Therapy-induced tumour secretomes promote resistance and tumour progression

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Drug resistance invariably limits the clinical efficacy of targeted therapy with kinase inhibitors against cancer1,2. Here we show that targeted therapy with BRAF, ALK or EGFR kinase inhibitors induces a complex network of secreted signals in drug-stressed human and mouse melanoma and human lung adenocarcinoma cells. This therapy-induced secretome stimulates the outgrowth, dissemination and metastasis of drug-resistant cancer cell clones and supports the survival of drug-sensitive cancer cells, contributing to incomplete tumour regression. The tumour-promoting secretome of melanoma cells treated with the kinase inhibitor vemurafenib is driven by down-regulation of the transcription factor Fra1. In situ transcriptome analysis of drug-resistant melanoma cells responding to the regressing tumour microenvironment revealed hyperactivation of several signalling pathways, most prominently the AKT pathway. Dual inhibition of RAF and the PI3K/AKT/mTOR intracellular signalling pathways blunted the outgrowth of the drug-resistant cell population in BRAF mutant human melanoma, suggesting this combination therapy as a strategy against tumour relapse. Thus, therapeutic inhibition of oncogenic drivers induces vast secretome changes in drug-sensitive cancer cells, paradoxically establishing a tumour micro-environment that supports the expansion of drug-resistant clones, but is susceptible to combination therapy.

Kinase inhibitors such as vemurafenib, erlotinib or crizotinib have shown clinical efficacy in melanoma with BRAF mutations, or in lung adenocarcinoma with EGFR mutations or ALK translocations, respectively3–6. Although complete responses are rare, the vast majority of patients show partial tumour regression or disease stabilization. However, dual inhibition of RAF and the PI3K/AKT/mTOR intracellular signalling pathways blunted the outgrowth of the drug-resistant cell population in BRAF mutant human melanoma, suggesting this combination therapy as a strategy against tumour relapse. Thus, therapeutic inhibition of oncogenic drivers induces vast secretome changes in drug-sensitive cancer cells, paradoxically establishing a tumour micro-environment that supports the expansion of drug-resistant clones, but is susceptible to combination therapy.

Circulating tumour cells can infiltrate and colonize tumours. This phenomenon, termed self-seeding20, may contribute to the distribution of resistant clones to several metastatic sites. Mice implanted with sensitive A375 tumours were treated with vehicle or vemurafenib, and intracardially injected with TGL-labelled A375R cells (Fig. 1g). A375R cells were more efficiently attracted to vemurafenib-treated regressing tumours compared to vehicle-treated controls, with 95% (21 out of 22) and 12.5% (2 out of 16) efficiency, respectively, exhibiting substantial accumulation of resistant cells in regressing tumours by day 5 (Fig. 1g and Extended Data Fig. 1n). To evaluate the contribution of seeding by resistant circulating tumour cells to disease relapse, we intracardially injected resistant A375R cells or vehicle into tumour-bearing mice and compared effects of vemurafenib on cancer or stromal cells.
the tumour volume during vemurafenib treatment (Fig. 1h). Whereas the unseeded tumours in the control group showed extensive tumour regression, seeding by A375R cells led to rapid tumour relapse (Fig. 1h). These results suggest that tumours regressing on targeted therapy are potent attractors of resistant circulating tumour cells that may contribute to rapid tumour progression.

Tumours consist of a complex microenvironment composed of immune, stromal and cancer cells. Soluble mediators from this microenvironment can foster cancer growth and therapy resistance. Considering that drug-sensitive cancer cells are the main population affected by targeted therapy, we proposed that signals derived from sensitive cancer cells in response to kinase inhibitors drive the outgrowth of drug-resistant cells. To test this hypothesis, we established an in vitro co-culture system and monitored the growth of TGL-expressing resistant cells (A375R, H2030) in the absence or presence of sensitive cells treated with kinase inhibitors or vehicle (Fig. 2a). Mimicking our in vivo findings, co-culture with vemurafenib-, crizotinib- or erlotinib-treated sensitive cells significantly enhanced the growth of resistant cancer cells (Fig. 2a and Extended Data Fig. 2a–c).

We derived conditioned media (CM) from vemurafenib-sensitive melanoma cells cultured in the absence (CM-vehicle) or presence of vemurafenib (CM-vemurafenib). CM-vemurafenib accelerated the proliferation of drug-resistant cells, with different clinically relevant resistance mechanisms, as determined by cell viability assays and Ki67 staining (Fig. 2b and Extended Data Fig. 2d–f). Similarly, conditioned media from crizotinib- or erlotinib-treated sensitive lung adenocarcinoma cells stimulated proliferation of lung adenocarcinoma cells with intrinsic or acquired resistance (Fig. 2c) and across different cell lines (Extended Data Fig. 2g). In addition, CM-vemurafenib elicited increased cell migration in transwell migration and monolayer gap-closing assays (Fig. 2d and Extended Data Fig. 2h–k). CM-vemurafenib was also active on vemurafenib-sensitive cancer cells, increasing survival and suppressing the apoptotic caspase activity up to 100-fold in these cells when treated with vemurafenib in vitro (Fig. 2e, f). Because all biologically active conditioned media was collected before cell death or senescence, it is likely that the secretome is actively produced as a result of oncogene inhibition (Extended Data Fig. 2l, m). These results demonstrate that BRAF, ALK and EGFR mutant cells respond to therapeutic stress under targeted therapy by secreting factors that support the survival of drug-sensitive cells and accelerate the growth of drug-resistant minority clones. The effects of this reactive secretome may augment previously reported resistance mechanisms including relief of feedback inhibition of intracellular signalling, upregulation of receptor tyrosine kinases, or the supply of stromal cytokines that protect the drug-sensitive cells.

To identify relevant components and regulators of the reactive secretome, we analysed gene expression changes in sensitive A375 melanoma cells at different time points after vemurafenib exposure (Fig. 3a, b, Extended Data Fig. 3a, b and Supplementary Table 1). After 48 h, more than one-third of the transcriptome was differentially expressed (FDR < 0.05, 405 genes encoding for proteins in the extracellular region, Gene Ontology (GO) accession 0005576), significantly overlapping with the gene expression changes of A375
Figure 2 | The secretome of RAF and ALK inhibitor-treated tumour cells increases proliferation and migration of drug-resistant cells and supports the survival of drug-sensitive cells. 

a. Schematic (left) and representative BLI images (right) after 7 days of co-culture. Average fold change (FC) of BLI signal from A375β-TGL cells in vemurafenib-treated wells relative to vehicle-treated control wells is depicted on the right (n = 4 biological replicates). 
b, c. Conditioned media (CM) was derived from drug-sensitive cells, treated with vehicle, vemurafenib or crizotinib. Drug-resistant cells were grown in conditioned media as indicated. n = 3 (b) and 6 (c) biological replicates. 
d. Schematic diagram of the migration assay (top) and relative migration of A375β cells towards conditioned media from different sources as indicated (bottom, n = 10 fields of vision (FOV)). **P < 0.0001, two-tailed Mann–Whitney U test. 
e. Survival assay of drug-sensitive A375 cells cultured in conditioned media and treated with vemurafenib, assessed on day 3 (n = 3 biological replicates). 
f. Apoptosis rate of A375 cells cultured in conditioned media and treated with vemurafenib (3 μM) (n = 3 biological replicates). 
Data are mean and s.e.m.

Figure 3 | FRA1 downregulation during RAFi treatment drives the reactive secretome. 

a. Principal component (PC) analysis of drug-sensitive A375 cells treated in vitro with vehicle or vemurafenib for 6 or 48 h. 
b. Volcano plots show genes significantly deregulated by vemurafenib treatment after 6 h (left) or 48 h (right). Transcription factors (TF) and gene products in the extracellular region are depicted in green (downregulated) and red (upregulated) (n = 3 tumours). 
c. Relative mRNA levels of FRA1 during vemurafenib exposure [0.1–1 μM]. 
d. Representative immunofluorescence staining of A375/A375β tumours for GFP (A375β, green) and FRA1 (red) after vehicle or vemurafenib treatment (5 days). DAPI, 4′,6-diamidino-2-phenylindole. Scale bars, 50 μm. 
e. Top, representative immunofluorescence staining for FRA1 (red) of melanoma biopsy sections of patient 1. Original magnification, ×20. Bottom, nuclear FRA1 staining was quantified in three melanoma patients before (B) and early-on therapy. RAFi and MEKi denote RAF and MEK inhibitors, respectively. 
f. Bioluminescent signal of A375β-TGL cells 6 days after subcutaneous co-implantation with A375 cells expressing control (shCtrl) or two independent shRNAs for FRA1 (shFRA1-1 and shFRA1-2) (n = 16 tumours). g. Seeding of A375β-TGL cells to unlabelled tumours expressing control or two independent shRNAs for FRA1, determined by BLI (vehicle, n = 10; shFRA1-1, n = 10; shFRA1-2, n = 8 tumours). 
Data are mean and s.e.m. *P < 0.05, **P < 0.01, ****P < 0.0001, Student’s t-test.
The therapy-induced secretome in melanoma promotes relapse

Figure 4 | The therapy-induced secretome in melanoma promotes relapse by activating the AKT pathway in resistant cells. a, Schematic diagram showing the isolation of polysome-associated transcripts from resistant cells by translating ribosome affinity profiling (TRAP) from tumours during treatment. IP, immunoprecipitation. b, Ingenuity upstream regulator analysis of gene expression profiles from A375R cells responding to a regressing tumour lysate. c, Tumour lysate IP RNA-seq normalized photon flux of A375R cells showed significant overlap between the secretome of melanoma and lung adenocarcinoma cells (P < 9.11 × 10⁻⁵) (Extended Data Fig. 3e–h and Supplementary Table 1). Furthermore, changes in the secretome of vemurafenib-sensitive melanoma cells coincided with changes in the immune cell composition (Extended Data Fig. 4a, b), and with changes of soluble mediators derived from murine stromal cells such as IFG1 and HGF (Extended Data Fig. 4c, d). These data indicate a therapy-induced secretome (TIS), a response that consists of many up- and downregulated secreted factors, permeates the regressing tumour microenvironment and stimulates cancer cells, probably also stromal cells.

To identify molecular drivers of the A375-TIS in response to vemurafenib, we integrated the data of differentially expressed transcription factors after 6 h of vemurafenib treatment with the transcription factor binding motifs that were enriched at the promoters of differentially expressed genes in the secretome after 48 h (Fig. 3a, b). This analysis highlighted FRA1 (also known FOSL1), a member of the AP1 transcription factor complex and effector of the ERK pathway, as one of the putative upstream regulators of the TIS (Extended Data Fig. 5a). FRA1 was downregulated in all drug-sensitive cells, but not in resistant cells, treated with vemurafenib, crizotinib and erlotinib (Fig. 3c, d and Extended Data Fig. 5b–d). Biopsies from melanoma patients early during RAFi treatment confirmed RAFi-induced FRA1 downregulation in clinical samples (Fig. 3e, Extended Data Fig. 5e and Extended Data Table 1).

To test the functional role of FRA1 in modulating the TIS, we used RNA interference (RNAi) to inhibit FRA1 expression. Co-culture and conditioned media assays using A375 cells expressing short hairpin RNAs targeting FRA1 (shFRA1) showed similar growth-accelerating and chemotactic activity on A375R cells as vemurafenib treatment (Extended Data Fig. 6a–d). In line with these results, FRA1 knockdown in A375 cells induced transcriptional changes similar to those induced by vemurafenib (Extended Data Fig. 6e). A375R cells co-implemented with A375 or UACC62 cells expressing shFRA1 also demonstrated increased growth in vivo (Fig. 3f and Extended Data Fig. 6f). A375-shFRA1 tumours attracted significantly more resistant cells from the circulation than tumours expressing the control vector (Fig. 3g). Thus, FRA1 downregulation drives the induction of the tumour-promoting secretome of vemurafenib-treated cancer cells.

To determine the effect of the reactive secretome on the drug-resistant tumour subpopulation in a regressing tumour, we expressed the ribosomal protein L10a (RPL10a) fused to enhanced green fluorescent protein (eGFP–RPL10a) in A375R cells, allowing the specific retrieval of transcripts from A375R cells by polisome immunoprecipitation for subsequent RNA-sequencing (RNA-seq) analysis (Fig. 4a). In line with the in vivo phenotype of accelerated growth, the gene expression pattern of resistant cells in the regressing microenvironment was enriched for biological processes involved in cell viability, proliferation and cell movement (Extended Data Fig. 7a). Pathway analysis of the expression data suggested activation of several pathways including PI(3)K/AKT, BMP-SMAD and NFKB (Fig. 4b). The hyperactivity of the PI(3)K/AKT pathway in this context also suggested a potential vulnerability of the cells to PI(3)K/mTOR inhibitors (Extended Data Fig. 7b). The pathway-analysis-based prediction of PI(3)K/AKT activation was also reflected at the protein level in both resistant and sensitive cells in the presence of CM-vemurafenib in vitro and under vemurafenib treatment in vivo (Fig. 4c and Extended Data Fig. 7c, d). Moreover, PI(3)K/AKT emerged as the dominant TIS responsive pathway in a targeted immunoblot analysis of survival pathways in vitro (Extended Data Fig. 7e).

**Figure 4** | The therapy-induced secretome in melanoma promotes relapse by activating the AKT pathway in resistant cells. a, Schematic diagram showing the isolation of polysome-associated transcripts from resistant cells by translating ribosome affinity profiling (TRAP) from tumours during treatment. IP, immunoprecipitation. b, Ingenuity upstream regulator analysis of gene expression profiles from A375R cells responding to a regressing tumour microenvironment (5 days of treatment; n = 3 tumours). c, Phosphorylation status of AKT (pAKT) in A375R cells, stimulated for 15 min with various conditioned media, as indicated by immunoblotting. tAKT, total AKT. d, Phosphorylation status of AKT in A375R cells after stimulation with positive regulators of the AKT pathway, upregulated in the melanoma TIS: ANGPTL7 (5 µg ml⁻¹, 30 min; upregulated in A375, Colo800, UACC62), PDGFD (10 ng ml⁻¹, 10 min; upregulated in Colo800), EGF (10 ng ml⁻¹, 10 min; upregulated in A375) and IGF1 (10 ng ml⁻¹, 10 min; upregulated in UACC62), e, Mice bearing A375/A375-TG tumours were treated with drugs, and growth of A375R cells was followed by BLI (vehicle, n = 14; vemurafenib, n = 16; vemurafenib and BEZ235, n = 16; vemurafenib and MK2206, n = 8 tumours). f, Graphical summary of the findings. Data are mean and s.e.m. P values calculated using a two-tailed Mann–Whitney U test.

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The TIS contained many mediators directly or indirectly activating the AKT pathway. Positive mediators that were upregulated during therapy included IGF1, EGF, ANGPTL7 and PDGF, each of which activated the AKT pathway in vitro (Fig. 4d). IGF1, one of the most potent activators of the AKT pathway, is also abundantly expressed in the tumour stroma and is further upregulated during targeted therapy (Extended Data Figs 4c and 7f). In addition, levels of IGFBP3, a negative regulator of IGF1, were markedly reduced in the TIS of all investigated cell lines, favouring increased AKT pathway activation in the presence of IGF1 and stimulation of proliferation of resistant cells in vivo (Extended Data Fig. 7f–k).

To test the role of AKT activation as a mediator of TIS-induced tumour proliferation, we combined vemurafenib with AKT/Pi(3)K/mTOR inhibitors. In co-culture and proliferation experiments using conditioned media, dual inhibition of the MAPK and AKT pathway diminished the growth benefit of the TIS (Extended Data Fig. 8a, b). We then treated mice with A375/A375R or A375R tumours with vemurafenib and AKT (MK2206) or Pi(3)K/mTOR inhibitors (BEZ235). The combined inhibition of MAPK and Pi(3)K/AKT/mTOR pathways significantly blunted the outgrowth of vemurafenib-resistant cells in the A375/ A375R tumours (Fig. 4e). This inhibition was specific for the amplified proliferation in the regressing tumour microenvironment and had no effects on the growth of resistant cells alone (Extended Data Fig. 8c). Furthermore, the outgrowth of resistant A375R cells in tumour seeding assays was significantly reduced when regressing tumours were co-treated with BEZ235 (Extended Data Fig. 8d). Thus, the TIS-induced proliferation is susceptible to therapeutic targeting.

The limited effectiveness of targeted therapies has been attributed to intracellular feedback loops and specific cytokines that support the survival of drug-sensitive cells. From these residual tumours, clones emerge that are intrinsically resistant to targeted therapy and are ultimately responsible for clinical relapse. Our work demonstrates that targeted inhibition of a cancer driver pathway can paradoxically promote these two aspects of drug resistance via induction of a complex, reactive secretome. This TIS not only enhances the survival of drug-sensitive cells, but also acutely accelerates the expansion and dissemination of drug-resistant clones. Rather than a cell death by-product, the TIS-induced proliferation is susceptible to therapeutic targeting.

Online Content Methods, along with any additional Extended Data display items, and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.
METHODS
Cell culture. A375, M249 (ref. 8) and B16 cells were cultured in DMEM media; Colo800, UACC62, SKMEL239-droneX, LOX, PKH, H2030, H3122 and HCC827 cells were cultured in RPMI media. YUMM1.1 and YUMM1.7 were cultured in DMEM/F12 media. GFP29 and 29T3 cells were used for retrovirus and lentivirus production, respectively. Both were maintained in DMEM media. All media contained 10% FBS, 2 mM L-glutamine, 100 IU ml\(^{-1}\) penicillin/streptomycin and 1 \(\mu\)g ml\(^{-1}\) amphotericin B, the media for GFP29 contained in addition 0.3 mg ml\(^{-1}\) G418, 20 \(\mu\)g ml\(^{-1}\) doxycycline and 2 \(\mu\)g ml\(^{-1}\) puromycin. All cells were grown in a humidified incubator at 37 °C with 5% CO\(_2\) and were tested regularly for mycoplasma contamination. All cell lines used were negative for mycoplasma.

To generate vemurafenib-resistant melanoma cell lines, vemurafenib-sensitive cell lines were seeded at low density and exposed to 1–3 \(\mu\)M vemurafenib (LC-Labs). After around 6–7 days, cells were analysed by flow cytometry for drug resistance (Extended Data Fig. 9a–j). Vemurafenib-resistant cell lines were selected by exposure to 1–3 \(\mu\)M vemurafenib for 1 month in several independent experiments. 

The media was then replaced by low serum media containing vehicle or vemurafenib (0.1 \(\mu\)M) and cells were grown until confluence. A tip was used to generate a gap, cells were washed and conditioned media was added. Images were acquired over time to monitor for cap formation in the vehicle-treated dish at time of conditioned media collection.

To generate crizotinib-resistant melanoma cell lines, crizotinib-sensitive cell lines were seeded at low density and exposed to 1–3 \(\mu\)M crizotinib (LC-Labs). After around a week, cells were analysed by flow cytometry for drug resistance. Vemurafenib-resistant cell lines were selected by exposure to 1–3 \(\mu\)M vemurafenib for 1 month in several independent experiments. 

The media was then replaced by low serum media containing vehicle or vemurafenib (0.1 \(\mu\)M) and crizotinib (0.1 \(\mu\)M) and cells were grown until confluence. A tip was used to generate a gap, cells were washed and conditioned media was added. Images were acquired over time to monitor for cap formation in the vehicle-treated dish at time of conditioned media collection.

To generate crizotinib-resistant melanoma cell lines, crizotinib-sensitive cell lines were seeded at low density and exposed to 1–3 \(\mu\)M vemurafenib (0.1 \(\mu\)M) and crizotinib (0.1 \(\mu\)M) and cells were grown until confluence. A tip was used to generate a gap, cells were washed and conditioned media was added. Images were acquired over time to monitor for cap formation in the vehicle-treated dish at time of conditioned media collection.

xCELLigence migration assay. Experiments were performed using the xCELLigence RTCA DP instrument (Roche Diagnostics GmbH) placed in a humidified incubator at 37 °C with 5% CO\(_2\). Cell migration experiments were performed using modified 16-well plates (CIM-16, Roche Diagnostics GmbH) according to the manufacturer’s instructions. The experiment was performed twice. A representative experiment is shown.

Animal studies. All experiments using animals were performed in accordance to our protocol approved by MSKCC’s Institutional Animal Care and Use Committee (IACUC). 5–7-week-old, female NOD-SCID-NOG (NCR) or NOD-SCID NCR-NU (NCI) mice were used for animal experiments with human cell lines. Primary YUMM1.1 and YUMM1.7 cell lines were isolated from melanomas developed in mice (Tyraseer; Braf^CA; Cdkn2a^−/− Pten^lox/lox^) treated with 4-hydroxytamoxifen and were subsequently implanted in female C57BL/6J (JAX) mice aged between 5 and 7 weeks. Tumour formation, outgrowth and metastasis were monitored by BLI of TGL-labelled tumour cells as described previously\(^{12}\). In brief, anesthetized mice (150 mg kg\(^{-1}\)) ketamine, 15 mg kg\(^{-1}\) xylazine or isoflurane) were injected retro-orbitally with r-luciferin (150 mg kg\(^{-1}\)) and imaged with an IVIS Spectrum Xenogen machine (Caliper Life Sciences). Bioluminescence analysis was performed using Living Image software, version 4.4. For co-implantation experiments, mice were anesthetized (150 mg kg\(^{-1}\) ketamine, 15 mg kg\(^{-1}\) xylazine) and 1 \(\times\) 10\(^5\) TGL-labelled resistant tumour cells were injected subcutaneously with 2 \(\times\) 10\(^5\) sensitive tumour cells in 50 ml growth-factor-reduced Matrigel/PBS (1:1) (BD Biosciences). For the control groups in which the effects of drug treatment on resistant cells alone were tested, 2 \(\times\) 10\(^5\) resistant cells were injected in growth-factor-reduced Matrigel/PBS. Two-to-four sites on the flanks were injected per mouse. After tumours reached a size of 50–150 mm\(^3\), the BLI signal of resistant cells was determined. To compensate for minor growth differences of the GFP\(^{-}\) resistant cell population between mice, the mice were assigned to the cohorts so that the overall BLI intensity (and consequently the cell number) was equal in the treatment and control groups. Each group received vehicle or drug treatment as indicated (vemurafenib/PLX4032, 25 mg kg\(^{-1}\) twice daily for YUMM1.1 and YUMM1.7 tumours, and 75 mg kg\(^{-1}\) twice daily for all other RAF mutant tumours, LC-Labs or Selleckchem; 100 mg kg\(^{-1}\) crizotinib once daily, LC-Labs; 50 mg kg\(^{-1}\) erlotinib once daily, LC-Labs; 100 mg kg\(^{-1}\) MK-2206 once daily, Chemietek; 50 mg kg\(^{-1}\) BEZ235 once daily, LC-Labs). Growth of the resistant population in the different groups was monitored by BLI, quantified and normalized to BLI signal at start of treatment. Tumour seeding and metastasis assays were performed as described with minor modifications\(^{12}\). In brief, sensitive tumour cells were injected subcutaneously on two sites per mouse. Once tumours were established (50–150 mm\(^3\)) mice were treated with vehicle or vemurafenib (75 mg kg\(^{-1}\) twice daily for 3 days, and 1 \(\times\) 10\(^5\) TGL-labelled drug-resistant cells were injected in the left cardiac ventricle. Treatment was continued, and metastatic burden and tumour seeding were determined in vivo and ex vivo by BLI. Tumour volume was determined using caliper measurements and calculated using the following formula: tumour volume = (D \(\times\) d\(^2\))/2, in which D and d refer to the long and short tumour diameter, respectively. All experiments with A375 cells were independently performed at least three times, except animal experiments in Fig. 3, which were performed twice. All other animal experiments were independently performed at least twice. Representative experiments are shown, except where noted and where instead the average of three experiments is shown.

Gene expression analysis. Whole RNA was isolated from cells using RNAeasy Mini Kit (QIAGEN). The Transcriptor First Strand cDNA synthesis kit (Roche) was used to generate cDNA. Differential RNA levels were assessed using Taqman gene expression assays (Life technologies). Assays used for human genes are: Hs04187685_g1, Hs00365742, Hs00605382, Hs0061975, Hs01099999, Hs00990100, Hs01029057, Hs00234244, Hs0095117, Hs01008482, Hs00989733, Hs00234140, Hs01019559, Hs01027691, Hs00999141, Hs01117294, Mm00607939_m1, Mm0047999995 and Mm004207958. Relative gene expression was normalized to internal control genes: Hs01035697_m1, GAPDH (Hs00224512_m1) and ACTB (Mm00474004_m1). Quantitative PCR reactions were performed on an ABI7500 RT-PCR system and analysed using VILIA software (Life Technologies). All data points represent at least four technical replicates and experiments were performed independently three times. A representative experiment is shown.

Cancer-cell-specific TRAP and sequencing. To investigate the gene expression changes specifically of drug-sensitive tumours during vemurafenib treatment, or gene expression changes of resistant cells exposed to a regressing tumour microenvironment, A375 and A375\(^{5}\) cells, respectively, were modified to express eGFP, RPL10a. Tumours derived from implanted A375-eGFP-RPL10a and A375\(^{5}\)-eGFP-RPL10a cells were homogenized and processed with the TRAP protocol as previously described\(^{17}\) with the following modifications: fresh tumour was homogenized with a Model PRO 200 homogenizer at speed 5 for four cycles of 15 s, RNAsin Plus RNase inhibitor (Promega, N2615) was used as RNase inhibitor, and anti-eGFP antibody coated sepharose beads (GE Healthcare) were used for immunoprecipitation. Polyosome-associated RNA was purified with RNAqueous micro kit (Life Technologies).
Technologies, AM1391). Ribogreen and the Agilent BioAnalyzer technologies were used to quantify and control the quality of RNA; 500 ng RNA (RNA integrity number (RIN) > 8.5) from each sample was used for library construction with TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer’s instructions. The samples were barcoded and run on a HiSeq 2000 platform in a 50-base-pair (bp)/50-bp or 75-bp/75-bp paired-end run, using the TruSeq SBS Kit v3 (Illumina). An average of 40 million paired reads was generated per sample.

RNA-seq analysis. For drug-sensitive A375, Colo800, UACC63 and H3122 cells, in vitro, raw paired-end sequencing reads were mapped to the human genome (build hg19) with STAR 2.0.3e (ref. 34) using standard options. Uniquely mapped reads were counted for each gene using HTSeq v0.5.4 (ref. 35) with default settings. Read counts of each sample were normalized by library size using the "DESeq"35 package of Bioconductor. Differential gene expression analysis between any two conditions was performed based on a model using the negative binomial distribution36. Genes with false discovery rate (FDR) < 0.05, fold change larger than 1.5 or smaller than 0.667-fold, and average read counts larger than 10 were treated as differentially expressed genes. RNA-seq data from in vivo xenograft TRAP samples were processed with the following modifications to avoid potential mRNA contamination from host mouse tissue: raw sequencing reads were mapped to a hybrid genome consisting indexes of both human (build hg19) and mouse (build mm9) genomes. Only reads that uniquely mapped to human genome indexes were preserved and counted using HTSeq v0.5.4 (ref. 35).

Bioinformatics analysis. Heatmap visualization of data matrices was performed using the ‘ggplot’ package of R. Principle component analysis of RNA-seq results was performed with the variance stabilizing transformation methods in ‘DESeq’ package of Bioconductor and the first two principal components were plotted. Volcano plots were derived from ‘DESeq’-based differential gene expression analysis. Differentially expressed genes with transcription factor activity (GO:0030706) at 6 h of vemurafenib treatment and gene products located in the extracellular region (GO:00005576) at 48 h of vemurafenib treatment were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID)36 v6.7 (http://david.abcc.ncifcrf.gov/) and enriched GO terms were visualized using REVIGO37 (http://revigo.irb.hr). Enriched transcriptional regulators for the list of differentially expressed gene products in the extracellular region were predicted with DAVID v6.7 and this list compared to the gene expression levels of transcription factors after 6 h of vemurafenib treatment in A375 cells. Upstream regulators, functions associated with the gene expression profile and potential drug vulnerabilities were determined by interpretative phenomenological analysis (IPA) analysis on differentially expressed genes from A375-eGFP-RPL10a cells in different tumour microenvironments in vivo.

Immunoblotting. RIPA buffer (Cell Signaling) was used for cell lysis, according to the manufacturer’s instructions, and the protein concentrations were determined by BCA Protein Assay Kit (Pierce). Proteins were separated by SDS-PAGE using Bis-Tris 4–12% gradient polyacrylamide gels in the MOPS buffer system (Invitrogen) and transferred to nitrocellulose membranes (BioRad) according to standard protocols. Membranes were immunoblotted with antibodies against pERK (4267), MET (8198), PDGFRβ (3169), pFRA1 (3880), caspase3 (9662), pPRAS40T246 (3033), p-CateninS33/37/T41 (9561), pSTAT-3Y705 (9145), pSTAT-5C11C5 (9359), pCREBS133/pATF-1 (9196) (Cell Signaling, 1:1,000), FRA1 (sc605, Santa Cruz, 1:500), EGFR (226, Santa Cruz, 1:100) or IRDye 680RD goat-anti-rabbit IgG (LI-COR) secondary antibody (1:20,000). Membranes were hybridized on the antibody arrays overnight (4°C) and detected with IRDye-labelled streptavidin (LI-COR) at a dilution of 1:5,000 was used for the detection, slides were scanned using a Odyssey CLx scanner (LI-COR) and analysed using Image Studio 2.0 software. The results were then normalized using internal controls, and the relative protein levels determined across four biological replicates.

Senescence β-galactosidase staining. A375 cells were grown in low-serum media and treated with vehicle or vemurafenib (0.1 μM) for 3 or 8 days, β-galactosidase staining was performed according to the manufacturer’s instructions (Cell Signaling). All experiments were performed independently at least two times. Representative experiments are shown.

Flow cytometry. Flow cytometry was performed as described previously22, with minor modifications. In brief, whole tumours were dissected, cut into smaller sections and dissociated for 1–3 h with 0.5% collagenase type III (Worthington Biochemical) and 1% dispase II (Roche) in PBS. Resulting single cells suspensions were washed with PBS supplemented with 2% FBS and filtered through a 70-μm nylon mesh. The resulting single cell suspension was incubated for 10 min at 4°C with anti-mouse Fc-block (BD) and anti-mouse antibody or antibodies to detect immune cells diluted in PBS supplemented with 1% BSA. Cells were subsequently washed with PBS/BSA and stained with control antibodies or antibodies to detect immune cells diluted in PBS supplemented with 1% BSA and 2% FBS/0.1% sodium azide. The following antibodies against mouse antigens were used: CD45-PE-Cy7 (clone 30-F11, BD Pharmingen, 1:200), CD11b-AF647 (clone: M1/70, BD Pharmingen, 1:100), Gr-1-PE (MACS, 1:10), CD31-APC (clone: 390, eBioscience, 1:100), F4/80-PE (clone: BM8, eBioscience, 1:50). To determine the level of Edu incorporation in A37552 cells within vehicle- or vemurafenib-treated A375/52552 tumours, Edu (50 mg kg1−1) (Life Technologies) was injected intra-peritoneally, after 2 h tumours were collected, single-cell suspensions generated as described above and further processed according to the manufacturer’s protocol (Click-IT Plus Edu Alexa Fluor 647 Flow Cytometry Assay Kit, Life Technologies). Data were acquired using a FACs Calibur (BD Biosciences). All experiments were performed independently at least two times. Representative experiments are shown.

Antibody arrays. Cytokines and cytokine receptors of murine stromal and immune cells, in A375 tumours treated with vehicle or vemurafenib for 5 days, were measured using the Mouse Cytokine Array G2000 (RayBio, AAH- CYT-G2000, detecting 174 proteins), according to the recommended protocols. In brief, tumours were homogenized with a Mini Immersion Blender (Pro Scientific) in Raybio Lysis buffer with protease inhibitors. Lysates were centrifuged for 5 min at 10,000g, the supernatant was collected and protein concentration was measured using the BCA assay (ThermoFisher, AM1931). Ribogreen and the Agilent BioAnalyzer Technologies were used to map the paired-end sequencing reads to the human genome (build hg19, 2008). Data were acquired using a FACs Calibur (BD Biosciences). All experiments were performed independently at least two times. Representative experiments are shown.

Statistical analysis. Data are generally expressed as mean ± s.e.m., or in box plots in which the centre line is the median, and whiskers are minimum to maximum values. Group sizes were determined based on the results of preliminary experiments and no statistical method was used to predetermine sample size. Group allocation and outcome assessment were not performed in a blinded manner. All samples that met proper experimental conditions were included in the analysis. Statistical significance was determined using a two-tailed Mann–Whitney U test or Student’s t-test using Prism 6 software (GraphPad Software), or using a hypergeometric variability test (http://www.geneprof.org). Significance was set at P < 0.05.

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Extended Data Figure 1 | Targeted therapy or oncogene knockdown leads to regression of sensitive melanoma and lung adenocarcinoma tumours but accelerates the proliferation and seeding of residual drug-resistant cells in vivo. a, FACS analysis of sensitive A375 and vemurafenib-resistant A375R cells expressing TGL, at tumour implantation and after 2 weeks at start of therapy (n = 8 tumours). Plots depict representative images. b, Tumour volume of A375 cells treated with vehicle or vemurafenib over time (vehicle, n = 8; vemurafenib, n = 12 tumours). c, Representative sections of A375/ A375R-TGL tumours at 0, 1, 3 and 6 days of vemurafenib treatment analysed with immunofluorescence against GFP. Arrowheads indicate emerging clusters of GFP+ resistant cells. Scale bars, 2 mm. d, Quantification of BrdU incorporation into vemurafenib-resistant A375R-TGL cells in A375/A375R tumours treated with vehicle or vemurafenib for 6 days (vehicle, n = 13 FOV of 3 tumours; vemurafenib n = 18 FOV of 4 tumours). Original magnification, ×20. e, Fold change of photon flux of TGL-expressing A375R cells in A375 tumours or A375R tumours alone treated with vehicle or dabrafenib for 8 days (A375/A375R: vehicle, n = 15; dabrafenib, n = 14; A375R: vehicle, n = 8; dabrafenib, n = 7 tumours). f, Tumour volume of doxycycline-inducible BRAF knockdown A375-i-shBRAF-derived xenograft tumours (in which ‘A375-i’ denotes expression of doxycycline-inducible hairpin) treated with vehicle or doxycycline over time (vehicle, n = 5; doxycycline, n = 4 tumours). g, Photon flux of TGL-expressing A375R cells mixed in A375-i-shBRAF tumours treated with vehicle or doxycycline (vehicle, n = 10; doxycycline, n = 11 tumours). h, Fold change of photon flux of TGL-expressing vemurafenib-resistant M249R cells mixed in previously treated A375R tumours or M249R tumours alone treated with vehicle or vemurafenib (n = 16 tumours). i–k, Co-implantation assay of tumours treated with vehicle or corresponding targeted therapy with BLI quantification after 5–8 days. i, Fold change of photon flux of TGL-expressing vemurafenib-resistant YUMM1.7R cells mixed in unlabelled, vemurafenib-sensitive YUMM1.7 tumours or YUMM1.7R tumours alone (YUMM1.7/YUMM1.7R: n = 24; YUMM1.7R: n = 20 tumours). j, Fold change of photon flux of TGL-expressing, intrinsically vemurafenib-resistant B16 cells mixed in vemurafenib-sensitive YUMM1.1 tumours or B16 tumours alone (YUMM1.1/B16: vehicle, n = 12; vemurafenib, n = 16; B16: n = 20 tumours). k, A375R mixed in crizotinib-sensitive H3122 cells or A375R tumours alone (H3122/A375R: vehicle, n = 14; crizotinib, n = 13; A375R: n = 12 tumours). l, Photon flux of tumours established from intrinsically resistant drug-resistant cells alone, treated with vehicle, crizotinib or erlotinib (crizotinib-resistant PC9, H2030- or erlotinib-resistant A375R) (n (from left to right) = 12, 12, 7, 12, 16 and 16 tumours, respectively). m, Summary of the model systems and conditions used in vivo. n, Left, representative immunofluorescence images of vemurafenib-treated, sensitive tumours 7 h or 5 days after intracardiac injection with A375R-TGL cells; sections stained for GFP (A375R, green), collagen type IV (blood vessels, red), and DAPI (nuclei, blue). Right, quantification of A375R single cells and cell clusters (>2 cells) infiltrating an A375 tumour treated with vehicle or vemurafenib after intracardiac injection of A375R cells (GFP+ cells were scored in at least 10 whole sections of at least 4 tumours). Original magnifications, ×20. Data in b, e–l and n are mean and s.e.m., in f, centre line is median, whiskers are minimum to maximum. P values calculated by a two-tailed Mann–Whitney U test.
a) Percent gap closure following treatment with Vemurafenib.

b) Photin flux of H2030-TGL.

c) Photin flux of A375-TGL.

d) Relative cell number.

e) YUMM1.7.

f) Relative cell number.

g) CM source and Test cells.

h) CM-Vehicle and CM-Vemurafenib.

i) Normalized cell migration.

j) A375 Normalized cell index.

k) CM-Vehicle and CM-Vemurafenib.

l) A375 at harvest of CM.

m) A375 8d on Vem. positive control.
Extended Data Figure 2 | The secretome of vemurafenib-treated melanoma and crizotinib- or erlotinib-treated lung adenocarcinoma cells stimulates the proliferation and migration of drug-resistant cells in vitro and occurs before apoptosis and senescence. 

a, Quantification of the co-culture assay, depicted in Fig. 2a, 7 days after addition of resistant A375<sup>R</sup>-TGL cells (n = 4 biological replicates). P values calculated using a Student’s t-test. 

b, Drug-sensitive cells were pre-treated with vehicle or drug (crizotinib or erlotinib) for 48 h before 5 x 10<sup>5</sup> TGL-expressing, drug-resistant cells were added. Growth was monitored by BLI and quantified 7 days after addition of the resistant cell population (n = 8 biological replicates), P values calculated using a Student’s t-test. 

c, Relative number of vemurafenib-resistant LOX<sup>R</sup> cells after 3 days in the presence of conditioned media derived from A375 and UACC62 cells (n = 3 biological replicates). 

d, Representative immunofluorescence for Ki67 in drug-resistant YUMM1.7<sup>R</sup> cells cultured in conditioned media from YUMM1.7 cells. Original magnification, ×20. 

f, Relative number of vemurafenib-resistant melanoma cells with different, clinically relevant resistance mechanisms after 3 days in the presence of conditioned media derived from A375 cells. SKMEL239-3 expressing the p61 BRAFV600E splice variant, A375 expressing NRAS<sup>Q61K</sup> or the constitutively active MEK variant MEK-DD (n = 5 biological replicates). 

g, Relative cell number of intrinsically vemurafenib-resistant lung adenocarcinoma cells (H2030, PC9) or crizotinib- and erlotinib-resistant melanoma cells (A375<sup>R</sup>) after 3 days cultured in the presence of conditioned media from vemurafenib-treated melanoma or crizotinib- and erlotinib-treated lung adenocarcinoma (n = 6 in all, except for A375<sup>R</sup> with HCC827-CM, n = 4 biological replicates). 

h, Representative image of A375<sup>R</sup> cells migrated towards A375-derived CM-vehicle or CM-vemurafenib. Original magnification, ×10. 

i, Relative migration towards conditioned media from different sources and different resistant test cells as indicated (n = 10 FOV). **P < 0.01, ****P < 0.0001, two-tailed Mann–Whitney U test. 

j, Representative graph and quantification of real-time migration of A375<sup>R</sup> cells in the presence of conditioned media derived from A375 cells as measured by the xCELLigence system (n = 4 biological replicates). P value calculated using two-tailed Mann–Whitney U test. 

k, Monolayer gap closing assay of A375<sup>R</sup> cells in the presence of conditioned media derived from A375 cells with representative light microscopy images and quantification of gap closure over time. 

l, Immunoblotting for cleaved caspase-3 and phosphorylated ERK protein levels in vemurafenib-sensitive melanoma cell lines after 72 h of vemurafenib treatment. 

m, β-galactosidase staining of A375 cells treated with vemurafenib for 72 h or 8 days. Original magnification, ×13. Data are presented mean and s.e.m.
Extended Data Figure 3 | The therapy-induced secretome of sensitive cells overlaps significantly in melanoma and lung adenocarcinoma cells and appears after gene expression changes enriched for transcriptional regulators. a, b, GO analysis (http://revigo.irb.hr) of gene expression changes after 6 h of vemurafenib treatment of A375 cells with spatial representation of enriched GO terms (a) and the molecular functions significantly affected (b). c, Heat map representing the expression levels of commonly up- and downregulated genes in vemurafenib-treated A375-derived xenograft tumours (5 days) and A375 cells in vitro (48 h). d, Principal component analysis of vemurafenib-sensitive Colo800 and UACC62 melanoma cells and crizotinib-sensitive H3122 lung adenocarcinoma cells treated in vitro with vehicle or vemurafenib or crizotinib for 48 h. e, Venn diagram indicating the overlap of genes in the extracellular region (GO:0005576) upregulated after 48 h of vemurafenib treatment in A375, Colo800 and UACC62 melanoma cell lines. f, Venn diagram indicating the overlap of genes in the extracellular region (GO:0005576) upregulated after 48 h of vemurafenib treatment in at least 2 out of 3 melanoma models and after 48 h of crizotinib treatment in the H3122 lung adenocarcinoma cell line. g, Venn diagram indicating the overlap of genes in the extracellular region (GO:0005576) downregulated after 48 h of vemurafenib treatment in A375, Colo800 and UACC62 melanoma cell lines. h, Venn diagram indicating the overlap of genes in the extracellular region (GO:0005576) downregulated after 48 h of vemurafenib treatment in at least 2 out of 3 melanoma models and after 48 h of crizotinib treatment in the H3122 lung adenocarcinoma cell line. P values calculated using a hypergeometric probability test.
Extended Data Figure 4 | Vemurafenib treatment induces widespread changes in the intra-tumour immune cell composition and stromal cytokine composition in tumours regressing during targeted therapy. a, b, FACS analysis of murine immune cell populations in A375-derived xenograft tumours treated with vehicle or vemurafenib for 5 days. a, b, Representative image (a) and quantification (b) of intra-tumour composition of indicated immune cell populations (vehicle, n = 4; vemurafenib, n = 6 tumours). c, d, Cytokine array of murine stroma-derived cytokines within A375-derived xenograft tumours treated with vehicle or vemurafenib for 5 days. Representative image (c) and quantification (d) of down- and upregulated cytokines (n = 4 tumours). P values calculated by a two-tailed Mann–Whitney U test. Data are mean and s.e.m.
Extended Data Figure 5 | Targeted therapy induces downregulation of FRA1 in drug-sensitive tumour cells. a, List of transcription factors predicted to regulate the vemurafenib-induced reactive secretome in A375 cells, and a heat map of the corresponding transcription factor gene expression levels in these cells. Red represents high, yellow medium and blue low relative expression on the colour scale. b, Immunoblotting of phosphorylated and total FRA1 protein levels in A375 and YUMM1.7 melanoma cell lines treated with vemurafenib for 24 h. c, Relative mRNA levels of FRA1 in H3122 cells treated with crizotinib (500 nM) and HCC827 treated with erlotinib (10 nM) at different time points (n = 4 technical replicates). d, Relative mRNA levels of FRA1 in A375<sup>5</sup> cells treated with vemurafenib at different time points (n = 4 technical replicates). e, Immunofluorescence staining of FRA1 (red) and DAPI (blue) in biopsies from melanoma patients before and after vemurafenib treatment (clinical information can be found in Extended Data Table 1). Original magnification, ×20.
Extended Data Figure 6 | The secretome of melanoma cells with FRA1 knockdown stimulates proliferation and migration of A375R cells in vitro and in vivo. a, Immunoblotting of phosphorylated and total FRA1 protein levels in A375 cells transduced with control shRNA, with or without vemurafenib treatment, or shRNAs targeting FRA1. b, Photon flux and representative BLI images of TGL-expressing A375R cells co-cultured with A375 cells expressing control shRNA (with or without vemurafenib treatment) or FRA1-targeting shRNAs after 7 days (n = 9 biological replicates). c, Relative number of A375R cells after 3 days in the presence of conditioned media derived from A375 cells transduced with control shRNA, with or without additional vemurafenib treatment, or FRA1 shRNAs (n = 3 biological replicates). d, Migration of A375R cells towards conditioned media derived from A375 cells transduced with control shRNA (with or without vemurafenib treatment) or FRA1 shRNAs using a Boyden chamber assay (shCtrl, n = 15; all other groups n = 10 FOV) e, Relative mRNA levels of selected secreted factors and transcription factors of A375 cells expressing control shRNA or an shRNA targeting FRA1 (shFRA1-1), treated with vehicle or vemurafenib (24 h). f, Bioluminescent signal of A375R-TGL cells 8 days after subcutaneous co-implantation with UACC62 cells expressing a control or an shRNA for FRA1 (shCtrl, n = 12; shFRA1, n = 20 tumours). Data are mean and s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test.
Functions Annotation  |  p-Value  |  Z-score
---|---|---
Cell movement  |  3.28E-07  |  2.158
Quantity of cells  |  7.49E-05  |  2.029
Proliferation/tumor cell lines  |  1.73E-04  |  3.238
Leukocyte migration  |  2.69E-04  |  2.456
Cell survival  |  5.31E-04  |  2.363
Homing  |  5.52E-04  |  2.410
Cell viability  |  8.55E-04  |  2.438
Chemotaxis  |  9.62E-04  |  2.194
Organismal death  |  3.99E-05  |  3.589
Apoptosis/cancer cell lines  |  4.72E-03  |  2.078

| Target  | Inhibitor  | Z-Score  | P-value |
|---|---|---|---|
| PI3K  | LY294002  | -2.287  | 2.87E-03|
| PKC  | bisindolylmaleimide I  | -2.200  | 1.26E-03|
| mTOR  | sirolimus  | -2.750  | 1.94E-02|

**a**

| Vem  | pAKT^S473  | pERK^T202/Y204  | Tubulin |
|---|---|---|---|
| 12 h  |  |  | |
| 24 h  | +  |  | |
| 72 h  | +  |  | |

**b**

| A375  | CM-Vehicle  | CM-Vemurafenib |
|---|---|---|
| 5  |  |  |
| 10  |  |  |
| 30  |  |  |

**c**

**d**

**e**

**f**

**g**

**h**

**i**

**j**

**k**
Extended Data Figure 7 | The TIS includes upregulated positive regulators and a loss of negative regulators of the PI(3)K/AKT/mTOR pathway, which is activated in sensitive and resistant cells in vitro and in vivo.

a, b. Enriched biological processes (a) and inferred drug vulnerabilities (b) as determined by Ingenuity pathway analysis of gene expression data from vemurafenib-resistant A375<sup>R</sup> cells responding to signals from the reactive tumour microenvironment of a tumour regressing during targeted therapy in vivo (for experimental set-up see Fig. 1a and Methods). c, Left, immunoblotting of phosphorylated AKT<sup>S473</sup> and phosphorylated ERK protein levels in A375 cells treated with vehicle or vemurafenib at different time points during the generation of conditioned media. Right, immunoblotting of phosphorylated AKT<sup>S473</sup> and phosphorylated ERK protein levels in A375 cells after short-term exposure to conditioned media derived from A375 cells treated with vehicle or vemurafenib. d, Immunoblotting of phosphorylated AKT<sup>S473</sup> and phosphorylated FRA1 protein levels in A375-derived xenograft tumours treated with vehicle or vemurafenib for 5 days. Normalized quantification of phospho-AKT<sup>S473</sup>/tubulin in the bottom panel.

e. Immunoblotting of a range of pathway nodes in A375<sup>R</sup> cells treated with CM-vehicle or CM-vemurafenib, derived from A375 cells, for 15, 30, 60 or 120 min. f. Cancer cell-derived IGFBP3 levels (left) and murine stromal IGF1 levels (right) in A375-derived xenograft tumours treated with vehicle or vemurafenib for 5 days as determined by ELISA (n = 4 tumours). g. Cancer-cell-derived IGFBP3 levels in conditioned media from indicated melanoma cell lines treated with vehicle or vemurafenib as determined by ELISA (n = 3 technical replicates of conditioned media derived from at least two biological replicates). h, IGFBP3 levels in conditioned media derived from A375 cells expressing control shRNA or shRNAs targeting IGFBP3 (shIGFBP3-1 and -2) as determined by ELISA (n = 3 technical replicates). i, Immunoblotting of phosphorylated AKT<sup>S473</sup> in A375<sup>R</sup> cells after incubation with conditioned media of A375 cells expressing control shRNA or shRNAs targeting IGFBP3. j, Phosphorylation status of AKT<sup>S473</sup> in A375<sup>R</sup> cells after incubation for 15 min with conditioned media, IGF1 and IGFBP3 as indicated. k. Bioluminescent signal of A375<sup>R</sup>-TGL cells 10 days after co-implantation with A375 cells expressing a control shRNA or an shRNA targeting IGFBP3 (shIGFBP3-1) (n = 10 tumours). P values calculated by a two-tailed Mann–Whitney U test. Data are mean and s.e.m.
Extended Data Figure 8 | Dual inhibition of RAF and the AKT/mTOR pathway blunts the effects of the regressing tumour environment on the resistant cell population. a, Relative photon flux and representative BLI images of GFP/luciferase expressing A375R cells co-cultured with A375 cells and treated with vehicle, vemurafenib or the combination of vemurafenib and either MK2206 (AKTi, 2 μM) or BEZ235 (PI(3)K/mTORi, 300 nM) for 7 days (n = 2–3 biological replicates). b, Relative number of A375R cells after 3 days in the presence of CM-vehicle or CM-vemurafenib with additional BEZ235 (300 nM) (n = 3 biological replicates). c, Mice bearing tumours consisting of A375/A375R cells or A375R cells alone were treated with drugs as indicated. Bioluminescent signal of TGL-expressing A375R cells was determined on day 5 of treatment (n = 16, 16, 12, 12 and 16 tumours, respectively). d, Mice bearing tumours consisting of unlabelled A375 cells were pre-treated for 3 days with drugs as indicated and 1 × 10⁵ TGL-expressing A375R cells were inoculated in the arterial circulation. Drug treatment was continued and seeding of resistant cells to the primary tumour was quantified by BLI. Representative BLI images on the right (vehicle, n = 4; vemurafenib n = 10, vemurafenib plus BEZ235, n = 10 tumours). P values calculated by a two-tailed Mann–Whitney test. Data are mean and s.e.m.
Extended Data Figure 9 | Characterization of cell lines in response to targeted therapy. a–h, Relative survival of human melanoma cell lines (A375, Colo800 and UACC62) (a, c, e), and the murine melanoma cell line YUMM1.7 (g) and corresponding vemurafenib-resistant derivatives (A375R, Colo800R, UACC62R and YUMM1.7R) under increasing concentrations of vemurafenib. Immunoblotting of phosphorylated ERK protein levels in indicated melanoma cell lines in the presence of increasing concentrations of vemurafenib (b, d, f, h). i, Immunoblotting of phosphorylated ERK protein levels in HCC827 lung adenocarcinoma cells in the presence of increasing concentrations of erlotinib. j, Immunoblotting of phosphorylated ERK protein levels in H3122 lung adenocarcinoma cells in the presence of increasing concentrations of crizotinib. k, Immunoblotting of protein levels of MET, EGFR, BRAF, PDGFRb, phosphorylated AKT and phosphorylated ERK in vemurafenib-sensitive and -resistant pairs of human melanoma cell lines (A375, Colo800 and UACC62).
## Extended Data Table 1 | Clinical data for tissue donor subjects

| Study site | Pt # | Bx samples | Age & Sex | Stage | Dose (mg) | BOR  | PFS (days) | Bx site               |
|------------|------|------------|-----------|-------|-----------|------|------------|-----------------------|
| UCLA       | 1 TG | Baseline  | 51M       | M1c   | 960 bid vemurafenib | -21% | 108        | SC, scalp             |
|            |      | Day 7      |           |       |            |      |            | SC, scalp             |
|            | 2 JCC| Baseline  | 44M       | M1c   | 960 bid vemurafenib +60 qd cobimetinib | -63% | Current response | SC, abdomen SC, abdomen |
|            |      | Day 15     |           |       |            |      |            | SC, abdomen SC, abdomen |
|            | 3 YAU| Baseline  | 26F       | M1c   | 960 bid vemurafenib +60 qd cobimetinib | -46% | 145        | Dermal/SC, abdomen     |
|            |      | Day 22     |           |       |            |      |            | Dermal/SC, clavicle   |

bid, twice daily; BOR, best overall response; Bx, biopsy; F, female; M, male; PFS, progression-free survival; Pt, patient; qd, daily; SC, subcutaneous; UCLA, University of California, Los Angeles.