**Supplementary materials and methods to**

**BRAF fusion gene partners influence oncogenic BRAF activity**

**Authors**

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***BRAF (fusion) gene cloning***

To ensure controlled and selective expression, *BRAF* (fusion) genes were introduced into the pInducer20 vector48, which encompasses a “Tet-on” system, where gene expression is only activated by the addition of dox (**Figure 1C**). In addition, a C-terminal HA-tag was linked different BRAF (fusion) genes to facilitate protein quantification and *in situ* detection by immunohistochemistry. *BRAF* (fusion) genes were cloned into the vector pInducer2048 by using the Gateway Cloning System (Invitrogen). In brief, patient RNA6 was reverse-transcribed with the High Capacity cDNA RT Kit (Applied Biosciences). BRAF (fusion) constructs were amplified from cDNA with the Phusion Polymerase (Biolabs) utilizing forward and reverse primers with the respective 3’ or 5’ attB overhang (**List of Primers, Supplementary Table 4**). PCR products were run on a 1% agarose gel, the expected bands excised and gel purified with the Wizard SV Gel and PCR clean-up system (Promega). *TRIM24-BRAF* was ordered from Twist Bioscience with the appropriate attB overhangs. Entry-vectors (attL) were generated by combining the attP donor-vector pDonr201 (150 ng) with the purified attB-PCR products (100 fmol) and mixed with BP clonase II. The mixture was incubated for 60 mins at 25° C and the reaction was stopped with Proteinase K for 10 mins at 37° C. DH5ɑ- library efficient cells (Invitrogen) were transformed with 1 ul of the BP reactions according to manufacturer's protocol and plated on kanamycin-LB agar plates (50 µg/ml). Bacteria were grown overnight at 37° C and single colonies were picked the next day for growing a 3 ml LB culture for a mini-prep. Plasmid DNA was extracted with the QIAPrep Spin Miniprep Kit (Qiagen) and correct insertion of fusion gene into the pDonr201 (now pEntry-vector) was verified by Sanger Sequencing. Generation of an expression-vector is achieved by combining the attR destination-vector pInducer20 (150 ng) with pEntry (150 ng, attL) and mixed with LR clonase II. The mixture was incubated for 60 mins at 25° C and the reaction was stopped with Proteinase K for 10 mins at 37° C. DH5-alpha library efficient cells were transformed with 1 ul of the LR reaction and plated on ampicillin LB agar plates (50 µg/ml). Bacteria were grown overnight at 37° C and single colonies were picked the next day for growing a 3 ml LB culture for a mini-prep. Plasmid DNA was extracted with the Qiagen MiniPrep Kit and correct insertion of fusion gene into the pInducer20 (now expression-vector) was verified by Sanger Sequencing.

***Patient-derived CRC organoid and HEK293 culture and maintenance***

The P18T patient-derived organoids were previously established and characterized13,49. P18T CRC organoids were cultured as described previously13,32. Culture medium contained advanced DMEM/F12 medium (Invitrogen) with 1% Penicillin/Streptomycin (P/S, Lonza), 1% Hepes buffer (Invitrogen) and 1% Glutamax (Invitrogen), 10% R-spondin conditioned medium, 10% Noggin conditioned medium, 1x B27 (Invitrogen), 1.25 mM n-Acetyl Cysteine (Sigma-Aldrich), 10 mM Nicotinamide (Sigma-Aldrich), 50 ng/ml EGF (Invitrogen), 500 nM A83-01 (Tocris), and 10 µM SB202190 (ApexBio). Organoids were split through Trypsin-EDTA (Sigma-Aldrich) treatment. Culture medium after splitting was supplemented with 10 µM Y-27632 dihydrochloride. For selection of organoids stably expressing *BRAF* (fusion) genes, organoids were grown in culture medium containing 400 µg/ml G418 (Santa Cruz).

HEK293 cells were cultured in DMEM medium (Lonza) supplemented with 10% FBS, 1% Penicillin/Streptomycin (P/S, Lonza) and 1% Glutamax (Invitrogen). For selection of HEK293 cells stably expressing *BRAF* (fusion) genes, HEK293 cells were grown in culture medium containing 400 µg/ml G418 (Santa Cruz).

P18T organoid lines were confirmed mycoplasm-negative with the mycoplasm PCR ELISA kit (Roche; last test was performed 12/9/2019) and organoids were kept in culture for 10 passages until final experiments were performed. HEK293 were kept in culture for 16 passages until final experiments were performed.

***Lentiviral organoid and HEK293 transduction***

Each *BRAF* (fusion) gene construct was stably integrated into the genome of patient-derived P18T organoids or HEK293 cells utilizing lentiviral transduction resulting in polyclonal *BRAF* (fusion) gene expressing lines. The P18T organoid line, which is deficient in the WNT and TP53 pathway, is derived from a non-hypermutated colorectal tumor. Furthermore, it is wild-type for the RAS signaling pathway and therefore dependent on EGF-mediated growth factor signaling for growth and survival13. For virus production, HEK293T cells were transfected with pHDM.Hgpm2 (1 µg), Rev (1 µg), Tat (1 µg), HDM.G (2 µg) and the respective pInducer20-construct plasmid (5 µg) using the Xtreme Gene transfection reagent (Roche). Medium was refreshed the following day. On day 3 medium was collected, sterile filtered (45 µM) and concentrated in a Ultracentrifuge at 35.000 rpm for 2.5 h at 4° C. Virus from one 10 cm dish of HEK 293T cells was resuspended in 250 µl infection medium (CRC medium + 10 µM Y-27632 + Polybrene (Millipore) and used for the transduction of one well of a 24-well plate of P18T organoids. Pre-infection, organoids were trypsinized to small clumps of single cells, pelleted in a 15 ml tube and resuspended in 15 µl infection medium per virus infection. Resuspended virus was added to the cells and cells were spin-infected at 600 rpm for 1 hour at room temperature. Thereafter, organoid-virus mixture was incubated at 37° C for 2 hours and spun down at 1400 rpm for 5 mins at 4° C. Supernatant was carefully removed and organoids were plated in 40 µl Matrigel (Corning Life Sciences BV) and supplemented with CRC medium plus 10 µM Y-27632. Three days after infection, selection with G418 (400 µg/ml) (Corning Life Sciences BV) was started and organoids were kept under antibiotic pressure for 1 month.

***Reverse Transcription and Breakpoint PCR***

To confirm selective fusion gene expression upon dox treatment, organoids were cultured with dox (1 µg/ml) or vehicle (ddH20) for 24 hours and RNA was extracted with the RNAeasy MiniKit (Qiagen) according to the manufacturer’s protocol. 500 ng of DNA-free RNA was reverse transcribed into cDNA using the High Capacity cDNA RT Kit (Thermofisher). Breakpoint PCR was performed with AmpliTaqGold (Applied Biosystems) utilizing specific breakpoint primers for each *BRAF* fusion gene or primers targeting the junction between *BRAF* and the HA-tag (**Supplementary Table 4**). PCR products were loaded and visualized on an 2% agarose gel.

***RT-qPCR***

To assess differences in *BRAF* (fusion) gene expression levels, 500 ng DNA-free RNA was transcribed into cDNA as described above. Thereafter, RT-qPCR was performed with the Power-up SYBR green assay (Applied Biosystems) on a CFX cycler (Bio-Rad). All reactions were performed in triplicate and in a total volume of 10 µl comprising 5 µl 2x MM, Primer (final 5 µM) and 1 µl cDNA (approx. 10 ng). Expression levels were normalized to glucose-6-phosphatase isomerase (GPI) housekeeping gene expression and fold change was calculated with respect to the expression levels of induced *BRAFWT* expressing organoids (= set to 1).

***Western blot assay***

Prior to cell lysis, organoids were incubated with 1 mg/ml dispase II (Invitrogen) for 10 minutes at 37°C to digest the BME. HEK293 cells were washed with PBS prior to cell lysis. Western blot samples for were lysed using RIPA buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1% NP-40) containing Complete protease inhibitors (Roche). Protein content was quantified using a BCA protein assay kit (PierceTM) and analyzed by Western blotting. Membranes were blocked and probed with antibodies directed against HA (RRID:AB\_631618), Vinculin (RRID:AB\_477629), GAPDH (RRID:AB\_2107445), beta-catenin (RRID:AB\_397555), pERK (RRID:AB\_331646), ERK (RRID:AB\_390779), pMEK (RRID:AB\_331648), MEK (RRID:AB\_823567), pCDK2 (AB\_2078685), tCDK2 (RRID:AB\_631215), pAKT (RRID: AB\_2315049) and AKT (RRID: AB\_1147620).

Organoid treatments: dox (MP Biomedicals) 1 µg/ml, 24 h or dd H20. Dox 1 µg/ml 24 h followed by treatment with afatinib (Selleck Chemicals) 1 µM, 24 h or DMSO.

***Phenotypic drug screen***

Four days after organoid trypsinization, 1 µg/ml dox or ddH2O was added to the organoid cultures. After 24 hours, 1 mg/ml dispase II (Invitrogen) was added to the medium of the organoids and these were incubated for 15 min at 37° C to digest the BME. Subsequently, organoids were mechanically dissociated by pipetting, filtrated using a 40 µm nylon cell strainer (Falcon), resuspended in 75% BME/growth medium (40 organoids/ml) and plated as two 10 µl drops on NuncTM Lab-TekTM II Chamber SlideTM Systems. After plating, culture medium containing either 1 µg/ml dox or ddH2O, together with 1 µM of afatinib or DMSO was added. The labtek plates were mounted on an inverted confocal laser scanning microscope (Leica SP8X) and imaged using a 10X objective. For visualization of cell viability, organoids were incubated with 16.2 mM Hoechst 33342 (Life Technologies) and 1.5 mM DRAQ7TM (Cell Signaling #7406) for 30 min at 37° C prior imaging. For calculating organoid viability and size, organoids were scored by morphology and analyzed by automated brightfield morphometry using Organoseg51.

***Immunofluorescence***

For immunofluorescence, organoids and HEK293 cells were washed in PBS, fixed and permeabilized with 4% PFA (Aurion) containing 0.25% glutaraldehyde (Sigma), and blocked with PBS containing 0.3% Triton X-100 (Sigma), 1 mg/ml BSA (Sigma Aldrich), 5% NGS (Life Technologies), and incubated with antibodies at 4°C overnight.

For immunofluorescence of HEK293 cell cultures, cells were washed in PBS, fixed with 4% PFA, permeabilized with PBS containing 0.2% Triton X-100, blocked in PBS containing 3% NGS, 2% BSA and 50 mM ammonium chloride, and incubated with antibodies directed against HA (RRID:AB\_390929) at 4°C overnight. Hoechst 33342 was added together with secondary antibodies to stain for DNA.

Images were captured with a Leica SP8X microscope using a 40X objective. Post-acquisition analyses of phenotypes were performed manually using ImageJ.

Treatments: Culture medium containing 1 µg/ml dox or ddH2O for 24 h.

***Drug screen and viability assessment***

Five days after organoid trypsinization to single cells, 1 mg/ml dispase II (Invitrogen) was added to the medium of the organoids and these were incubated for 15 min at 37° C to digest the BME. Subsequently, organoids were mechanically dissociated from the BME by subtle pipetting, filtrated using a 40 µm nylon cell strainer (Falcon), resuspended in 2% BME/growth medium (15–20,000 organoids/ml) prior plating of 40 µl (Multi-dropTM Combi Reagent Dispenser) on BME pre-coated 384-well plates. The drugs and their combinations were added 3 hrs after plating the organoids by using the Tecan D300e Digital Dispenser. Drugs were dispensed in a non-randomized manner and DMSO end concentration was 0.6% in all wells. 96 hrs after adding the drugs organoids were fixed with 4% PFA (Merck) and stained with Hoechst (Invitrogen). Organoids were screened by automated microscopy of whole wells (CX5 High Content Screening (HCS) platform (Thermo Scientific), equipped with an Olympus UPLFLN U Plan Fluorite 4x Microscope Objective). Organoid size was measured by integrating Hoechst signal and contrast using Columbus Cellular imaging and analyses (Perkin Elmer). Relative survival was determined by normalization of the results to controls (average of all 5 nM drug concentrations = 100% alive) and 20 µM Navitoclax (= 0% alive), which induces maximal killing within 96 hours after treatment. Multiple identical drug combinations were averaged.

***Targeted inhibitors***

Afatinib, Selumetinib, Encorafenib and Navitoclax were purchased from Selleck Chemicals. SCH772984 was obtained from MedChem Express. Dabrafenib was obtained from Bio-Connect. These compounds were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored as 10 mM aliquots.

***Curve fitting and drug sensitivity***

Dose-response curves were generated using GraphPad software by performing nonlinear regression (curve fit), assuming a standard Hill equation (chosen method: log(inhibitor) vs. Response, constrain top=100).

***RNA Sequencing***

Organoids were treated with dox (1 µg/ml) or ddH20 for 24 hours and RNA was extracted with the RNAeasy MiniKit (Qiagen) according to the manufacturer’s protocol. 1 µg of total RNA was shipped to Macrogen (Korea). RNA-libraries were prepared for sequencing with the Illumina TruSeq Stranded Total RNA (+ Ribo-Zero HMR) Kit and sequenced on the NovaSeq platform (2x100bp, 60M reads per sample). Data were processed with our in-house RNA analysis pipeline (<https://github.com/UMCUGenetics/RNASeq>, v.2.4.0, default settings) by utilizing STAR (v.2.4.2a) to map the reads. Samples were normalized for sequencing depth based on the sum of the read counts over all genes for each sample. Expressed genes were selected by excluding all genes where ≥ 3 samples had less than 10 reads. Principal Component Analysis (PCA), Euclidean Distance-based clustering and Differential Expression (DGEA) calculations were performed with the DESeq2 package52. P-adjusted (padjusted) was calculated by multiplying the p-value with the number of genes (=expressed genes) tested. Unsupervised hierarchical clustering was performed on 300 intermediately expressed genes (mean gene expression > 100 and max gene expression 500). DGEA was performed on induced BRAF fusion lines vs. all other lines or each induced line vs. controls (= all uninduced lines and induced GFP line). Geneset overrepresentation analysis was performed on Webgestalt25, with the settings set to Reactome pathway analysis and the reference gene set as all protein-coding genes. The top 50 up- and downregulated genes upon *DLG1-BRAF* fusion gene expression were based on the DGEA analysis between the DLG1-BRAF expressing organoid line and the control lines (padjusted <0.05). The cohort of CRC patients (n=233) was selected from a cohort of primary CRC patients6 by excluding all patients with a mutation in *KRAS* or *BRAFV600E*.

***Mass spectrometry sample preparation and enrichment of phosphopeptides***

For SILAC labeling, HEK293 cells were cultured in high-glucose (10% dialyzed FBS (BioWest)) DMEM (Thermo) lacking lysine and arginine supplemented with Lys-0/Arg-0 or Lys-8/Arg-10 (Silantes). After 4 hours of doxycycline or ddH2O administration, cells were lysed in 8 M Urea, 1M Ammonium-BiCarbonate (ABC) containing 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 40mM 2-chloro-acetamide supplemented with protease inhibitors (Roche, complete EDTA-free) and 1% (v/v) phosphatase inhibitor cocktails 2 and 3 (Sigma, Cat. No. P5726 and Cat. No. P0044). After ultra-sonication, cell lysates were mixed 1:1 and proteins (20 mg total) were overnight in solution digested with trypsin (1:50) (Worthington). Peptides were desalted using SepPack columns (Waters) and eluted in 80% acetonitrile (ACN), 0.1% Formic Acid (FA) (buffer B) after which TFA was added to 1%. To enrich phosphopeptides for, 50 mg titanium dioxide (TiO2) beads (Sachtopore-NP, 5 µM, 300 Å, Zirchrom) were washed with 5% NH3 and equilibrated 3 times with 6% TFA in 80% ACN after which the phospho-peptides were allowed to bind at 37 ˚C for 5 minutes on a shaker. After centrifugation, remaining phospho-peptides were enriched from the supernatant sequentially. After loading the beads on an in-house made C8 stage-tip (Empore, 3M) they were washed 3 times with 1% TFA in 80% ACN. Peptides were eluted with 200 µl 5% NH3 into 40µl 20% FA followed by elution with 5 µl buffer B. Peptides were loaded on in-house made C18 stage-tips and divided with high PH elution into three fractions (100 mM NH3/FA PH=10 in 5%, 10% or 50% ACN).

***LC-MS/MS analysis***

After elution from the stage tips, acetonitrile was removed using a SpeedVac and the remaining peptide solution was diluted with buffer A (0.1% FA) before loading. Peptides were separated on a 30 cm pico-tip column (75µm ID, New Objective) in-house packed with 1.9 µm aquapur gold C-18 material (dr. Maisch) using 240 gradient (7% to 80% ACN 0.1% FA), delivered by an easy-nLC 1000 (Thermo), and electro-sprayed directly into an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Scientific). The latter was set in data dependent Top speed mode with a cycle time of 1 second, in which the full scan over the 400-1500 mass range was performed at a resolution of 240000. Most intense ions (intensity threshold of 5000 ions) were isolated by the quadrupole and fragmented with an HCD collision energy of 30%. The maximum injection time of the ion trap was set to 50 milliseconds with injection of ions for all available parallelizable time.

***Mass spectrometry data analysis***

Raw files were analyzed with the Maxquant software version 1.6.1.53 with phosphorylation of serine threonine and tyrosine as well as oxidation of methionine set as variable modifications, and carbamidomethylation of cysteine set as fixed modification. The Human protein database of Uniprot was searched with both the peptide as well as the protein false discovery rate set to 1%. The SILAC quantification algorithm was used in combination with the ‘match between runs’ tool (option set at two minutes). Peptides were filtered for reverse hits and standard contaminants.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifiers PXD013461.

Resulting phosphorylation sites were analyzed in Perseus software (Version 1.5) using MaxQuant normalized H/L ratios. Reverse hits, common contaminants, and sites with localization probability below 0.75 were deleted. Furthermore, identified phosphopeptides were log2-transformed and accounted for quantification only if they were present in both forward and reverse (label-swap) experiments. *p*-values were calculated in a Student’s *t*-test (p-value < 0.05, S0=0) with Perseus 1.5 software. Subsequently, phosphoproteomics data were normalized to relative protein expression and ratios were deemed significantly changed if they were <-1.5 or >1.5 fold. Kinase activity scores were calculated using Kinase-Substrate Enrichment Analysis (KSEA) analysis24 in which we used both PhosphoSitePlus and NetworKIN databases. The NetworKIN cutoff score was set to 5, the *p*-value cutoff was set to 0.05, andthe substrate count cutoff was to 5. Geneset overrepresentation analysis was performed on Webgestalt25, with the settings set to Reactome pathway analysis and the reference gene set as all protein-coding genes.