Inventory of Supplemental Data.

SUPPLEMENTARY FIGURES

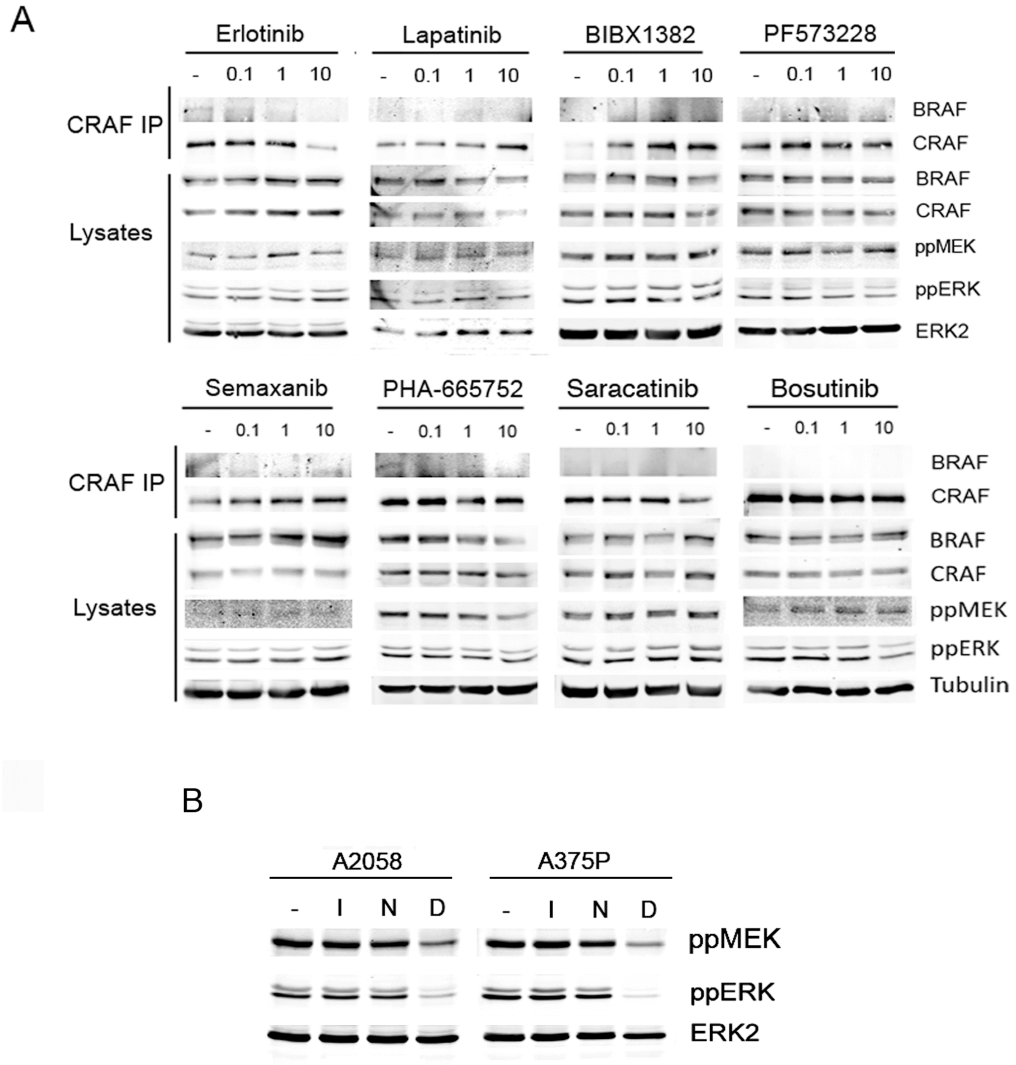

Figure S1. Examining the effects of kinase inhibitors on MEK and ERK activity in RAS and BRAF mutant cells.

Related to Figure 1.

A. Western blots for BRAF, CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK) and tubulin (loading control) in D04 cells treated with DMSO (-) or the indicated inhibitors at the concentrations indicated in μM.

B. Western blots for phospho-MEK (ppMEK), phospho-ERK (ppERK) and ERK (loading control) in A2058 and A375P cells treated with imatinib (I; 10μM), nilotinib (N; 1μM) or dasatinib (D; 5μM).

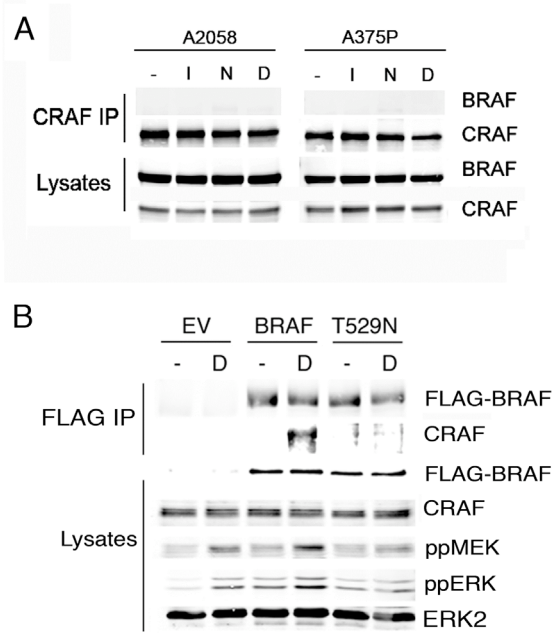

Figure S2. Drug binding to BRAF drives BRAF binding to CRAF.

Related to Figure 2.

A. Western blots for BRAF and CRAF in CRAF immunoprecipitates (CRAF IP) and cell lysates from A2058 and A375P cells treated with DMSO (-), imatinib (I; 10μM), nilotinib (N; 1μM) or dasatinib (D; 5μM).

B. Western blots for FLAG-tagged BRAF (FLAG-BRAF), endogenous CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK) and ERK2 (loading control) in FLAG immunoprecipitates (FLAG IP) and cell lysates from D04 cells stably expressing empty vector (EV), FLAG-tagged BRAF (BRAF) or FLAG-tagged ^{T529N}BRAF (T529N) and treated with DMSO (-) or dasatinib (D; 5μM).

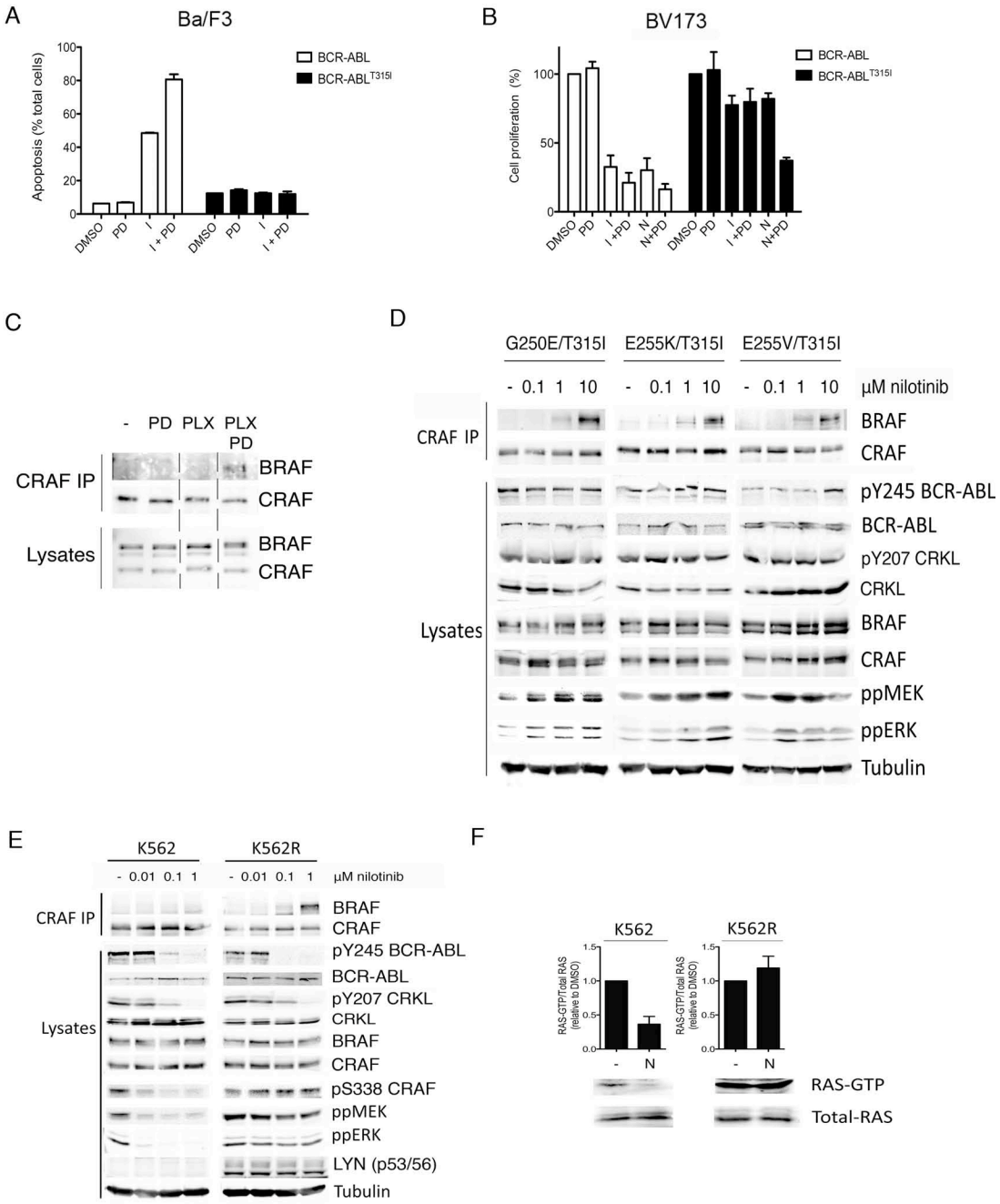

Figure S3. Nilotinib induces RAF dimers and paradoxical RAF/MEK/ERK pathway activation in a broad spectrum of drug-resistant cells.

Related to Figure 5.

A. Percentage of apoptotic cells in BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with DMSO, PD184352 (PD; 2µM), imatinib (I; 3µM) or the indicated combinations for 96 hr. Apoptosis was detected by staining cells with annexin V and propidium iodide. The mean percentage of apoptotic cells is shown, as determined by triplicate samples, along with the SD.

B. Cell proliferation was measured in BV173 (BCR-ABL) and BV173R (BCR-ABL^{T315I}) treated with PD184352 (PD; 0.3μM), imatinib (I; 3μM), nilotinib (N; 0.1μM) or the indicated combinations for 6 days. Growth, determined in triplicate, is expressed as a percentage of DMSO controls with the SD.

C. Western blots for BRAF and CRAF in CRAF immunoprecipitates (CRAF IP) and cell lysates from BCR-ABL^{T315I} Ba/F3 cells treated DMSO (-), PD184352 (PD; 2 μM) or PLX4720 (PLX; 1 μM).

D. Western blots for BRAF, CRAF, phospho-Y245 BCR-ABL (pY245 BCR-ABL), BCR-ABL, phospho-Y207 CRKL (pY207 CRKL), CRKL, phospho-S338 CRAF (pS338 CRAF), phospho-MEK (ppMEK), phospho-ERK (ppERK) and tubulin (loading control) in CRAF imunoprecipitates (CRAF IP) and cell lysates from BCR-ABL$^{G250E/T315I}$, BCR-ABL$^{E255K/T315I}$ and BCR-ABL$^{E255V/T315I}$ Ba/F3 cells treated with nilotinib at the indicated concentrations.

E. Western blots for BRAF, CRAF, phospho-Y245 BCR-ABL (pY245 BCR-ABL), BCR-ABL, phospho-Y207 CRKL (pY207 CRKL), CRKL, phospho-S338 CRAF (pS338 CRAF), phospho-MEK (ppMEK), phospho-ERK (ppERK) and ERK2 (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from K562 and K562R cells treated with nilotinib at the indicated concentrations.

F. Graph showing RAS-GTP levels, determined by RAS-capture assay in K562 and K562R treated with DMSO (-) or nilotinib (N; 1μM, 1 hr). The data are relative to the DMSO treated cells and are the mean of triplicate experiments with error bars to indicate SD. The western blot below the graph provides an example of the raw data.

SUPPLEMENTARY EXPERIMENTAL METHODS

Cell Line	**Tumour type**	**Mutation**
A375	Melanoma	$BRAF^{V600E}$
A2058	Melanoma	$BRAF^{V600E}$
D04	Melanoma	$NRAS^{Q61L}$
Panc1	Pancreatic cancer	$KRAS^{G12D}$
H460	Lung cancer	$KRAS^{Q61H}$
SW620	Colorectal carcinoma	$KRAS^{G12V}$
BV173	Leukemia	BCR-ABL
BV173R	Leukemia	$BCR\text{-}ABL^{T315I}$
K562	Leukemia	BCR-ABL
K562R	Leukemia	BCR-ABL with Lyn overexpression

Table S1. Cell lines used in this study. The cell lines used in this study are shown, together with their primary genetic aberration.

Buffer	Composition
NP40 protein extraction	50mM Tris-Cl pH 7.5, 150mM NaCl, 0.5% (v/v) Igepal, 5mM NaF, 0.2mM Na_3VO_4, 5μg/ml aprotinin, 5μg/ml leupeptin
WASH	30mM Tris-Cl pH 7.5, 0.2mM EDTA, 0.3% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 0.2mM Na_3VO_4, 1M/0.1M/no KCl
MKK	30mM Tris-Cl pH 7.5, 0.1mM EDTA, 10mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5mM NaF, 0.2mM Na_3VO_4, 0.3% (v/v) β-ME, 6.5μg/ml (~0.093 μM) GST-MEK, 400μg/ml (~6.68 μM) GST-ERK, 5mM ATP
KILL	30mM Tris-Cl pH 7.5, 9.0mM EDTA, 0.1% (v/v) Triton X-100, 5mM NaF, 0.2mM Na_3VO_4, 0.3% (v/v) β-ME
MBP	50mM Tris-Cl pH 7.5, 0.1mM EDTA, 12mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5mM NaF, 0.2mM Na_3VO_4, 0.3% (v/v) β-ME, 200μg/ml BSA, 1mg/ml MBP, 0.12MBq [γ^{32}P]-ATP (5.55 x 10^8MBq/mmol; PerkinElmer)
RAS extraction buffer	25mM Tris-Cl pH 7.5, 10% (v/v) glycerol, 100mM KCl, 5mM $MgCl_2$, 5mM NaF, 0.2mM Na_3VO_4, 1mM EDTA, 1% (v/v) Triton X-100, 0.3% (v/v) beta-mercaptoethanol, 5μg/ml aprotinin, 5μg/ml leupeptin, 1mM benzamidine.

Table S2. Composition of buffers used in this study.

Reagents. For western blotting the following antibodies were used: rabbit anti-ppMEK1/2 and rabbit anti-phospho-c-Abl (Tyr245), rabbit anti-c-Abl, rabbit anti-phospho-Crkl (Tyr207), rabbit anti-phospho-CRAF (Ser338) (Cell Signaling Technology); mouse anti-NRAS (C-20), rabbit anti-ERK2 (C-14), mouse anti-BRAF (F-7) (Santa Cruz Biotechnology); mouse anti-FLAG, mouse anti-Tubulin, and mouse anti-ppERK1/2 (Sigma); mouse anti-CRAF (for Western blotting), mouse-anti RAS and mouse anti-MEK1 (BD Transduction Laboratories). For immunoprecipitation, the following antibodies were used: rabbit anti-myc (Abcam); rabbit anti-CRAF (C-20;

Santa Cruz Biotechnology). Imatinib, nilotinib, dasatinib, erlotinib, gefitinib, lapatinib, bosutinib, sorafenib and saracatinib were obtained from LC Laboratories (Woburn, MA, USA). Semaxanib and GNF-2 were obtained from Sigma. SB590885 was obtained from Symansis (Timaru, New Zealand); L779,450, BIBX1382, PF573228 and PHA-665752 were obtained from Tocris Bioscience (Ellisville, MO, USA). RAF265 was obtained from American Custom Chemicals (San Diego, CA, USA). All drugs were prepared in DMSO. PD184352 was synthesised in-house, synthetic routes are available on request.

Cell culture techniques. Human cell lines were cultured in DMEM (A375, A2058, COS-7, Panc1, SW620, H460, K562) or RPMI (D04, BV173, K562R) supplemented with 10% fetal bovine serum. African green monkey kidney COS-7 cells were cultured in DMEM and murine Ba/F3 cells were cultured in RPMI supplemented with 10% fetal bovine serum. All cells were incubated at 37°C and 10% CO_2. For inhibitor treatment, the drugs were dissolved in DMSO and added to the medium for 3 hours, unless otherwise stated.

Cell proliferation assay. For proliferation determination, cells were seeded in 96-well plates at a density of 4,000-8,000 cells per well in 100µL medium. Cells were treated with drug in triplicate for 3-6 days. Cell growth as measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Briefly, cells were incubated with 20 µL of a 20:1 mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) for 2 hours. The reduction of MTS into a formazan product was measured at 490nm using a Spectramax M5 plate reader (Molecular Devices, Wokingham, UK).

Detection of cell death. Apoptotic cells were detected by staining with FITC-Annexin V and propidium iodide (PI) with the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen). Briefly, cells were collected by centrifugation, washed twice in cold PBS and resuspended in 100µl 1X Binding Buffer. Cells were stained with 5µl Annexin V and 5µl PI, incubated for 15 min at room temperature. Binding Buffer (400µl) was added to the cells for analyis by analysis on a BD LSRII flow cytometer. For detection of the cellular sub-G_1 fraction cells were harvested and fixed as a single cell suspension in 70% ethanol at -20°C. Cells were recovered by centrifugation,

stained with propidium iodide and subjected to analysis on a BD LSRII flow cytometer.

siRNA transfections. D04 cells were plated at a density of 2 x 10^5 cells per well of 6-well plate and the following day were transfected with lipofectamine (Invitrogen, Paisley, UK) and 10nM of the following siRNAs: BRAF1 (5'-AAA GAA TTG GAT CTG GAT CAT-3'), BRAF2 (5'-TCA GTA AGG TAC GGA GTA A-3'), CRAF1 (5'-AAG CAC GCT TAG ATT GGA ATA-3'), CRAF2 (5'-TGG GAA ATA GAA GCC AGT GAA-3'), NRAS1 (5'-GTG GTG ATG TAA CAA GAT A-3'), NRAS2 (5'-GCA CTG ACA ATC CAG CTA A-3') NRAS (5'-CAT GGC ACT GTA CTC TTC TCG-3') or scrambled control (5'-AAA CCG TCG ATT TCA CCC GGG-3'). The following day cells were split 1:2 and reseeded in 6-well plates. After another 24 hours cells were transfected again with a further 10nM of each appropriate siRNA. Cells were harvested 24 hours following this second siRNA transfection using NP40 buffer (Table 2) as previously described (Wan et al., 2004).

DNA transfections. For transient transfection of K562 cells, 2μg of DNA was mixed with 1x10^6 cells resuspended in 100μl of Nucleofection Solution V in an Amaxa-certified cuvette and transfected using programme T-016 of the Lonza Nucleofector (Lonza, Cologne AG). Cell lysates were collected 48 hours post-transfection.

For transient protein expression in D04 cells, 2μg of DNA was mixed with 4x10^6 cells resuspended in 100μl of Nucleofection Solution V and transfected as above using programme T-030. The cells were re-plated into 100mm diameter tissue culture wells and incubated for 48 hours before preparation of cell extracts.

For generation of stable lines, D04 cells were transfected using Effectene (Invitrogen) and selected in G418. Briefly, 3-4x10^5 cells were plated in 35mm diameter wells and incubated overnight. 0.4μg of DNA diluted into 100μl of DNA condensation buffer (EC) and 3.2μl enhancer were mixed vigorously and incubated for 2-5 minutes. 10μl Effectene reagent was added and the mixture was incubated for another 5-10 minutes. The cells were washed with 2ml PBS and 1.6ml fresh medium containing serum was added. The DNA complexes were diluted with 600μl of culture medium and the mixture added to the cells drop-wise. After six hours, the medium was replaced with 2ml of fresh growth medium. After 48 hours, the cells were replated into several 10cm dishes in a 10-fold dilution series and incubated in G418

(1mg/ml) for selection. The medium was refreshed weekly and after 2-3 weeks, single colonies were selected and expanded.

For transient expression in COS-7 cells, Lipofectamine (Invitrogen) was used. $2x10^5$ cells were plated into 35mm diameter wells and incubated overnight. 75 to 200ng of expression plasmid (depending on construct) was mixed with 4µl of Lipofectamine in 200µl of serum-free media and incubated for 20 minutes at room temperature. The cells were washed twice with 1ml serum-free DMEM, and then overlaid with 800µl of serum free DMEM. The DNA:Lipofectamine mix was added drop-wise to cells. Media was changed six hours later. Cell extracts were prepared two days following transfection.

Preparation of cell lysates. Culture medium was aspirated from cells and cells were placed on ice and washed three times in ice-cold PBS. The cells were scraped into 50-200µl Nonidet P40 (NP40) extraction buffer (Table S2) and incubated on ice for five minutes. The cells were sheared by passing through a pipette tip several times and the samples were centrifuged at 20,000 *g* for 5 minutes at 4°C and the soluble fraction was harvested.

Expression constructs. The expression vectors for myc-BRAF (pEFm-BRAF), HA-BRAF (pEF-HA-BRAF), myc-CRAF (pEFm-CRAF), HA-CRAF (pEF-HA-CRAF), myc-$BRAF^{T529N}$, myc-$BRAF^{R188L}$, myc-$CRAF^{T421N}$, myc-$CRAF^{R89L}$, FLAG-BRAF (pMCEF-FLAG-BRAF), FLAG-$BRAF^{T529N}$, FLAG-CRAF (pMCEF-FLAG-CRAF), FLAG-$CRAF^{T421N}$, $HRAS^{G12V}$, $HRAS^{S17N}$ and Src^{Y527F} have been described (Heidorn et al., 2010; Marais et al., 1995; Marais et al., 1998). Briefly, the vector backbone is pUC19 and the elongation factor 1a (EF1a) promoter is used to drive exogenous protein expression. The vector includes the first intron from human EF1α to assist mRNA processing during expression. The b-globin 5' and 3' untranslated regions (UTRs) are used to provide a strong translation start site (5' UTR), and also to provide mRNA stability and a poly adenylation signal (3' UTR). The vector introduces a variety of amino-terminal monoclonal antibody epitope tags onto the expressed protein (myc tag: EQKLISEEDL; FLAG tag: DYKDDDKGS; HA tag YPYDVPDYA). The BRAF coding region includes the alternatively spliced exons 1 and 2 but not exons 8b or 10 and various modifications were introduced to provide additional restriction sites (without changing the amino acid sequence) and alterations to the 3'-UTR to allow easier manipulation of this construct. Standard

PCR-directed mutagenesis approaches were used to generate the various mutations used in the study and all mutations were verified by automated dideoxy sequencing. The expression vector pMCEF-FLAG-CRAF uses the same expression cassette, but the backbone also possesses a neo resistance cassette to facilitate selection in the presence of G418.

RAF co-immunoprecipitations. Immunoprecipitations were performed in 300µl cell lysates from one 35mm diameter well for endogenous protein or from 2-3 wells for transfected protein. Endogenous CRAF was immunoprecipitated with 5µg CRAF C-20 and myc-tagged BRAF and CRAF with 2µg rabbit anti-myc antibody (Abcam). The antibody-protein complex was captured using 20µl of a 1:1 Protein G sepharose 4B beads (Sigma-Aldrich) mixture in NP40 lysis buffer (Table S2) and immunoprecipitates (IPs) were mixed for 2 hours at 4°C on a rotation wheel. Thereafter, the IPs were washed three times with 300µl of NP40 lysis buffer (Table S2) before analysis on standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Specific bands were detected using fluorescent-labelled secondary antibodies (Invitrogen; Li-COR Biosciences) and analyzed using an Odyssey Infrared Scanner (Li-COR Biosciences).

RAF kinase assays. The in vitro kinase activity of endogenous RAF proteins or myc-tagged RAF proteins transiently expressed in COS-7 cells was measured using a coupled kinase cascade assay with GST-MEK, GST-ERK and myelin basic protein (MBP) (Sigma-Aldrich) as sequential substrates. ERK activation is quantified by measuring the incorporation of [^{32}P]-orthophosphate (PerkinElmer) into MBP. For measurement of endogenous BRAF kinase activity, D04 or Ba/F3 cells were harvested in 300µl of NP40 buffer (Table S2) as described above. Protein concentrations were determined and equal amounts of protein were immunoprecipitated as described above.

For measurement of mutant BRAF kinase activities, COS-7 cells were transiently transfected with myc-tagged BRAF and cells in one 35mm diameter well were harvested in 200µl of NP40 buffer (Table S2). The relative concentrations of exogenously expressed RAF in these cell lysates were determined by quantitative western blotting using the myc antibody (Cell Signalling Technology) specified above. Bands were quantified using the Odyssey infrared imaging system (LI-COR

Biosciences). Equivalent amounts of RAF were immunoprecipitated using rabbit myc antibody (Abcam) as specified above.

Endogenous and transiently transfected RAF proteins were immunoprecipitated in a total of 300μl NP40 buffer (Table S2) for 2 hours at 4^{o}C and immunoprecipitates were washed sequentially three times with chilled wash buffer (Table S2) containing decreasing concentrations of KCl (1M KCl, 0.1M KCl and no KCl). The first-step reaction was initiated by addition of 20μl MKK buffer (containing GST-MEK and GST-ERK; Table S2) to the beads and incubated at 30^{o}C for 10 minutes in the case of myc-tagged BRAF or 30 minutes for endogenous BRAF and CRAF. Reactions were terminated by the addition of 20μl KILL buffer (Table S2), which contains EDTA to chelate Mg^{2+} ions and inhibit kinase activity. The reaction supernatants were collected from the beads and transferred into fresh tubes for the second step reaction. 5μl of supernatant was incubated with 25μl MBP buffer containing [γ-^{32}P]ATP (PerkinElmer) for ten minutes at 30^{o}C in triplicate to measure ERK activity. The second reaction was terminated by spotting 20μl of reaction mix onto a 1cm^2 piece of P81 paper (VWR International), which was then dropped into 400ml 25mM orthophosphate solution. The papers were washed three times in 400ml 25mM orthophosphate solution to remove the unincorporated ATP and the [^{32}P]-orthophosphate incorporated into MBP was determined using Cerenkov counting. For transfected samples, the background counts were determined using lysates of cells transfected with the empty vector. For endogenous protein, samples in which no RAF was immunoprecipitated were used. Background values were removed and to ensure linearity, assays were used at below 50% saturation. To determine $BRAF^{WT}$, $BRAF^{T529N}$, $BRAF^{V600E}$ and CRAF sensitivity to imatinib, nilotinib and dasatinib, immunoprecipitated BRAF was pre-incubated with drug in KCl-free buffer for 30 minutes at room temperature prior to the first-step reaction.

RAS activity. Cells were treated with 1μM nilotinib for the times indicated. Cells were harvested in RAS extraction buffer (Table S2). 10% of protein extract was collected to measure the total RAS expression level. The remainder of the lysate was incubated with 25μl GST-RBD (RAF1 RAS binding domain) crude bacterial extract and 20μl glutathione-sepharose beads (Amersham Biosciences) for 1-2 hours at 4°C. Beads were washed 3 times with RAS extraction buffer and then separated on 12.5%

SDS-PAGE. Blots were probed with RAS (BD Transduction Laboratories) antibody at 1:50 dilution.

Kinase profiling. IC50 determinations were performed using commercial Z-lyte technology (Invitrogen) at 100uM ATP.

Cell viability assays for primary cells. Clinical samples were obtained following informed consent and protocol approval by the Oregon Health & Science University Institutional Review Board. Mononuclear cells from newly diagnosed and imatinib-resistant CML patients harboring native BCR-ABL or BCR-ABL^{T315I}, respectively (n=2/group), were isolated from bone marrow or peripheral blood by Ficoll centrifugation (GE Healthcare). Bone marrow CD34+ cells from healthy individuals (n=2; purchased from Lonza, Inc.) were included as controls. Cells were distributed in 96-well plates (4 x 10^4 cells/well) in the presence of PD184352 (0.3 μM) and nilotinib (1 μ M) alone or in combination in serum-free IMDM media (Invitrogen) supplemented with 20% BIT 9500 serum substitute (Stemcell Technologies), 40 μ g/mL human low-density lipoprotein (Sigma-Aldrich), and 100 μM β -mercaptoethanol. After 96 hours, cell viability was assessed by methanethiosulfonate (MTS)-based assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega). All treatment conditions were performed in quadruplicate; values are normalized to those of DMSO-treated control wells and represent the mean ± SEM.

Supplementary References

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