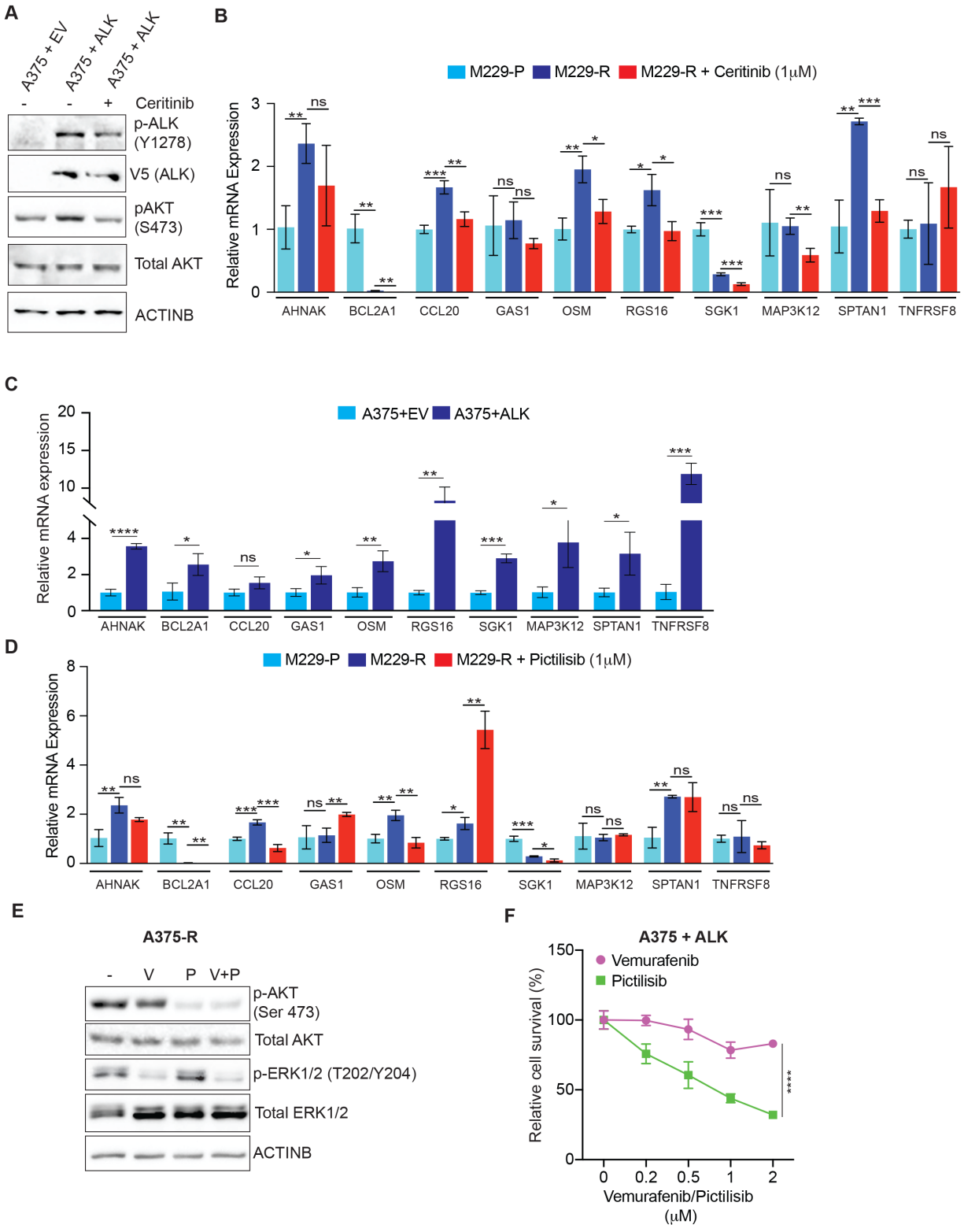

Figure S3. ALK-activated PI3K/AKT in melanoma cells, related to Figure 3 and 4. (A) A375 cells expressing an empty vector (A375+EV), ALK (A375+ALK), or ceritinib-treated A375+ALK

(1 μM) for 24 h were analyzed for the indicated proteins by immunoblotting. ACTINB was used as the loading control. **(B)** mRNA expression for the indicated ALK-activated genes was measured in M229-P, M229-R, and M229-R treated with ceritinib (1 μM) for 24 h. mRNA expression for indicated genes relative to M229-P cells is shown. ACTINB mRNA expression was used for normalization. (**C**) mRNA expression for the indicated ALK-activated genes was measured in A375 cells overexpressing ALK (A375+ALK) or empty vector (A375+EV). mRNA expression for indicated genes relative to A375+EV cells is shown. ACTINB mRNA expression was used for normalization. (**D**) mRNA expression for the indicated ALK-activated genes was measured in M229-P, M229-R, and M229-R treated with pictilisib (1 μM) for 24 h. mRNA expression for indicated genes relative to M229-P cells is shown. ACTINB mRNA expression was used for normalization. (**E**) A375-R cells were treated with indicated concentration of vemurafenib, pictilisib or combination and analyzed for ERK and AKT phosphorylation by immunoblotting. ACTINB was used as a loading control. (**F**) A375 cells expressing ALK (A375+ALK) were treated with indicated concentrations of vemurafenib or pictilisib for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) in reference to DMSO treated cells is shown. Data is presented as mean ± SD. ns= not significant p-value, *, **, *** and **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

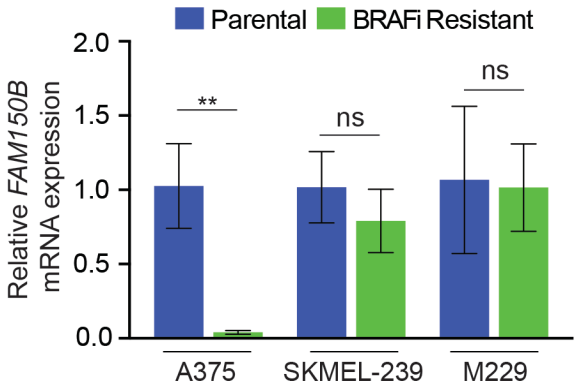

Figure S4. Analysis of FAM150B expression, related to Figure 5. mRNA expression for FAM150A was measured for the indicated pairs of parental and BRAFi-resistant cells (A375, SKMEL-239, M229). mRNA expression is shown relative to respective parental melanoma cells. ACTINB mRNA expression was used for normalization Data is presented as mean ± SD. ns= not significant p-value. ** represents $p<0.01$.

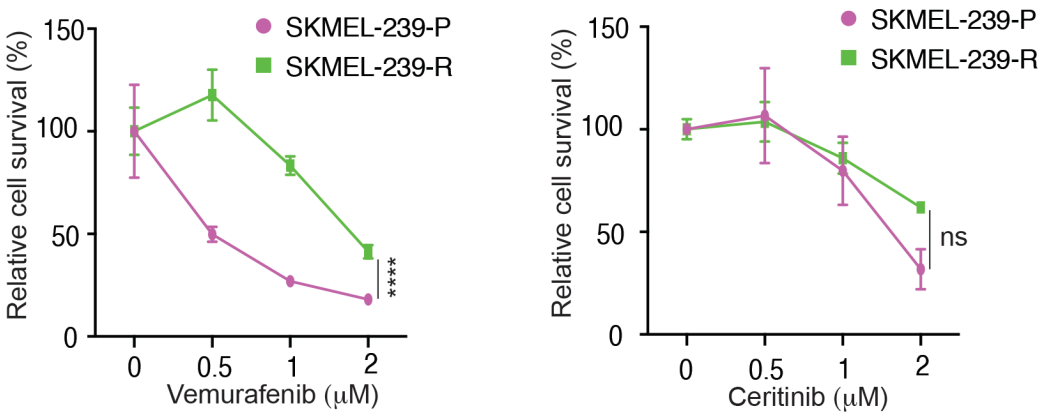

Figure S5. Measuring the sensitivity of BRAF inhibitor-resistant SKMEL-239 cells to ALK inhibitor, related to Figure 6. Parental and BRAFi-resistant SKMEL-239 melanoma cell lines were treated with DMSO or with the indicated concentrations of vemurafenib or ceritinib, and analyzed for survival using the MTT assay. Relative cell survival (%) in reference to DMSO treated cells is shown. Data is presented as mean ± SD. ns= not significant p-value, **** represents $p<0.0001$.

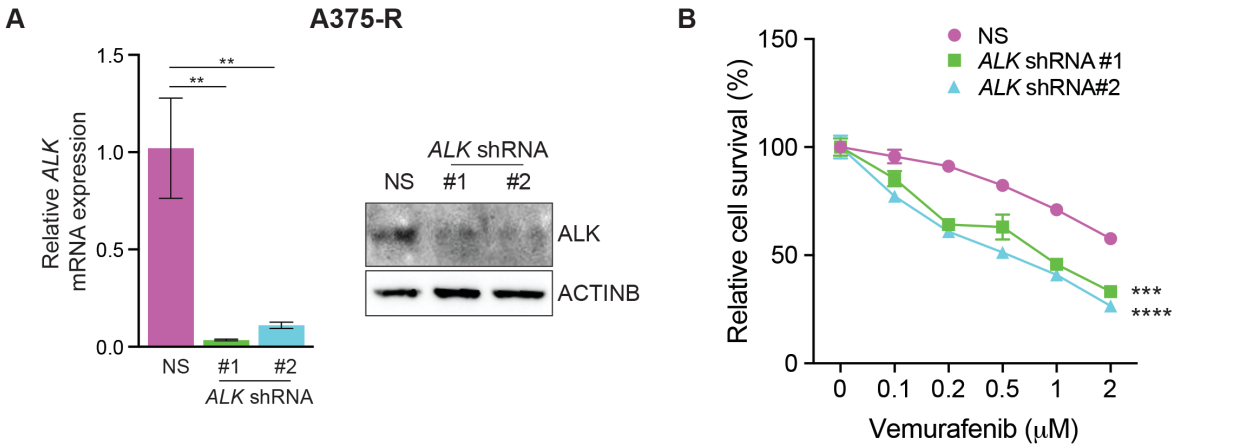

Figure S6. shRNA-mediated downregulation of ALK expression increased the sensitivity of A375-R cells to vemurafenib, related to Figure 6. (A) A375-R cells expressing ALK-specific shRNA were analyzed for (left) ALK mRNA expression using RT-qPCR or (right) protein expression by immunoblotting. ACTINB was used as a loading control. **(B)** A375-R cells expressing ALK-specific shRNA were treated with either DMSO or the indicated concentrations of vemurafenib for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) for each cell line in reference to DMSO treated cells is shown. Data is presented as mean ± SD. ns= not significant p-value, *** and **** represent $p<0.001$and $p<0.0001$ respectively.

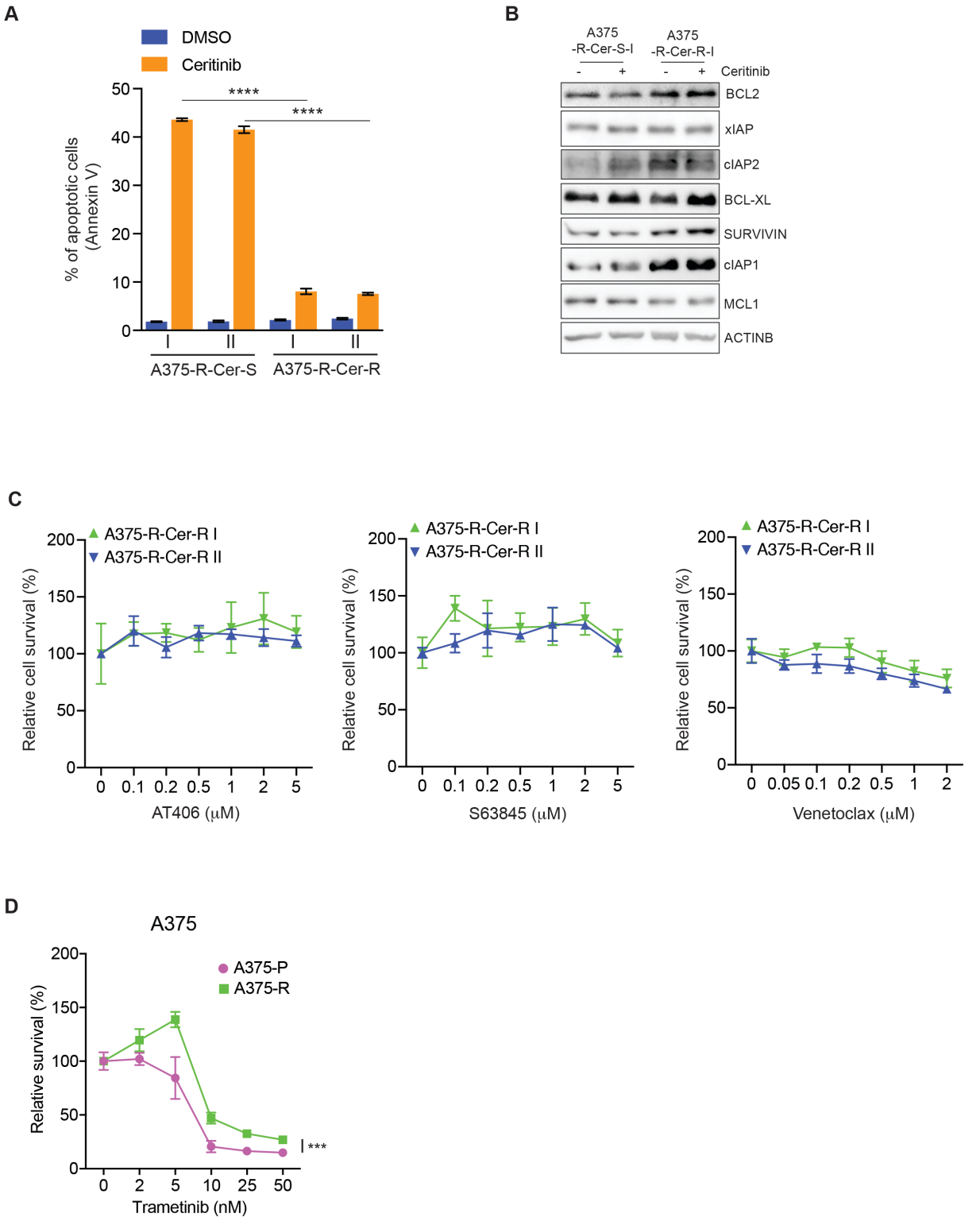

Figure S7. Measuring the sensitivity of A375 cells resistant to BRAF and ALK inhibitors (A375-R-Cer-R) to AT406, S63845, and Venetoclax, related to Figure 7. (**A**) Tumor-derived ceritinib-sensitive (A375-R-Cer-S) and ceritinib-resistant (A375-R-Cer R) cells were treated with DMSO or ceritinib and analyzed for Annexin V positive cells by flow cytometry. % of Annexin-

V positive cells is plotted under indicated conditions. (**B**) Tumor-derived ceritinib-sensitive (A375-R-Cer-S) and ceritinib-resistant (A375-R-Cer R) cells were treated with ceritinib and analyzed for expression of several anti-apoptotic proteins by immunoblotting. ACTINB was used as a loading control. (**C**) Tumor-derived ceritinib-sensitive (A375-R-Cer-S) and ceritinib-resistant (A375-R-Cer R) cells were treated with the indicated concentration of AT406 (IAP inhibitor), S63845 (MCL1 inhibitor), or Venetoclax (BCL2 inhibitor) for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) in reference to DMSO treated cells is shown. (**D**) Parental and BRAFi-resistant pairs of A375 (A375-P and A375-R) were treated with DMSO or indicated concentrations of trametinib for 3 days and analyzed for survival using the MTT assay. Relative cell survival (%) in reference to DMSO treated cells is shown. Data is presented as mean ± SD. *** and **** represent $p<0.001$ and $p<0.0001$, respectively.

Table S1: Primer sequences for RT-qPCR analysis; antibodies used; source and concentration of chemical inhibitors used, related to figures 1-7 and figures S1-S7.

Application	Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)
RT-qPCR	*ACTINB*	GCATGGAGTCCTGTGGCATC	TTCTGCATCCTGTCGGCAAT
	AHNAK	GTGGGCTGGAAGGAAAGATG	GACCCAAGGCTCAGATCCAC
	BCL2A1	TGGATCAGGTCCAAGCAAAA	TGATGCCGTCTTCAAACTCC
	CCL20	GAGTTTGCTCCTGGCTGCTT	CCGTGTGAAGCCCACAATAA
	GAS1	GCCGCTACCTGACCTACTGC	CGCTGCTCGTCATCGTAGTC
	OSM	CCCAGTGAGGAGACCCTGAG	GCTGCTCTAAGTCGGCCAGT
	RGS16	TGAGGCCCCTAAAGAGGTCA	CTTCAGGAAGCGTGGGTAGG
	SGK1	AGGAGGATGGGTCTGAACGA	AGGAGAAGGGTTGGCATTCA
	MAP3K12	TGAGGTGATCCGCAATGAAC	ACGGGCAGATGGAGACTGTT
	SPTAN1	TCTGCTGGAACTGGGTGAGA	CTTGTCAGAGCGGCTTCCTT
	TNFRSF8	CAGCAGAGACGGTCACCAAG	GGAGTCCACCAGCAAGCTCT
	FAM150A	CCGGTCACATTTTCACCAGA	TAGGTCTGGGAGCACAGTGG
	FAM150B	CCGGAGCAGCGAGTGGAAAT	CTGACAGCCAGCCGGGTAAG
	ALK	GAGAGCAAGGACGCTGCAAA	GCCTTTTGCGTTCCTTTTGG
Antibodies	**Protein name**	**Catalogue number**	**Source**
	ACTINB	#4970	Cell Signaling
	ALK	#3633	Cell Signaling
	pALK Y1278	#6941	Cell Signaling
	ERK	#9102	Cell Signaling
	pERK T202/Y204	#9101	Cell Signaling
	AKT	#9272	Cell Signaling
	pAKT S473	#9271	Cell Signaling
	V5	#13202	Cell Signaling
	PARP	#9542	Cell Signaling
	Bcl-2	#4223	Cell Signaling
	Bcl-xL	#2764	Cell Signaling
	Mcl-1	#5453	Cell Signaling
	c-IAP1	#7065	Cell Signaling
	c-IAP2	#3130	Cell Signaling
	Surviving	#2802	Cell Signaling
	XIAP	#2045	Cell Signaling
Pharmacological Inhibitors	**Inhibitors**	**Concentration**	**Source**
	Vemurafenib	Indicated concentrations	Selleckchem
	Dabrafenib	Indicated concentrations	Selleckchem
	Pictilisib	Indicated concentrations	Selleckchem
	Ceritinib	Indicated concentrations	Selleckchem
	AT406	Indicated concentrations	Selleckchem
	AT101	Indicated concentrations	Selleckchem
	S63845	Indicated concentrations	Selleckchem
	Venetoclax	Indicated concentrations	Selleckchem
	Trametinib	Indicated concentrartions	Selleckchem
Expression Constructs	**Gene symbol**	**Catalogue number**	**Source**
	ALK	OHS6271-213586819	Dharmacon
	FAM150A	OHS6085-213578147	Dharmacon

	DDR1	OHS6085-213578460	Dharmacon
	VEGFR2	OHS6271-213586949	Dharmacon
	PI3KCA	13339	Addgene
shRNAs	**Gene symbol**	**shRNA#1 ID**	**shRNA#2 ID**
	ALK	RHS3979-9569182	RHS3979-9569183

TRANSPARENT METHODS

Cell culture

A375, and SKMEL-28 cell lines were obtained from American Type Culture Collection (ATCC). The SKMEL-239 and M229 cell lines (parental and vemurafenib-resistant), which were a kind gift from Drs. David Solit, Neal Rosen and Roger Lo and have been described previously (Nazarian et al., 2010; Poulikakos et al., 2011). A375 and M229 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SKMEL-28 and SKMEL-239 cells were grown in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Human phospho-RTK array analysis

Human Phospho-RTK Array Kit (R&D Systems) was used determine the relative levels of tyrosine phosphorylation for 49 distinct RTKs, according to the manufacturer's protocol. Briefly, cell lysates were prepared from parental and BRAF inhibitor-resistant A375 cells using RIPA lysis buffer (Invitrogen) containing Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich). After blocking for 1 h with Array Buffer 1 (R & D Systems, Minneapolis, MN), the arrays were incubated with 200 μg of protein lysate overnight at 4°C. Arrays were washed in Wash Buffer (R & D Systems, Minneapolis, MN), and incubated with a horseradish peroxidase–conjugated phospho-tyrosine detection antibody (1:5000) for 1 h. Finally, arrays were developed using the SuperSignal West Pico Reagent to detect changes in phosphorylation of spotted RTKs (Pierce).

Lentivirus and retrovirus preparation

ALK, FAM150A, DDR1, and VEGRF2 expressing vectors and ALK shRNAs were obtained from Open Biosystems. The vector for expression of constitutively active PI3K were purchased from Addgene (Plasmid #13339) (McFall et al., 2001). Virus particles carrying cDNA were generated by co-transfecting the shRNA plasmids and lentiviral packaging plasmids, pSPAX2, and pMD2.G, or the retroviral packaging plasmids, pCMV-Gag-Pol and pCMV-VSV-G, into 293T cells using Effectene (Qiagen), according to the supplier's instructions. Information regarding all the plasmids is provided in **Table S1.**

Generation of BRAFi vemurafenib-resistant A375 cells

BRAFi vemurafenib-resistant A375 cells were generated from A375 cells by continuously culturing them with 2 μM vemurafenib. The medium was replaced every 3 days and supplemented with fresh vemurafenib until colonies formed. To isolate the resistant cells, individual colonies were trypsinized, expanded, and subjected to the proliferation assay in the presence of vemurafenib to confirm the resistant phenotype.

RNA Isolation, Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen) and purified using RNeasy Mini Columns (Qiagen), according to the manufacturer's instructions. We generated cDNA using ProtoScript first strand cDNA synthesis kit (New England Biolabs). Then, we performed qPCR using the Power SYBR Green (Master Mix) (Life Technologies).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Santra et al., 2009). Briefly, protein extracts were prepared in the Pierce lysis buffer (Cat.No.#87788) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were estimated using Pierce BCA Protein Assay according to the manufacturer's instructions. Protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with the primary antibodies listed in **Table S1** and appropriate secondary antibodies. SuperSignal West Pico or Femto Reagent was used for detecting the proteins (Pierce).

MTT assay

We determined the relative survival of melanoma cells under various conditions using thiazolyl blue tetrazolium bromide-based assay (MTT assay, Sigma-Aldrich, USA). MTT solution in PBS (5 mg/ml) was added to each sample and incubated for 1 h to allow the formation of MTT formazan. After aspirating the medium, we reduced the resulting formazan with DMSO and measured the absorbance of each sample at a wavelength of 590 nm with a reference wavelength of 630 nm using a microplate reader (Synergy™ Mx, BioTec).

Clonogenic assay

To analyze the colony formation ability of ALK-overexpressing cells, 5×10^6 cells were plated in 10 cm cell culture dishes and cultured in the presence of 2 μM vemurafenib. The medium was replaced every 3 days and supplemented with fresh 2 μM vemurafenib. After 6 weeks, the vemurafenib-resistant colonies were visualized by staining the cell culture dishes with Coomassie

brilliant blue staining solution (50% H_2O, 40% Methanol, 10% Acetic Acid, and 0.1% Coomassie® brilliant blue R-250).

Annexin V staining

Apoptotic rate was analyzed using Annexin V staining kit (BD Pharmingen™ #556547) as per manufacturers protocol. In brief, 500,000 cells were plated in a 6 well plate format and treated with DMSO or Ceritinib. After 48hrs, cells were trypsinized, washed 2 times with 1XPBS and resuspended in 1x Binding buffer. 100,000 cells were transferred into FACS tube, 5 μl FITC Annexin V and 5 μl PI was added and incubated for 15 min. in the dark. After incubation, 400 μl of 1x binding buffer were added and analyzed using flow cytometry.

Soft-agar assay

We used soft-agar assays to analyze anchorage-independent growth. Briefly, we seeded 5×10^3 cells from each parental or resistant melanoma cell line or melanoma cell lines stably expressing the indicated expression vectors into a single well in a 6-well plate. Cells were embedded into 0.4% low-melting agarose (Sigma-Aldrich) and layered on top of a 0.8% agarose base. After 2 weeks of growth, the cells were fixed, stained with crystal-violet, and analyzed. Colony size was analyzed automatically using the ImageJ program. The number of analyzed colonies was dependent on the treatment conditions. For untreated parallels, more than 800 colonies were scored for each cell line in each replicate. For inhibitors (vemurafenib, ceritinib and AT101) at least 100 colonies per replicate were analyzed.

Mouse tumorigenesis experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University and performed in accordance with the IACUC guidelines. Athymic nude (NU/J) mice (Stock No. 002019, Jackson Laboratory) were injected subcutaneously with 5 × 10^6 A375-R melanoma cells. Vehicle, vemurafenib (30 mg/kg) or ceritinib (25 mg/kg) was administered by oral gavage every second day starting on day 7 after the injection of cells, until the end of the experiment. We measured tumor volume every 7 days and calculated volume using the formula: length × width^2 × 0.5.

Chemical inhibitors

BRAFV600E inhibitor vemurafenib, dabrafenib and MEK inhibitor trametinib were obtained from Selleckchem. Ceritinib and Pictilisib were obtained from Selleckchem. AT101, AT406, S63845, and Venetoclax were purchased from Selleckchem. The treatment concentrations and time points details are described in **Table S1** and relevant figure legends.

Statistical analysis

All of the experiments were conducted in triplicate. The results of experiments are expressed as mean ± SD. An area under the curve (AUC) was calculated to allow for the comparison between two curves and used for p-value calculations when applicable. The p-values were calculated by t-tests using GraphPad Prism version 6.0h for Macintosh, GraphPad Software, San Diego California USA (www.graphpad.com). Differences were considered when the p-value was equals to lower than 0.05.

SUPPLEMENTAL REFERENCES

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