Supplementary Materials for

Patient-derived organoids model treatment response of metastatic gastrointestinal cancers

Georgios Vlachogiannis[1], Somaieh Hedayat[1], Alexandra Vatsiou[2], Yann Jamin[3], Javier Fernández-Mateos[1], Khurum Khan[1,4], Andrea Lampis[1], Katherine Eason[1], Ian Huntingford[1], Rosemary Burke[5], Mihaela Rata[3], Dow-Mu Koh[3,6], Nina Tunariu[3,6], David Collins[3], Sanna Hulkki-Wilson[1], Chanthirika Ragulan[1], Inmaculada Spiteri[2], Sing Yu Moorcraft[4], Ian Chau[4], Sheela Rao[4], David Watkins[4], Nicos Fotiadis[6], Maria Bali[3,6], Mahnaz Darvish-Damavandi[1], Hazel Lote[1,4], Zakaria Eltahir[1], Elizabeth C Smyth[4], Ruwaida Begum[4], Paul A Clarke[5], Jens C Hahne[1], Mitchell Dowsett[7], Johann de Bono[8], Paul Workman[5], Anguraj Sadanandam[1], Matteo Fassan[9], Owen J Sansom[10], Suzanne Eccles[5], Naureen Starling[4], Chiara Braconi[4,5], Andrea Sottoriva[2], Simon P Robinson[3], David Cunningham[4] & Nicola Valeri[1,4,*]

correspondence to: nicola.valeri@icr.ac.uk

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Other Supplementary Materials for this manuscript includes the following:

Tables S1 to S8 (provided as two separate microsoft excel files)

Materials and Methods

Experimental design

Research objectives: To establish and characterize a biobank of patient-derived organoids (PDOs), and investigate the potential of PDOs to recapitulate *ex vivo* responses to chemotherapeutic and targeted agents observed in the clinic.

Research subjects: Biopsies obtained from patients with metastatic gastrointestinal cancers enrolled in prospective phase I/II clinical trials were used in order to establish a biobank of PDOs. All PDOs were established as part of co-clinical trials (stated in the exploratory endpoints of the trial protocols; available upon request) supporting this study and allowing direct comparison between clinical and pre-clinical response. Informed consent was obtained from all patients described in the manuscript. The researchers were blinded to the patients' response in order to avoid biases

Experimental design: The histopathological and genomic profiling of PDOs were characterized in detail in comparison to their parental tumor, demonstrating high similarities between the two. The genomic and transcriptomic stability of PDOs *in vitro* was verified over time, and their response profile to a library of 55 clinically relevant compounds was investigated. *Ex vivo* responses to anticancer agents in PDOs and PDO-based orthotopic mouse tumor xenograft models were matched to patient response observed in the clinic (**fig. S1**).

Clinical trials

PROSPECT-C Trial: A Study of Biomarkers of Response or Resistance to Anti-EGFR Therapies in Metastatic Colorectal Cancer. ClinicalTrials.gov identifier: NCT02994888.

PROSPECT-C is a phase II study investigating the molecular markers of response or resistance to anti-epidermal growth factor receptor (EGFR) antibodies.

PROSPECT-R Trial: A Prospective Translational Study Investigating Molecular Predictors of Resistance and Response to regorafenib Monotherapy. ClinicalTrials.gov identifier: NCT03010722. PROSPECT-R is a single-center prospective biological translational research study involving the collection of tumor tissue, blood samples, and clinical data from patients being treated with regorafenib for metastatic colorectal cancer (mCRC) at the Royal Marsden Hospital. Patients were eligible for the study if they had a histological diagnosis of CRC, were refractory to standard available therapies with palliative intent for mCRC, had received prior treatment with at least one anti-VEGF antibody and chemotherapy drugs including fluorouracil (5-FU) or capecitabine, oxaliplatin, and irinotecan, and patients had *RAS* mutant tumors (*18*).

FOrMAT Trial: Feasibility of a Molecular characterization Approach to Treatment (REC: 13/LO/1274RM CCR 3994). FOrMAT is a single-center prospective translational research study involving the collection and analysis of tumor tissue, blood samples and clinical data from patients with locally advanced/metastatic gastrointestinal cancers.

FGFR Trial: FGFR (EudraCT No. 2011-003718-18) is a phase II, open-label, nonrandomized study of AZD4547 in patients with previously treated advanced *FGFR*-amplified cancer.

In all trials, biopsies were collected before, at time of best response, and at time of disease progression via image-guided or endoscopic procedures. Following harvesting, the patient specimens were placed in cold PBS and transported to the lab on ice, where they were processed

for the establishment of organoid cultures. In most cases, tissue-processing was performed between 20 min – 2 h post harvesting.

Establishment and culture of PDOs from GI cancers

Biopsies were minced, conditioned in 5 ml PBS/EDTA 5 mM for 15 min at room temperature, and digested in 5 ml PBS/EDTA 1 mM containing 2x TrypLe (Thermo Fisher Scientific) for 1 hr at 37 ^{o}C. Following digestion, mechanical force (pipetting) was applied in order to facilitate cell release in solution. Dissociated cells were collected in Advanced DMEM/F12 (Thermo Fisher Scientific), pelleted (1,200 rpm, 5 min, 4 ^{o}C), resuspended in 120 µl of growth factor reduced (GFR) matrigel (Corning), and seeded in a well of a 24- or 48-well flat bottom cell culture plate (Corning). The matrigel was then solidified by a 20-minute incubation in a 37 ^{o}C and 5% CO_2 cell culture incubator, and overlaid with 500 µl of complete human organoid media; complete media was subsequently refreshed every two days.

Passaging of PDOs was performed using TrypLe. Briefly, PDOs were mechanically harvested (pipetting) out of matrigel using PBS-EDTA 1mM containing 1x TrypLe, and incubated for 20 min at 37 ^{o}C. PDOs were then dissociated to single cells by applying mechanical force (pipetting), washed with HBSS (Thermo Fisher Scientific), pelleted (1,200 rpm, 5 min, 4 ^{o}C), resuspended in GFR matrigel, and re-seeded at an appropriate ratio.

PDOs were biobanked in FBS (Thermo Fisher Scientific), containing 10% DMSO (Sigma-Aldrich).

Human PDO culture media

GI PDOs were cultured in Advanced DMEM/F12 (Thermo Fisher Scientific), supplemented with 1x B27 additive (Thermo Fisher Scientific), 1x N2 additive (Thermo Fisher Scientific), 0.01% BSA (Roche), 2 mM L-Glutamine (Thermo Fisher Scientific), 100 units/ml penicillin-streptomycin (Thermo Fisher Scientific), and containing the following additives:

Additive	Supplier	Cat. No.	Concentration
EGF	PeproTech	AF-100-15	50 ng/ml
Noggin	PeproTech	250-38	100 ng/ml
R-Spondin 1	PeproTech	120-38	500 ng/ml
Gastrin	Sigma-Aldrich	G9145	10 nM
FGF-10	PeproTech	100-26	10 ng/ml
FGF-basic	PeproTech	100-18B	10 ng/ml
Wnt-3A	R&D Systems	5036-WN	100 ng/ml
Prostaglandin E_2	Tocris Bioscience	2296	1 µM
Y-27632	Sigma-Aldrich	Y0503	10 µM
Nicotinamide	Sigma-Aldrich	N0636	4 mM
A83-01	Tocris Bioscience	2939	0.5 µM
SB202190	Sigma-Aldrich	S7067	5 µM
HGF*	PeproTech	100-39	20 ng/ml

*HGF was only used for the cholangiocarcinoma organoids.

3D PDO drug assays

PDOs were harvested and dissociated into single cells following the passaging procedure described above. Cell pellets were resuspended in 500 µl of Advanced DMED/F12, cells were counted with the Countess automated cell counter (Thermo Fisher Scientific), and appropriate cell dilutions were done in GFR matrigel. 30 µl of GFR matrigel containing 4,500 – 6,000 cells were seeded in standard 96-well cell culture plates (Corning), and plates were incubated for 20 min in a 37 ^{o}C and 5% CO_2 cell culture incubator so that the matrigel solidifies; the matrigel was then overlaid with 70 µl of complete human organoid media. Complete media was refreshed once after 24 h.

3 days post seeding, media was removed and replaced by 50 µl of drug-containing complete human organoid media. In order to take into account the pre-existing volume of matrigel, the compound concentration during this first addition was calculated with the following formula: *[1st drug addition] = ([Desired concentration]*80 µl)/50 µl*. Drug-containing media was further refreshed every 2 days for another 3 or 4 times depending on the growth rate of the various organoid cultures, so that the necessary cytokines/additives for organoid growth were replenished. At the end of the treatment, media was removed and replaced with 100 µl of complete human organoid media containing 10% CellTiter-Blue cell viability assay (Promega). Plates were placed back in the incubator, and, for most experiments, at least two viability readings were obtained using the EnVision plate reader (PerkinElmer), between 2 – 4 hours after CellTiter-Blue addition.

3D PDO drug screens

The PDOs drug screens were conducted in 96-well cell culture plates following the procedure described above, and using a custom-made library of 55 compounds and 5 DMSO controls (**table S5**). Each screen was conducted in triplicate, using a concentration of 1 µM for all compounds.

Briefly, compounds (dissolved in DMSO) and vehicle controls were arrayed in polypropylene round-bottom 96-well plates (assay plates), using the ECHO 550 liquid dispenser (Labcyte). For each screen, two types of assay plates were generated: an assay plate for the first drug addition that accounted for the pre-existing volume of matrigel (as previously explained), each of its wells containing 280 nl of 1 mM compound; and 3 or 4 assay plates for the

subsequent drug additions, each of their wells containing 175 nl of 1 mM compound. These compound-containing assay plates were stored at -20 ^{o}C. Prior to treatment, the compounds in the assay plates were resuspended in 175 µl of complete human organoid media, and 50 µl of drug-containing complete media were added in each of the screen's three replicates.

DNA and RNA extractions

PDOs were harvested and pelleted using the passaging procedure described above, and their DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA/miRNA universal kit. PDO-matching tumor DNA was extracted from the undigested tissue that remained following the PDO establishment procedure using the Qiagen QIAamp Blood DNA Mini kit. In one case (R-006) PDO-matching tumor DNA was extracted from a matching FFPE biopsy using the Qiagen QIAamp DNA FFPE Tissue kit. Germline DNA was extracted from blood (500 µl), using the Qiagen QIAamp Blood DNA Mini kit. DNA and RNA from FFPE archival material were obtained using the Qiagen AllPrep DNA/RNA FFPE kit.

DNA-sequencing, mutation analysis, and copy number analysis

Targeted library preparation and DNA-sequencing were outsourced to GATC Biotech (Germany). In brief, DNA libraries were prepared with the ClearSeq Comprehensive Cancer panel (Agilent Technologies) that targets 151 cancer-related genes, using SureSelectV6 chemistry (Agilent Technologies). Paired-end sequencing (2 x 125 bp) was then performed using

Illumina technology. The annotation of the 2,505 regions sequenced was obtained through the Agilent Technologies website and is provided in **table S8**.

Mean depth coverage of 1,673x was achieved across samples (range: 626x – 4,482x). FastQC and bamQC were used for QC quality control. Adapter trimming was performed with Skewer v0.1.126 (*22*), with minimum read length after trimming 35 and mean quality value before trimming of 10. Paired-end alignment to the hg19 human reference genome was carried out with BWA v0.7.12 (*23*), and picard tools were used for sorting and indexing. SNVs per patient were called jointly with Platypus v0.8.1 using genotyping (*24*). SNVs with minimum coverage of 100, minimum number of 3 reads covering the variant and minimum genotype quality of 10, were filtered for further analysis. To avoid potentially false positive variants with low Variant Allele Frequency (VAF), a VAF threshold of ≥0.1 was applied. Variants present in at least one tumor sample that have germline frequency (if available) equal to 0 were classified as somatic. Annotation was performed with CAVA (*25*). Copy number log-ratios were computed with CNVkit (*26*), and correlation between the different organoid passages was calculated using Spearman correlation measure.

Whole-genome sequencing (WGS) libraries were prepared from single organoids using the NEBnext Ultra II DNA library prep kit for Illumina (New England Biolabs), following the manufacturer's recommendations. WGS was outsourced to BGI (China); libraries were sequenced on one HiSeq X-Ten lane to an average coverage of 30x. Copy number log-ratios were computed with CNVkit (*26*).

Sequence data have been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001002784. Further information about EGA can be found on https://ega-archive.org.

RNA sequencing and data analysis

RNA sequencing and data analysis were outsourced to Arraystar (USA). In brief, 1-2 µg of total RNA were enriched by oligo(dT) magnetic beads (rRNA removal), and sequencing libraries were prepared using the KAPA Stranded RNA-Seq library prep kit (Illumina) which incorporates dUTP into the second cDNA strand and renders the RNA-seq library strand-specific. The completed libraries were qualified with Agilent 2100 Bioanalyzer and quantified by absolute quantification qPCR method. To sequence the libraries on the Illumina HiSeq 4000 instrument, the barcoded libraries were mixed, denatured to single stranded DNA in NaOH, captured on Illumina flow cell, amplified *in situ*, and subsequently sequenced for 150 cycles for both ends on Illumina HiSeq instrument. Image analysis and base calling were performed using Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Sequence quality was examined using the FastQC software. The trimmed reads (trimmed 5',3'-adaptor bases using Cutadapt) were aligned to reference genome using Hisat2 software (v2.0.4) (*27*). The transcript abundances for each sample was estimated with StringTie (v1.2.3) (*28, 29*), and the FPKM value (*29*) for gene and transcript level were calculated with R package Ballgown (v2.6.0) (*30*).

The raw RNAseq data and analyzed FPKM values reported in **fig. S6** can be accessed through GEO/NCBI (GEO accession number GSE108391).

Antibodies

The primary antibodies, and the respective applications they were used in are shown in the table below:

Target	Supplier	Cat. No.	Application	Dilution
Actin	Sigma-Aldrich	A1978	WB	1:5000
AKT	Cell Signaling	4685	WB	1:1000
pAKT (Ser473)	Cell Signaling	4060	WB	1:1000
pC-RAF (Ser338)	Cell Signaling	9427	WB	1:1000
Caspase-3	Cell Signaling	9668	WB	1:1000
EGFR	Cell Signaling	4267	WB	1:1000
pEGFR (Tyr1068)	Cell Signaling	3777	WB	1:1000
ERBB2	Cell Signaling	4290	WB	1:1000
pERBB2 (Tyr1221/1222)	Cell Signaling	2243	WB	1:1000
ERK1/2	Cell Signaling	4695	WB	1:1000
pERK1/2 (Thr202/Tyr204)	Cell Signaling	4370	WB	1:1000
pH2A.X (Ser139)	Cell Signaling	9718	WB	1:1000
pHistone H3 (Ser10)	Cell Signaling	3377	WB	1:1000
MEK1/2	Cell Signaling	8727	WB	1:1000
pMEK1/2 (Ser217/221)	Cell Signaling	9154	WB	1:1000
p110α	Cell Signaling	4249	WB	1:1000
PARP	Cell Signaling	9546	WB	1:1000
PRAS40	Cell Signaling	2691	WB	1:1000
pPRAS40 (Thr246)	Cell Signaling	13175	WB	1:1000
pS6 (Ser235/236)	Cell Signaling	4858	WB	1:1000
α-SMA	Abcam	ab5694	IF	1:100
CK8	Abcam	ab107115	IF	1:200
CD31 (anti-human)	Novus Biologicals	NB600-562	IHC	1:100
CD31 (anti-mouse)	Cell Signaling	77699	IHC	1:100
CDX-2	Roche	760-4380	IHC	N/A
CK7	DAKO	M7018	IHC	1:300
ERBB2	Roche	790-4493	IHC	N/A
TK1	Abcam	ab76495	IHC	1:50

PDO/tissue histology, immunohistochemistry, CISH, and FISH

In order to maintain their 3D structure, PDOs were harvested out of matrigel by inoculating them with 1 ml of Cell Recovery solution (Corning) for 60 min at 4 ^{o}C. PDOs were then collected in cold PBS, pelleted (4,000 rpm, 4 min, 4 ^{o}C), and fixed in formalin 10% (Sigma-Aldrich) for 60 min. Following fixation, PDOs were washed with PBS, pelleted (4,000 rpm, 4 min), and resuspended in 200 µl of warm (~45 ^{o}C) agarose 2% (in H_2O). The agarose pellet was

left to set, dehydrated using ethanol (EtOH), and embedded in paraffin using a standard histological protocol. Alternatively, following pelleting the fixed PDOs were resuspended in 200 µl of OCT compound (Thermo Fisher Scientific), snap-frozen on dry ice, and stored at -80 ^{o}C.

Organoid and tissue H&E staining was conducted either using the Ventana BenchMark XT platform (Pathology department, Royal Marsden Hospital), or manually following a standard staining protocol. CDX-2, CK7, human-CD31, and ERBB2 immunohistochemistry stainings were conducted by the Royal Marsden's Pathology department, using the Ventana BenchMark XT platform. ERBB2 CISH was also conducted by the Royal Marsden's Pathology department, using the INFORM HER2 Dual ISH DNA Probe Cocktail Assay (Roche) and the Ventana BenchMark XT platform. FGFR2 FISH was conducted by the Royal Marsden's Molecular Diagnostics department, using the ZytoLight SPEC FGFR2 Dual Color Break Apart Probe (Zytovision).

Mouse CD31 immunohistochemistry, TK1 immunohistochemistry, and CK8/α-SMA dual immunofluorescence were conducted manually. Briefly, tissue sections were deparaffinized in xylene and hydrated in ethanol (EtOH) using standard histological procedures, and then subjected to a 30 min heat-mediated antigen retrieval step using a sodium citrate buffer (sodium citrate 10 mM, 0.05% tween 20, pH 6.0). For CD31 and TK1 immunohistochemistry: endogenous peroxidase activity was blocked using the peroxidase-blocking solution (Dako), sections were blocked with the protein block, serum free solution (Dako), the primary antibody was diluted in antibody diluent (Dako), and staining was performed overnight at 4 ^{o}C. Immunohistochemistry was then continued using the anti-rabbit EnVision+ system HRP (Dako), following the manufacturer's instructions. For the CK8/α-SMA dual immunofluorescence: the deparaffinized sections were blocked using 5% BSA (in PBS/triton X-100 0.15%), primary

antibodies were diluted in 1% BSA (in PBS/triton X-100 0.15%), and staining was performed overnight at 4 ^{o}C. Sections were then incubated with labelled secondary antibodies (goat anti-chicken Alexa Fluor 488, and goat anti-rabbit Alexa Fluor 594; Thermo Fisher Scientific); secondary antibodies were diluted 1:500 in 1% BSA (in PBS/tween-20 0.1%), and immunostaining was performed at room temperature for 60 min. Following washes, the sections were counterstained with Hoechst 33342 (10 ng/ml in PBS; Sigma-Aldrich) for 10 min, and mounted using Vectashield antifade mounting medium (Vector Laboratories).

Microscopy

Images of live PDOs cultures were obtained with a Leica DMi8 microscope. Immunohistochemistry, H&E, and CISH images were obtained using a Leica DM2000 microscope. Immunofluorescence images were obtained using a Zeiss LSM 700 Confocal microscope. FISH images were obtained using a Zeiss Axio Imager Z2.

Western blot

PDOs were mechanically harvested out of matrigel (pipetting) using a cold (4 ^{o}C) harvesting solution, comprised of Cell Recovery solution and HBSS at a 1:1 ratio. PDOs were washed with HBSS, pelleted (2,000 rpm, 3 min, 4 ^{o}C), and lysed immediately in RIPA buffer (Sigma-Aldrich) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich) and 1:100 phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Proteins were quantified with Bradford reagent (Sigma-Aldrich), using a BSA standard curve (10 – 0.15 µg/µl). 50 µg of protein lysates were run in NuPage 4-

12% Bis-Tris pre-cast gels (Thermo Fisher Scientific) using MOPS SDS running buffer (Thermo Fisher Scientific), and transferred onto PVDF membranes (GE Healthcare Life Sciences). Primary antibodies were diluted in PBS containing 1% BSA, and staining was conducted overnight at 4 ^{o}C. Staining with the secondary antibodies (anti-rabbit IgG HRP-linked, and anti-mouse IgG HRP-linked; Cell Signaling) was done at room temperature for 60 min; secondary antibodies were diluted 1:5,000 in PBS containing 5% skimmed milk. Luminescence signal was generated with Luminata Crescendo western HRP substrate (Merck Millipore), and captured with Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

Cell cycle analysis

F-014 BL and PD organoids were treated with 2.5 nM of paclitaxel (Sigma-Aldrich) or vehicle (DMSO) for 24 h, before being harvested and dissociated into single cells following the passaging procedure described above. Cell pellets were resuspended in 300 µl of cold (4 ^{o}C) PBS, and the resulting cell suspensions were passed through a 70 µm cell strainer (Falcon) in order to eliminate cell clumps; cells were then fixed by adding 700 µl of cold (4 ^{o}C) EtOH while gently vortexing. Fixed cells were incubated at 4 ^{o}C for at least 30 min, before being washed with PBS and stained with 40 ng/µl propidium iodide (in PBS, containing 5 ng/µl RNase A; Sigma-Aldrich) for 1 h at 37 ^{o}C. Stained cells were washed, resuspended in 500 µl of PBS, passed once more through a 70 µm cell strainer, and analyzed using a BD LSR II flow cytometer (BD Biosciences).

Copy number variation analysis using digital droplet PCR (ddPCR)

ddPCR copy number assays were set up with the ddPCR supermix for probes without dUTP (Bio-Rad), using 10-50 ng of DNA as template. PCR droplets were generated using the QX200 droplet generator (Bio-Rad), and the PCR reaction was run in a C1000 Touch thermo cycler (Bio-Rad) according to the manufacturer's protocol. Finally, the droplets were read with the QX200 droplet reader (Bio-Rad), and results were analyzed with the QuantaSoft software (Bio-Rad). The CNV probes used in this manuscript are listed in the table below; all assays were run using a probe against RPP30 as reference assay.

Target	Supplier	Cat. No.	Fluorophore
ABCB1	Bio-Rad	dHsaCP1000464	FAM
AKT1	Bio-Rad	dHsaCP2500336	FAM
EGFR	Bio-Rad	dHsaCP2500318	FAM
ERBB2	Bio-Rad	dHsaCP1000116	FAM
KRAS	Bio-Rad	dHsaCP1000033	FAM
MET	Bio-Rad	dHsaCP2500321	FAM
PIK3CA	Bio-Rad	dHsaCP2500445	FAM
RPP30	Bio-Rad	dHsaCP2500350	HEX

cDNA synthesis and real-time PCR (RT-PCR)

Total RNA (400 ng) from organoids was retrotranscribed to cDNA using the High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific). cDNA synthesis reactions were set up in a total volume of 20 ul, following the manufacturer's instructions. Newly synthesized cDNA was diluted 1:1 in PCR-grade H_2O, and 1 ul of diluted cDNA was used as template for RT-PCR. RT-PCR assays were set up in triplicate, using the SYBR Select Master Mix (Thermo Fisher Scientific) and the TK1 and GAPDH Quantitect Primer Assays (Qiagen). RT-PCR assays were

run in the StepOnePlus Real-Time PCR System (Applied Biosystems), using the fast protocol. Results were analyzed with the $\Delta\Delta C_T$ method, using GAPDH as internal reference gene.

Viral infection of PDOs

The Luc2 ORF was removed from pGL4.1 (Promega) as a Nhe1/Xba1 fragment and ligated into the MCS of pCDH-CMV-MCS2-EF1-Hygro (System Biosciences). Luc-viral supernatant was prepared by packaging with the pPACKH1 plasmid mix following the protocol described by the manufacturer.

PDOs were harvested, dissociated in single cells, and pelleted following the passaging procedure described above. Pellets were resuspended in complete organoid media and Luc-viral supernatant at a 1:1 ratio (500 µl each), with the addition of 8 µg/ml polybrene. The cell solution was transferred in a well of a 12-well plate, and left overnight in a 37 ^{o}C and 5% CO_2 cell culture incubator. The following morning cells were harvested using PBS-EDTA 1mM containing 1x TrypLe, pelleted, seeded in matrigel, and overlaid with complete medium. Two days post infection 200 ng/µl of hygromycin B (Sigma-Aldrich) was added to the complete organoid media, and selection of infected cells was done for a period of 1-2 weeks.

Animal experiments

All *in vivo* experiments were performed in accordance with the local ethical review panel, the UK Home Office Animals (Scientific Procedures) Act 1986, the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research (*31*), and the

ARRIVE guidelines (*32*).

Luc+ PDOs were harvested and dissociated into single cells following the passaging procedure described above. Cell pellets were resuspended in Advanced DMED/F12, cells were counted with the Countess automated cell counter, and 20,000 cells were seeded in 120 µl of GFR matrigel; cells were cultured *in vitro* for about a week, so that small organoids were formed. PDOs were then harvested using PBS-EDTA 1mM containing 1x TrypLe, washed immediately with HBSS, pelleted (1,500 rpm, 5 min, 4 ^{o}C), and resuspended in cold matrigel (Corning). 20 µl of matrigel containing ~20,000 small organoids were injected intrahepatically in the left liver lobe of 6-7 weeks old NOD *scid* gamma (NSG) animals (Charles River). Animals were housed in specific pathogen-free rooms in autoclaved, aseptic microisolator cages with a maximum of four animals per cage. Food and water were provided *ad libitum*. Successful engraftment and development of PDO-xenografts was monitored by longitudinal bioluminescence imaging. About 8 weeks post inoculation mice were randomized to treatment/control groups based on IVIS luminance or magnetic resonance imaging (MRI), and treated with regorafenib (Selleckchem) or vehicle control for 5 days; regorafenib was reconstituted in a cremophor/EtOH/H_2O solution at a 1:1:6 ratio, and was administered daily at 10 mg/kg via oral gavage.

MRI was performed on a 7T horizontal bore Bruker Biospec 70/20 (Ettlingen, Germany) using a 4cm abdominal volume coil. Anesthesia was induced with a 10 ml/kg intraperitoneal injection of fentanyl citrate (0.315 mg/ml) plus fluanisone (Hypnorm 10 mg/ml; Janssen), midazolam (Hypnovel 5 mg/ml; Roche), and sterile water, used at a ratio of 1:1:2. A lateral tail vein was cannulated with a 27G butterfly catheter (Hospira) for remote administration of contrast agent. Mice core temperature was maintained at 37°C with a mouse water heating pad.

Contiguous multi-slice T_2-weighted 1mm thick axial images were first acquired for tumor localization and volume determination. Functional vasculature was then interrogated prior to and 5 days following treatment with regorafenib or vehicle, using susceptibility-contrast MRI. Briefly, multiple gradient-recalled echo (MGRE) images were acquired from three 1mm-thick axial slices across each tumor using a 256x170 matrix over a 3cmx2cm field of view, a repetition time of 200ms, 8 echo times of 3 to 24ms spaced 3ms apart, and 8 averages, giving an overall acquisition time of ~7 minutes. A dose of 150 μmolFe/kg of the ultra-small superparamagnetic iron oxide (USPIO) particle preparation P904® (Guerbet Group, Villepinte, France) was then administered intravenously and, after 3 minutes to allow for equilibration, a second set of identical MGRE images were acquired. Image analysis was performed using in-house software (Imageview, developed in IDL, ITT Visual Information Systems, Boulder, CO, USA). Tumor volumes were determined using segmentation from regions of interest drawn on T_2-weighted images for each tumor-containing slice. Parametric maps of tumor fractional blood volume fBV (%) were subsequently calculated using the USPIO-induced change in the transverse relaxation rate R_2* (ΔR_2*), as previously described (*33*, *34*).

Following the post-treatment MRI scan, animals were culled, and their livers/tumors were excised, fixed in formalin (Sigma-Aldrich), and embedded in paraffin.

Survival study

Luc+ PDOs established from the BL and PD biopsies of patient R-011 were transplanted orthotopically in the liver of NSG mice (BL: n=18; PD: n=19), using the procedure described above. PDO-xenografts were monitored by longitudinal bioluminescence imaging, with mice

randomized to control (BL: n=9; PD: n=8) and treatment arms (BL: n=9; PD: n=11) based on luminance, and treated with vehicle or regorafenib (10 mg/kg) for 10 days. Following-treatment a random cohort of animals was culled for histopathological analysis (vehicle arm: BL n=4, PD n=3; regorafenib arm: BL n=4, PD n=6). The survival of the remaining animals (vehicle arm: BL n=5, PD n=5; regorafenib arm: BL n=5, PD n=5) was monitored over time.

Histological evaluation, CD31 scoring, and tumor growth pattern scoring

Histological evaluation was performed on H&E stained sections, and tumor grading was evaluated according to the 2010 World Health Organization Bosman criteria. Tumor-associated CD31 was evaluated in CD31-immunostained (IHC) sections. The CD31 scores shown represent the average number of CD31-positive vessels obtained by analyzing at least 10 high-power fields (HPFs); only positive endothelial structures were retained for scoring. Tumor growth pattern was evaluated and scored as previously described (*19*).

Supplementary Text

Targeted NGS analysis (related to **Fig. 1D** and **table S2**)

We found a total of 282 single nucleotide variants (SNVs; 58 missense, 16 stop-gained, 35 synonymous, 10 splice-site, 130 intronic) and 33 indels in PDOs using deep targeted sequencing; NGS data from 22 PDOs (germline DNA was not available for patient F-013), and for five cases from an additional PDO passage, were used in this analysis (mean depth: 1812x). At the copy number profile level, a mean of 9.8% and 13.2% of PDO genomes were amplified and deleted respectively; moreover, 6% and 24.1% of PDOs showed synchronous SNV/indels with amplification and deletion respectively.

Purity of PDOs (related to **table S2** and **table S3**)

Analysis of variant allele frequency (VAF) distribution and purity confirmed that PDOs represent a pure cancer cell population.

PDOs from mCRC retain their metastatic potential (related to **fig. S12C**)

Histopathological evaluation of the livers of animals carrying R-005 and R-009 PDO-xenografts revealed that the tumors these PDO-xenografts generated were not only localized at the injection site, but metastatic deposits were present in distant sites within the liver, as shown in **fig. S12C**.

Suppl. fig. S1

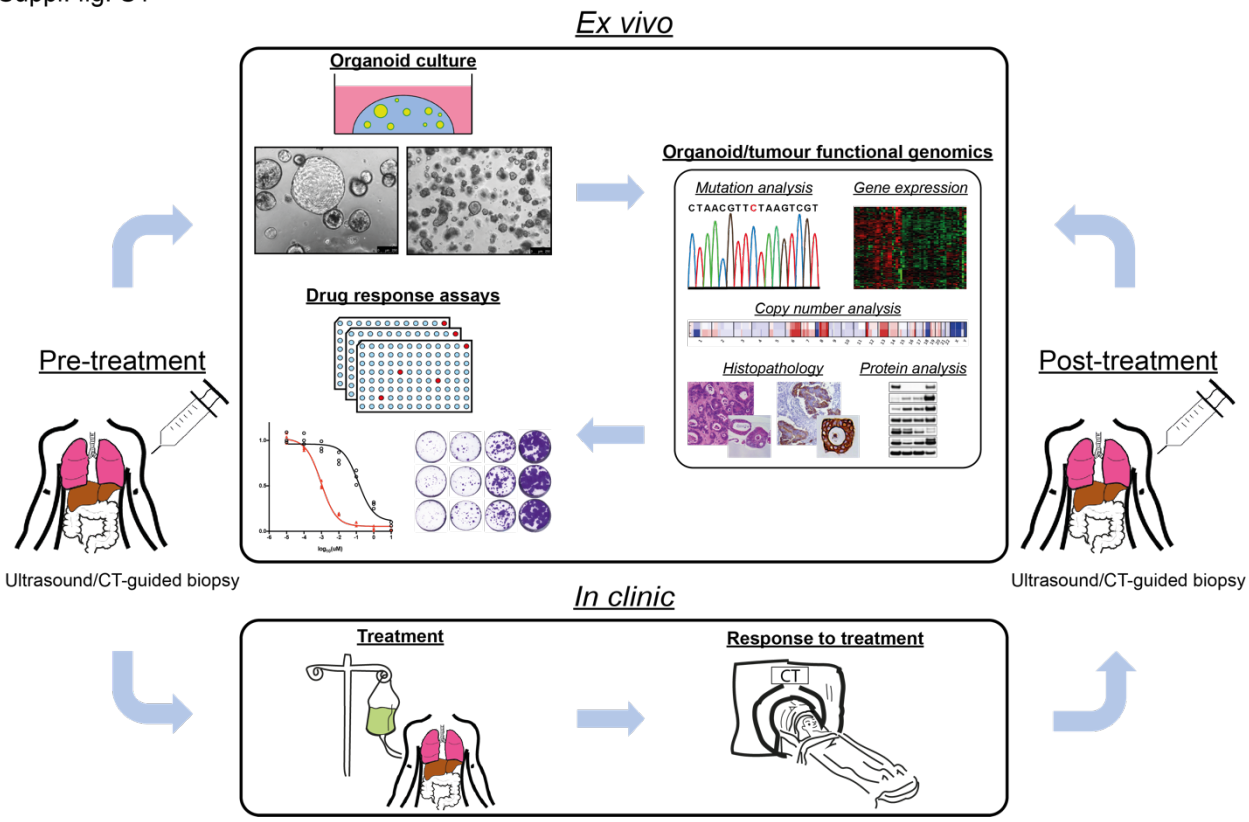


Fig. S1. Patient-derived organoids (PDOs) based co-clinical trials in metastatic gastrointestinal cancers. Image-guided biopsies were used to generate PDOs from metastatic chemo-refractory colorectal and gastroesophageal adenocarcinomas and a metastatic cholangiocarcinoma. PDOs were established from sequential biopsies collected before and after treatment, as well as at time of best response; multi-region PDOs were also generated in two patients. Molecular profiling of PDOs, their parental tissues, and archival material (primary cancer) was characterized and compared. PDOs were used in high-throughput drug screening, and response to anti-cancer agents was functionally matched with their genetic profile. Patient response to treatment in clinic was compared with *ex vivo* responses in their derivative PDOs.

Suppl. fig. S2

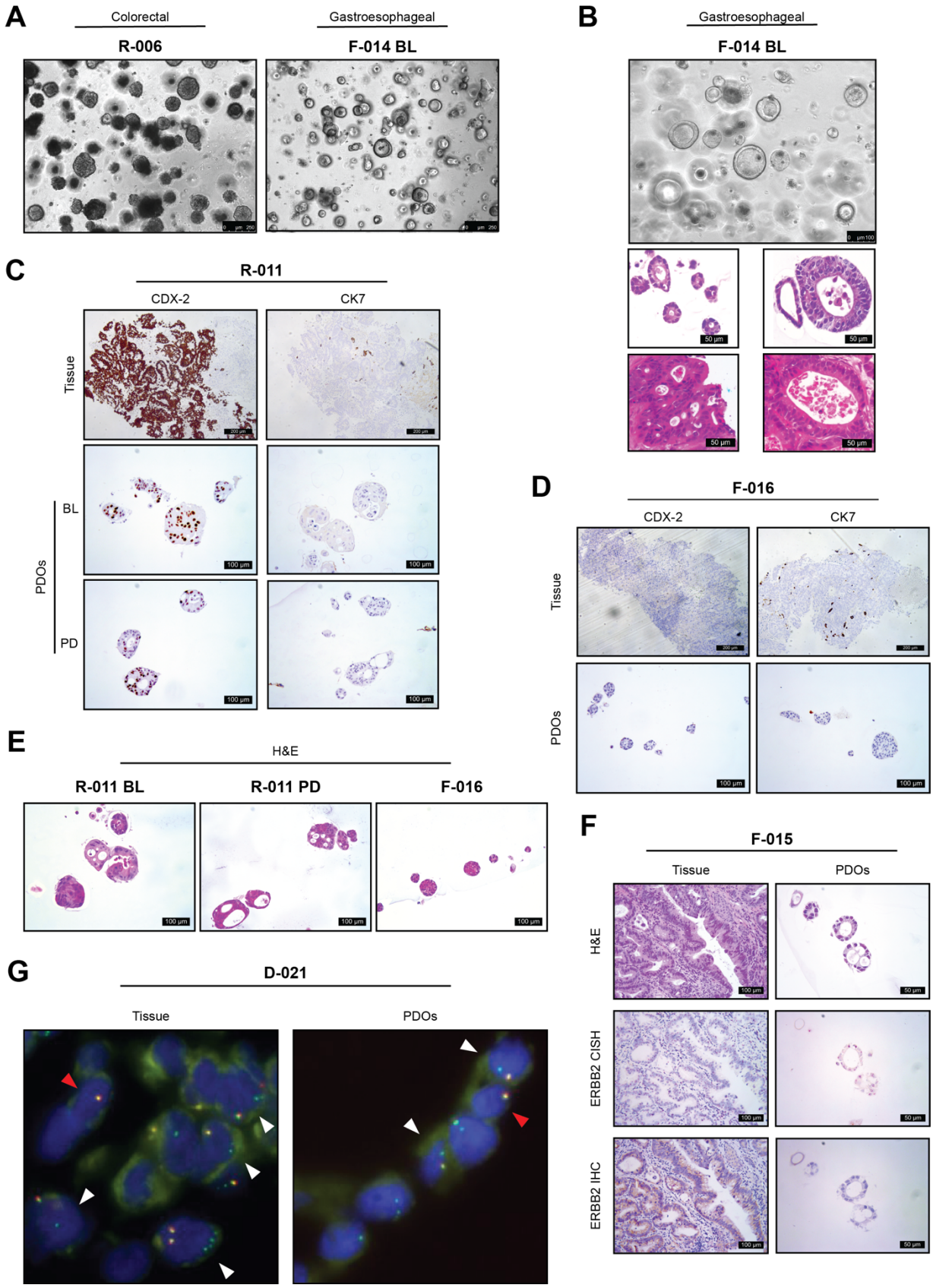

A
Colorectal
Gastroesophageal
R-006
F-014 BL
B
Gastroesophageal
F-014 BL
C
R-011
CDX-2
CK7
Tissue
PDOs
BL
PD
D
F-016
CDX-2
CK7
Tissue
PDOs
E
H&E
R-011 BL
R-011 PD
F-016
F
F-015
Tissue
PDOs
H&E
ERBB2 CISH
ERBB2 IHC
G
D-021
Tissue
PDOs

Fig. S2. Morphological and histopathological comparison of PDOs and parental biopsies. **(A)** Low magnification phase-contrast images of mCRC and mGOC PDOs. (**B**) Phase-contrast image of a mGOC PDO culture, and H&E staining comparing PDOs to their matching biopsy. (**C&D**) Immunostaining for the nuclear transcription factor CDX2 and epithelial cytokeratin CK7 in a CK7-positive [(**C**) (R-011)] and double-negative [(**D**) (F-016)] pair of parental tumor and matching PDOs. (**E**) H&E images of PDOs described in (**C**) and (**D**). (**F**) *ERBB2* CISH and IHC in *ERBB2* non-amplified mGOC PDOs and matching biopsy. (**G**) Break-apart FISH images of *FGFR2* rearrangement in PDOs and matching mCCA biopsy. White arrows indicate loss of the 5' *FGFR2* region, whereas red arrows indicate complete loss of one *FGFR2* allele; both genetic events are maintained in the PDOs.

H&E= hematoxylin and eosin; IHC= immunohistochemistry; PDOs= patient-derived organoids; mCRC= metastatic colorectal cancer; mGOC= metastatic gastroesophageal cancer; mCCA= metastatic cholangiocarcinoma; CISH= chromogenic *in situ* hybridization; FISH= fluorescence *in situ* hybridization.

Suppl. fig. S3

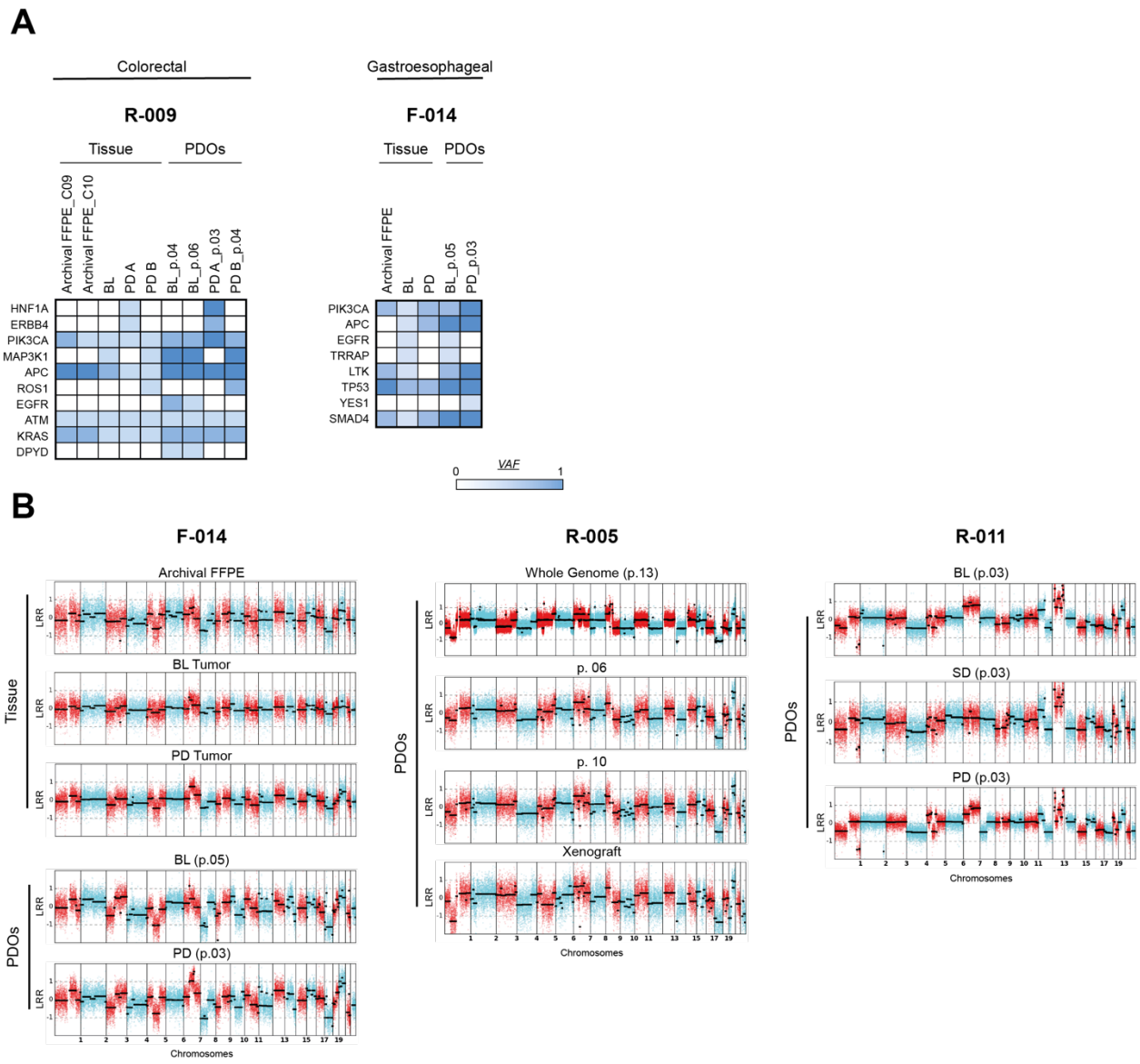


Fig. S3. Molecular landscape of PDOs and parental biopsies. (**A**) Heatmaps comparing mutational profiling and VAF in PDOs, parental biopsies, and archival material (primary cancers) in mCRC and mGOC. A VAF threshold of 0.5% was applied in order to eliminate false positives; intronic and synonymous mutations are excluded. (**B**) CNA plots comparing PDOs established from sequential biopsies (before and after paclitaxel treatment) to parental biopsies

and archival material (left panel); CNA plots from WGS and targeted NGS in PDOs at different passages, and in a liver orthotopic tumor generated from the same PDO (central panel); CNA plots of PDOs collected before regorafenib treatment (BL), at best response (SD), and at progression (PD) (left panel); numbers within brackets indicate the PDO passage number.

Suppl. fig. S4

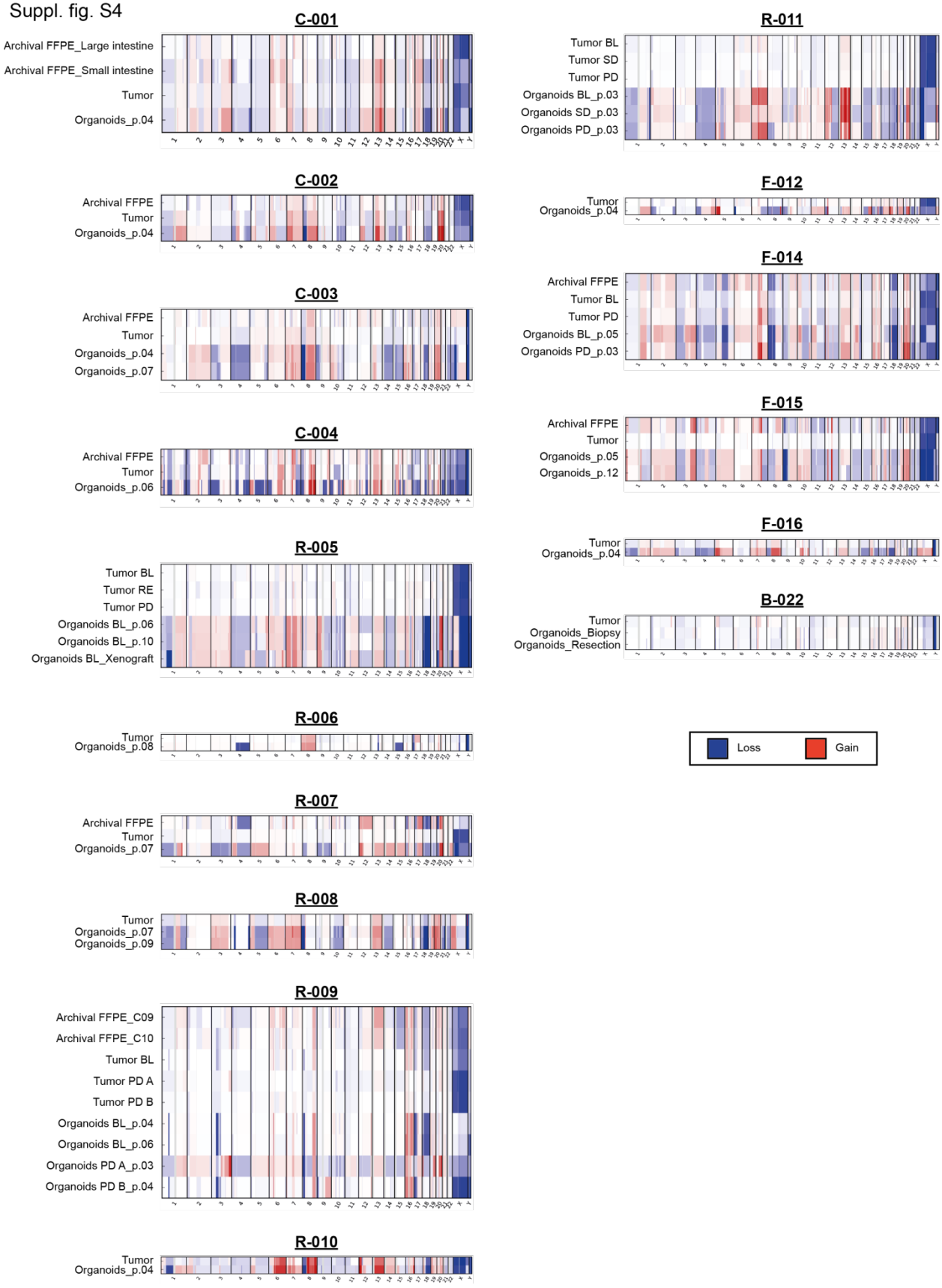

C-001
Archival FFPE_Large intestine
Archival FFPE_Small intestine
Tumor
Organoids_p.04
C-002
Archival FFPE
Tumor
Organoids_p.04
C-003
Archival FFPE
Tumor
Organoids_p.04
Organoids_p.07
C-004
Archival FFPE
Tumor
Organoids_p.06
R-005
Tumor BL
Tumor RE
Tumor PD
Organoids BL_p.06
Organoids BL_p.10
Organoids BL_Xenograft
R-006
Tumor
Organoids_p.08
R-007
Archival FFPE
Tumor
Organoids_p.07
R-008
Tumor
Organoids_p.07
Organoids_p.09
R-009
Archival FFPE_C09
Archival FFPE_C10
Tumor BL
Tumor PD A
Tumor PD B
Organoids BL_p.04
Organoids BL_p.06
Organoids PD A_p.03
Organoids PD B_p.04
R-010
Tumor
Organoids_p.04
R-011
Tumor BL
Tumor SD
Tumor PD
Organoids BL_p.03
Organoids SD_p.03
Organoids PD_p.03
F-012
Tumor
Organoids_p.04
F-014
Archival FFPE
Tumor BL
Tumor PD
Organoids BL_p.05
Organoids PD_p.03
F-015
Archival FFPE
Tumor
Organoids_p.05
Organoids_p.12
F-016
Tumor
Organoids_p.04
B-022
Tumor
Organoids_Biopsy
Organoids_Resection
Loss
Gain

Fig. S4. Copy number alteration (CNA) plots of PDOs and their matching biopsies. "Tumor" identifies the parental biopsy; "archival" identifies diagnostic material from primary resection or pre-treatment biopsies; "p" identifies the PDOs passage number; B-022 identifies PDOs from an endoscopic biopsy and subsequent surgical resection of a patient with locally advanced gastric cancer.

PDOs= patient-derived organoids; BL=baseline; RE= best response; SD= stable disease; PD= progressive disease.

Suppl. fig. S5

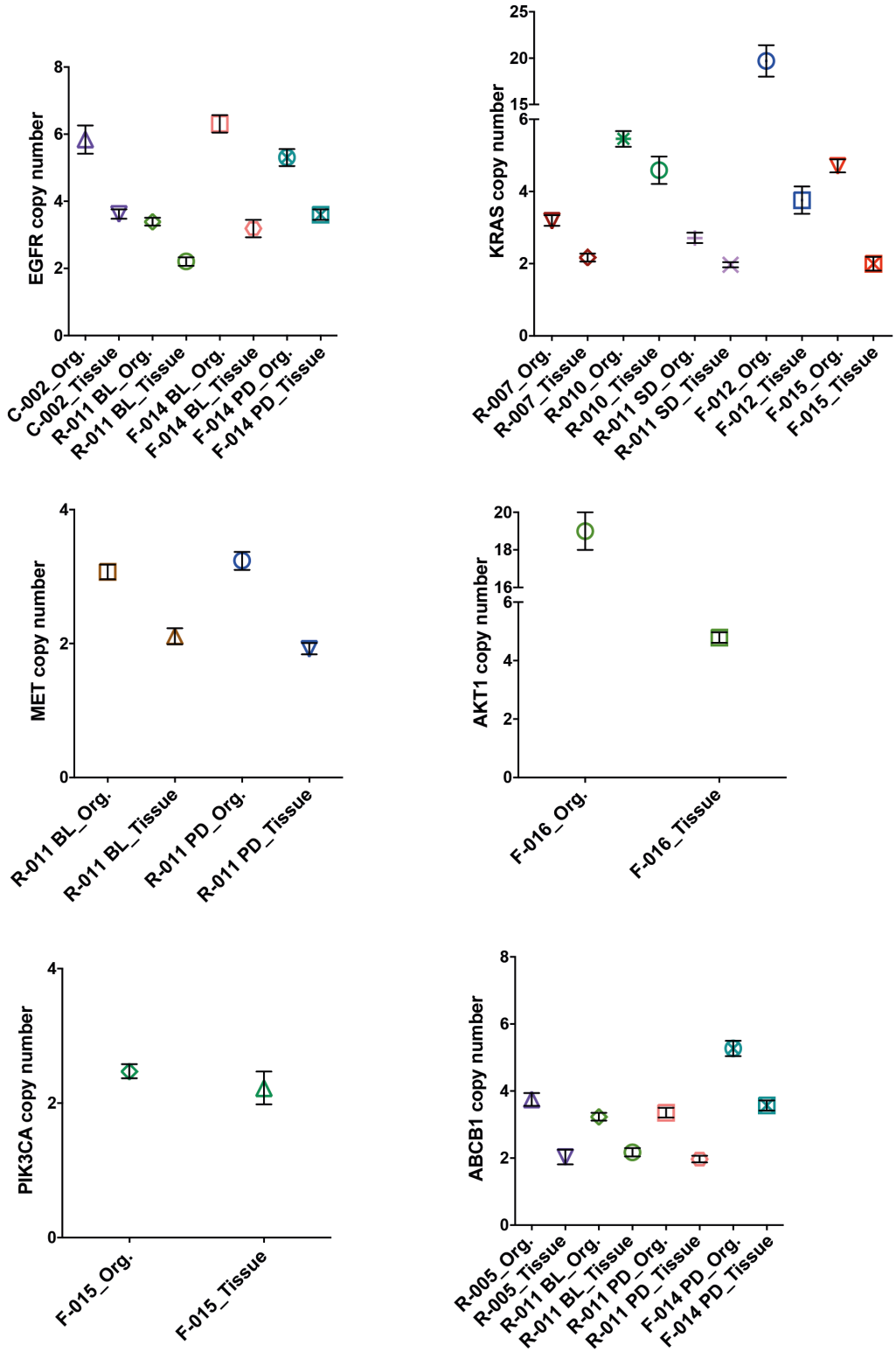

EGFR copy number
C-002_Org.
C-002_Tissue
R-011 BL_Org.
R-011 BL_Tissue
F-014 BL_Org.
F-014 BL_Tissue
F-014 PD_Org.
F-014 PD_Tissue
KRAS copy number
R-007_Org.
R-007_Tissue
R-010_Org.
R-010_Tissue
R-011 SD_Org.
R-011 SD_Tissue
F-012_Org.
F-012_Tissue
F-015_Org.
F-015_Tissue
MET copy number
R-011 BL_Org.
R-011 BL_Tissue
R-011 PD_Org.
R-011 PD_Tissue
AKT1 copy number
F-016_Org.
F-016_Tissue
PIK3CA copy number
F-015_Org.
F-015_Tissue
ABCB1 copy number
R-005_Org.
R-005_Tissue
R-011 BL_Org.
R-011 BL_Tissue
R-011 PD_Org.
R-011 PD_Tissue
F-014 PD_Org.
F-014 PD_Tissue

Fig. S5. Digital-droplet PCR validation of copy number alterations in PDOs and parental biopsies. "Tissue" identifies the parental biopsy.

PDOs= patient-derived organoids; BL=baseline; SD= stable disease; PD= progressive disease.

Suppl. fig. S6

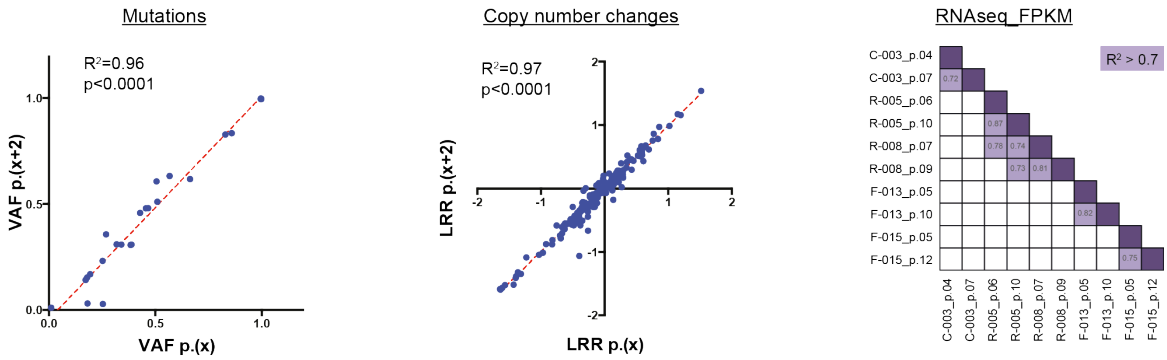


Fig. S6. Molecular features of PDOs show stability over time. Correlation between VAF (4 organoid cultures), CNA (5 organoid cultures), and gene expression (5 organoid cultures) in PDOs' DNA/RNA harvested over 1-4 months of continuous culture.

PDOs= patient-derived organoids; p= passage.

Suppl. fig. S7

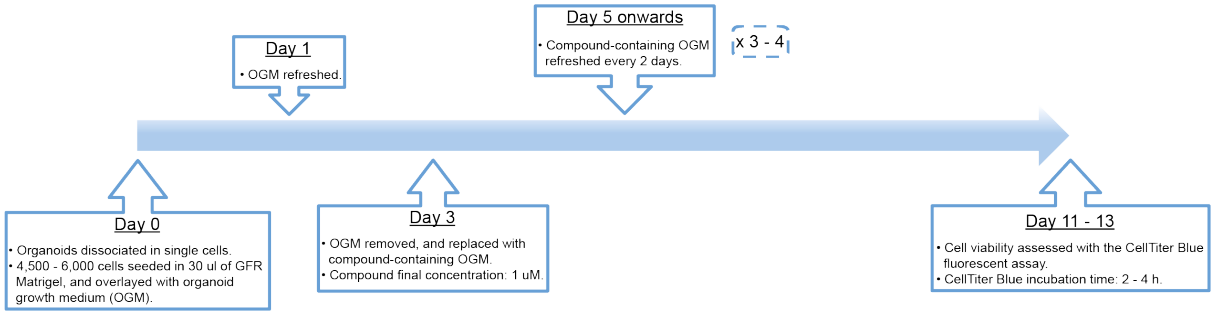


Fig. S7. Outline of the 3D drug screening assay for PDOs. PDOs were screened in a fully 3D assay, using a custom library of 55 drugs currently tested in phase I-III clinical trials, or used in clinical practice. Assay endpoint was PDOs cell viability.

PDOs= patient-derived organoids.

Suppl. fig. S8

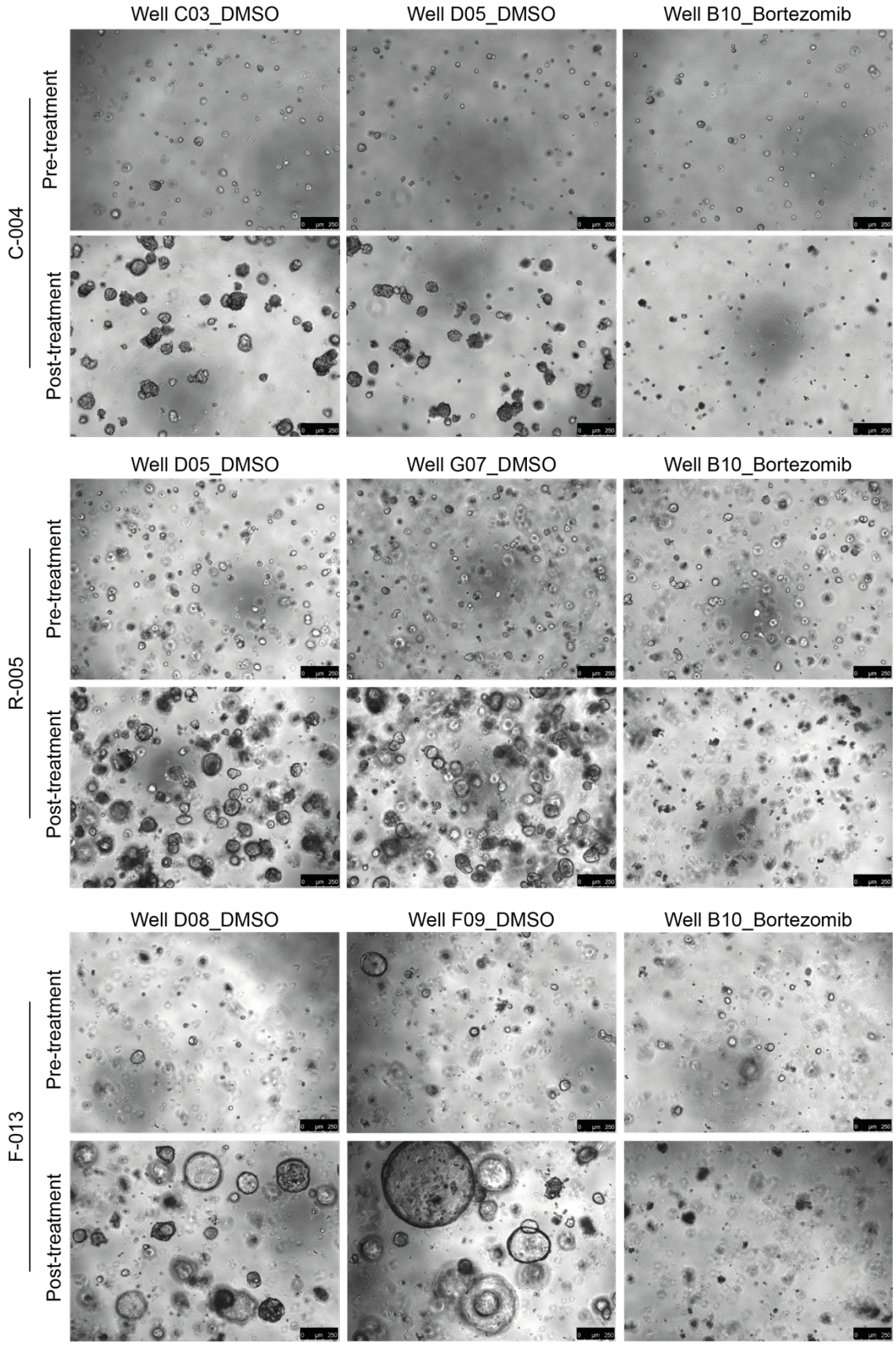

Well C03_DMSO
Well D05_DMSO
Well B10_Bortezomib
C-004
Pre-treatment
Post-treatment
Well D05_DMSO
Well G07_DMSO
Well B10_Bortezomib
R-005
Pre-treatment
Post-treatment
Well D08_DMSO
Well F09_DMSO
Well B10_Bortezomib
F-013
Pre-treatment
Post-treatment

Fig. S8. Examples of cell death in the 3D drug screening assays. Pre- and post-treatment phase-contrast images of two wells treated with DMSO, and one well treated with bortezomib (1μM), in three of our drug screens.

Suppl. fig. S9

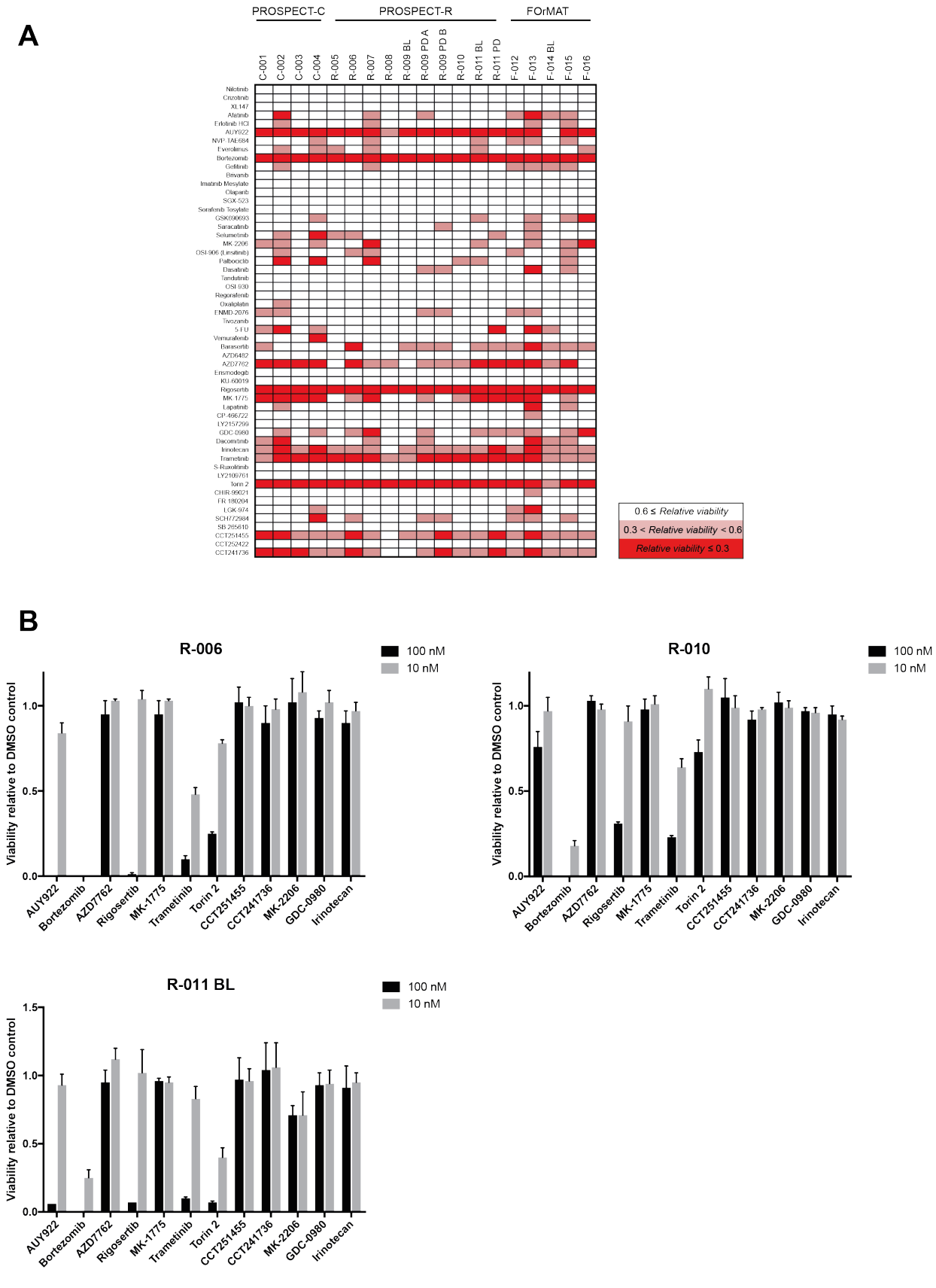
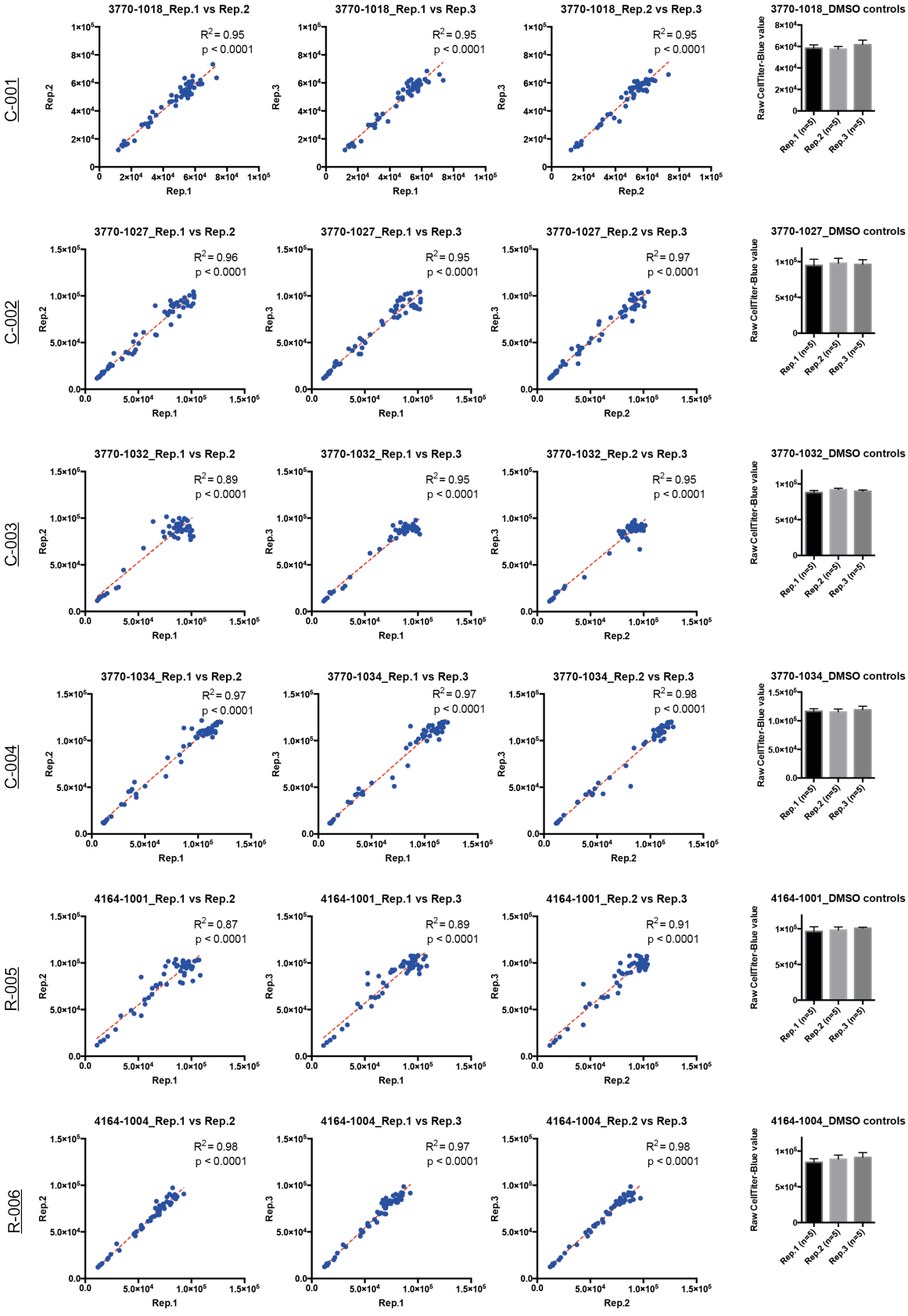
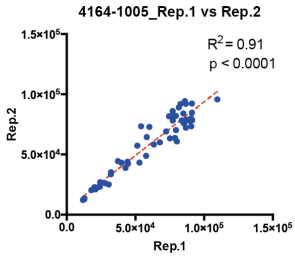

A
PROSPECT-C
PROSPECT-R
FOrMAT
C-001
C-002
C-003
C-004
R-005
R-006
R-007
R-008
R-009 BL
R-009 PD A
R-009 PD B
R-010
R-011 BL
R-011 PD
F-012
F-013
F-014 BL
F-015
F-016
Nilotinib
Crizotinib
XL147
Afatinib
Erlotinib HCl
AUY922
NVP-TAE684
Everolimus
Bortezomib
Gefitinib
Binimib
Imatinib Mesylate
Olaparib
SGX-523
Sorafenib Tosylate
GSK690693
Saracatinib
Selumetinib
MK-2206
OSI-906 (Linsitinib)
Palbociclib
Dasatinib
Tandutinib
OSI-930
Regorafenib
Oxaliplatin
ENMD-2076
Tivozanib
5-FU
Vemurafenib
Barasertib
AZD6482
AZD7762
Ensmodegib
KU-60019
Rigosertib
MK-1775
Lapatinib
CP-466722
LY2157299
GDC-0980
Dacomitinib
Irinotecan
Trametinib
S-Ruxolitinib
LY2109761
Torin 2
CHIR-99021
FR-180204
LGK-974
SCH772984
SB 265610
CCT251455
CCT252422
CCT241736
0.6 ≤ Relative viability
0.3 < Relative viability < 0.6
Relative viability ≤ 0.3
B
R-006
R-010
R-011 BL
100 nM
10 nM
Viability relative to DMSO control
0.0
0.5
1.0
1.5
AUY922
Bortezomib
AZD7762
Rigosertib
MK-1775
Trametinib
Torin 2
CCT251455
CCT241736
MK-2206
GDC-0980
Irinotecan

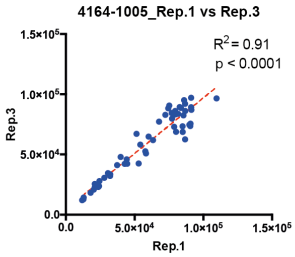
Fig. S9. Results of the 3D drug screening assay for PDOs. (**A**) Heatmap summarizing the results of the 3D drug screening assays using a custom library of 55 drugs tested in clinical trials, or used in clinical practice for tumors of various origins. **(B)** "Hit" validation at lower concentrations. 12 compounds that showed broad efficacy in our drug screening assays at 1 μM were tested at 100 nM and 10 nM in three different PDOs. Data are shown as mean ± standard deviation from single experiments performed in triplicate.

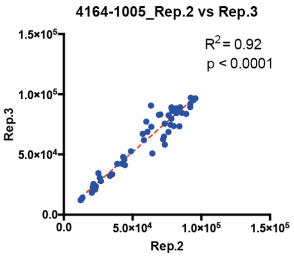
Suppl. fig. S10

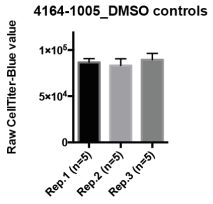


R-007

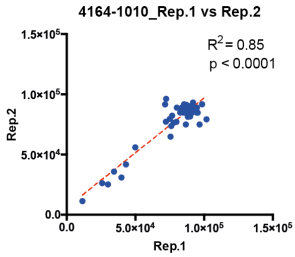


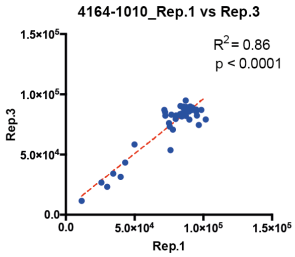


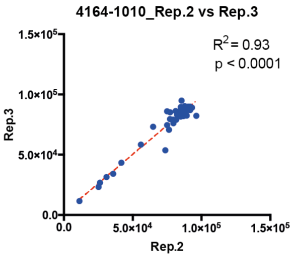


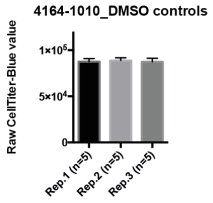


R-008

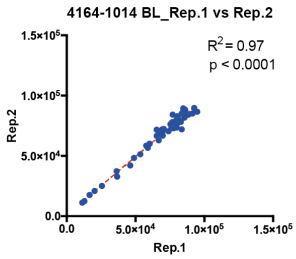


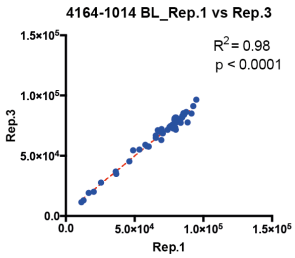


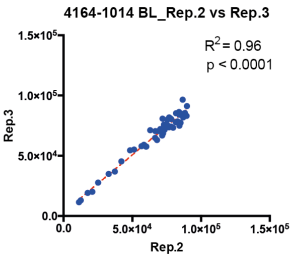


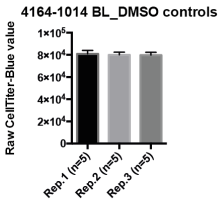


R-009 BL

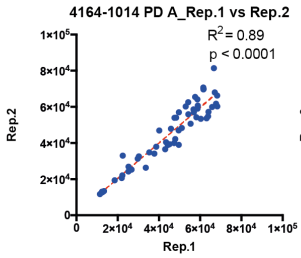


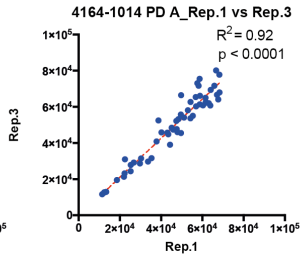


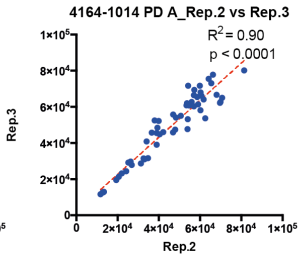


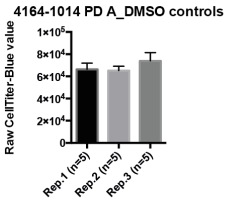


R-009 PD A

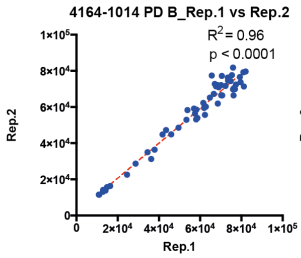


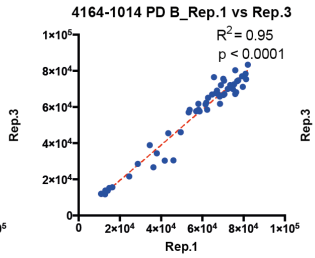


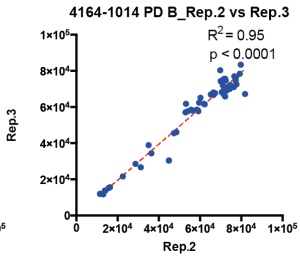


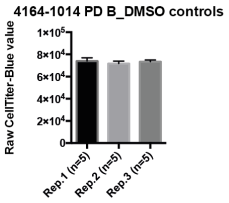


R-009 PD B

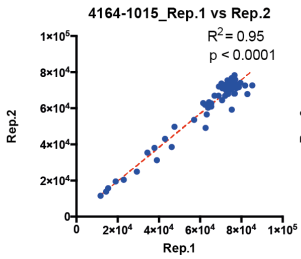


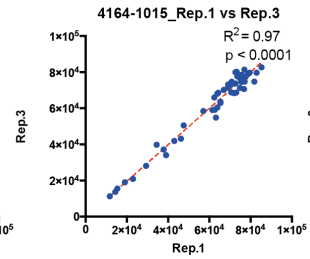


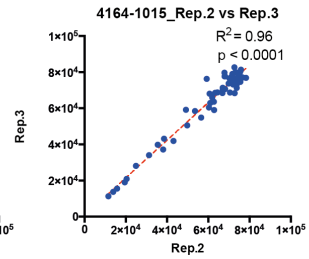


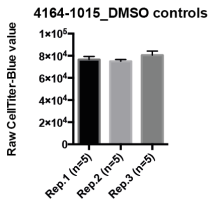


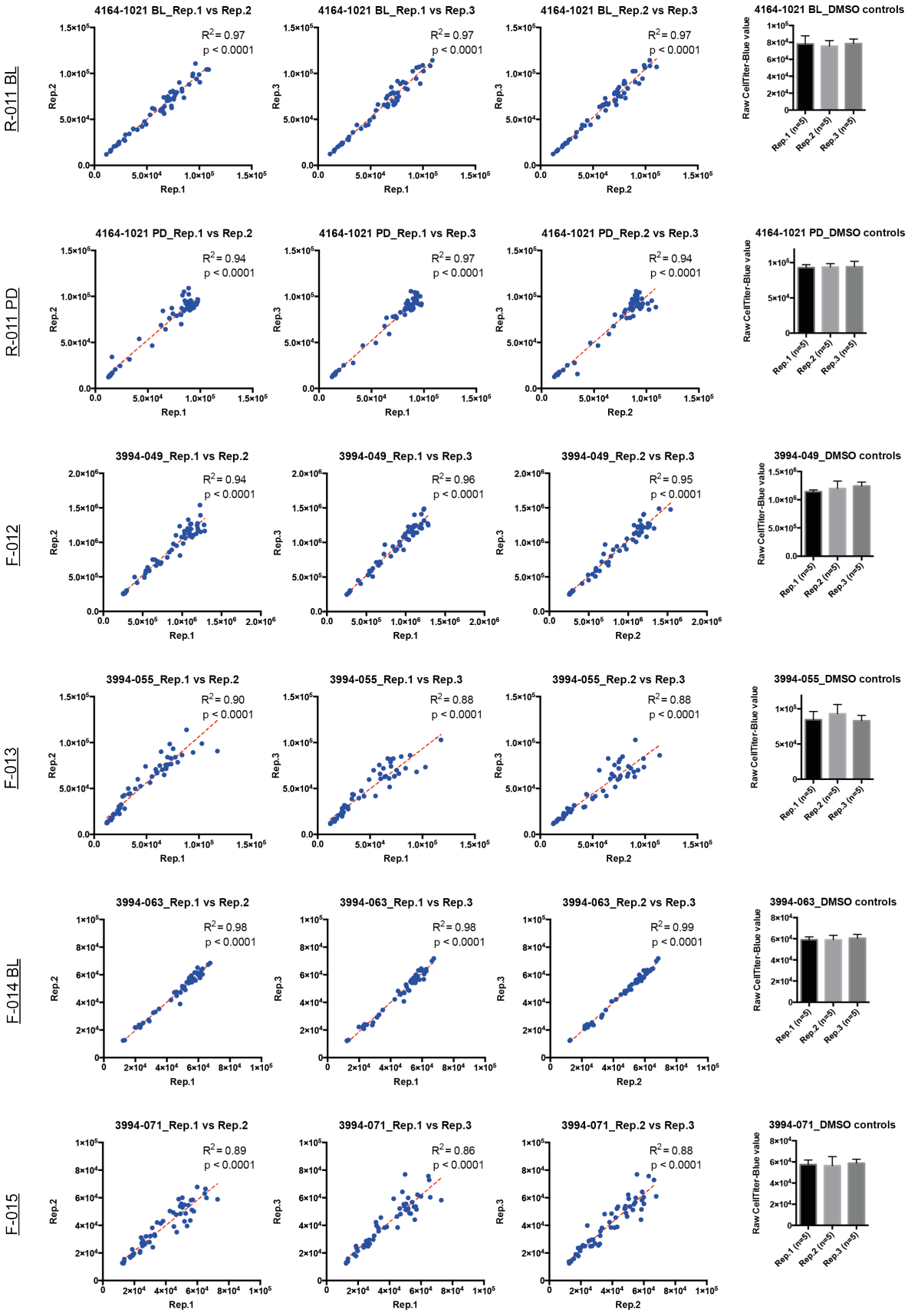
R-010










R-011 BL
4164-1021 BL_Rep.1 vs Rep.2
R² = 0.97
p < 0.0001
Rep.2
Rep.1
4164-1021 BL_Rep.1 vs Rep.3
R² = 0.97
p < 0.0001
Rep.3
Rep.1
4164-1021 BL_Rep.2 vs Rep.3
R² = 0.97
p < 0.0001
Rep.3
Rep.2
4164-1021 BL_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)
R-011 PD
4164-1021 PD_Rep.1 vs Rep.2
R² = 0.94
p < 0.0001
Rep.2
Rep.1
4164-1021 PD_Rep.1 vs Rep.3
R² = 0.97
p < 0.0001
Rep.3
Rep.1
4164-1021 PD_Rep.2 vs Rep.3
R² = 0.94
p < 0.0001
Rep.3
Rep.2
4164-1021 PD_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)
F-012
3994-049_Rep.1 vs Rep.2
R² = 0.94
p < 0.0001
Rep.2
Rep.1
3994-049_Rep.1 vs Rep.3
R² = 0.96
p < 0.0001
Rep.3
Rep.1
3994-049_Rep.2 vs Rep.3
R² = 0.95
p < 0.0001
Rep.3
Rep.2
3994-049_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)
F-013
3994-055_Rep.1 vs Rep.2
R² = 0.90
p < 0.0001
Rep.2
Rep.1
3994-055_Rep.1 vs Rep.3
R² = 0.88
p < 0.0001
Rep.3
Rep.1
3994-055_Rep.2 vs Rep.3
R² = 0.88
p < 0.0001
Rep.3
Rep.2
3994-055_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)
F-014 BL
3994-063_Rep.1 vs Rep.2
R² = 0.98
p < 0.0001
Rep.2
Rep.1
3994-063_Rep.1 vs Rep.3
R² = 0.98
p < 0.0001
Rep.3
Rep.1
3994-063_Rep.2 vs Rep.3
R² = 0.99
p < 0.0001
Rep.3
Rep.2
3994-063_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)
F-015
3994-071_Rep.1 vs Rep.2
R² = 0.89
p < 0.0001
Rep.2
Rep.1
3994-071_Rep.1 vs Rep.3
R² = 0.86
p < 0.0001
Rep.3
Rep.1
3994-071_Rep.2 vs Rep.3
R² = 0.88
p < 0.0001
Rep.3
Rep.2
3994-071_DMSO controls
Raw CellTiter-Blue value
Rep.1 (n=5)
Rep.2 (n=5)
Rep.3 (n=5)

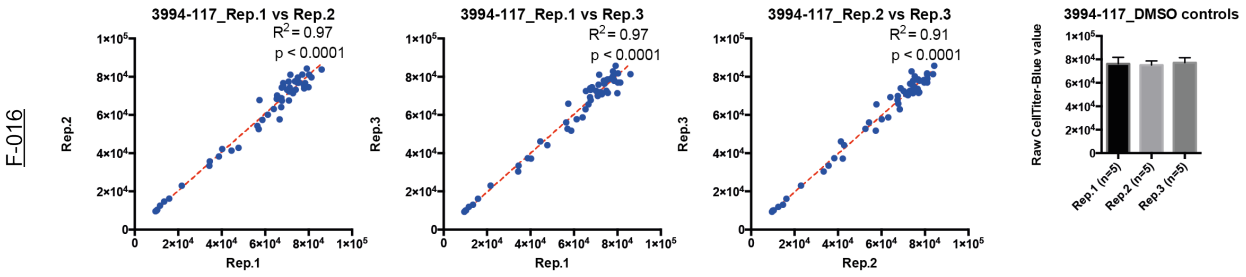


Fig. S10. Concordance among triplicates in the 3D drug screening assays. Concordance among viability readings obtained from the three replicates of each screening assay (for the 55 compounds tested). Bar charts show the mean ± standard deviation for the viability readings obtained from the DMSO-treated wells (n=5) present in each of the three replicates.

Suppl. fig. S11

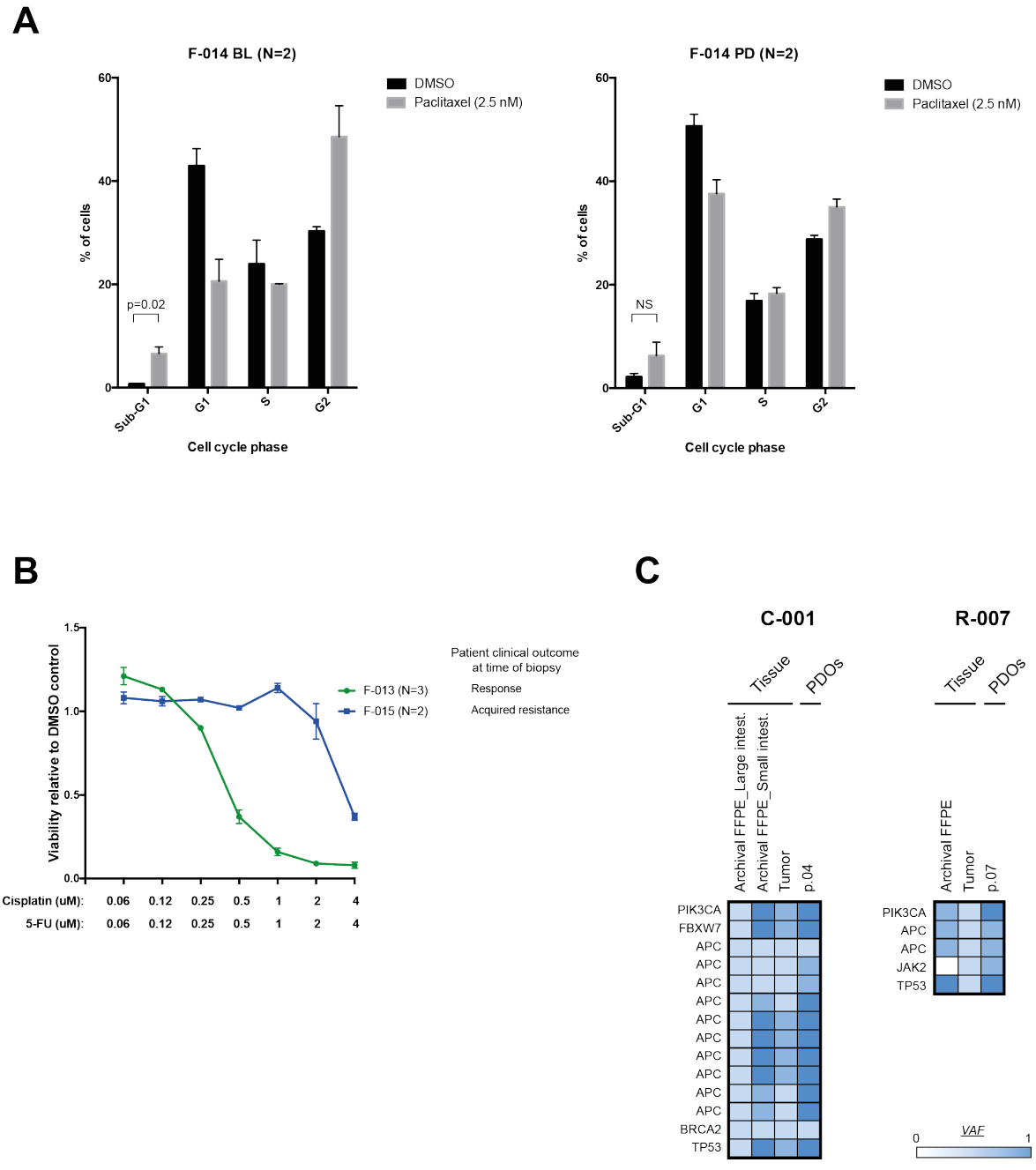


Fig. S11. ***Ex vivo*** **co-clinical trials in mGOC and mCRC.** (**A**) Cell cycle analysis in BL and PD PDOs from the same liver metastasis of a mGOC patient (F-014) treated with second line paclitaxel in the FOrMAT trial. Apoptosis (sub-G1) and significant G2 arrest were observed in

BL PDOs established from the metastasis that responded to paclitaxel, but not in the PD PDOs established at the time of disease progression to paclitaxel. The mean ± SD of two independent FACS experiments is shown. Statistical significance was determined using Student's t-test (**B**) *In vitro* response to chemotherapy combination for PR organoids established from a mGOC patient responding to chemotherapy (F-013), versus PD organoids collected from a second mGOC patient at time of progression to chemotherapy (F-015) in the FOrMAT trial. Data are shown as mean ± SEM from multiple independent experiments (N; indicated in the graph). (**C**) Molecular analysis of PDOs, matching biopsy (tumor), and primary bowel cancer (archival), for two additional mCRC PDOs treated with cetuximab *ex vivo*.

PDOs= patient-derived organoids; BL= baseline; PR= partial response; PD= progressive disease; mGOC= metastatic gastroesophageal cancer; SD= standard deviation; SEM= standard error mean.

Suppl. fig. S12

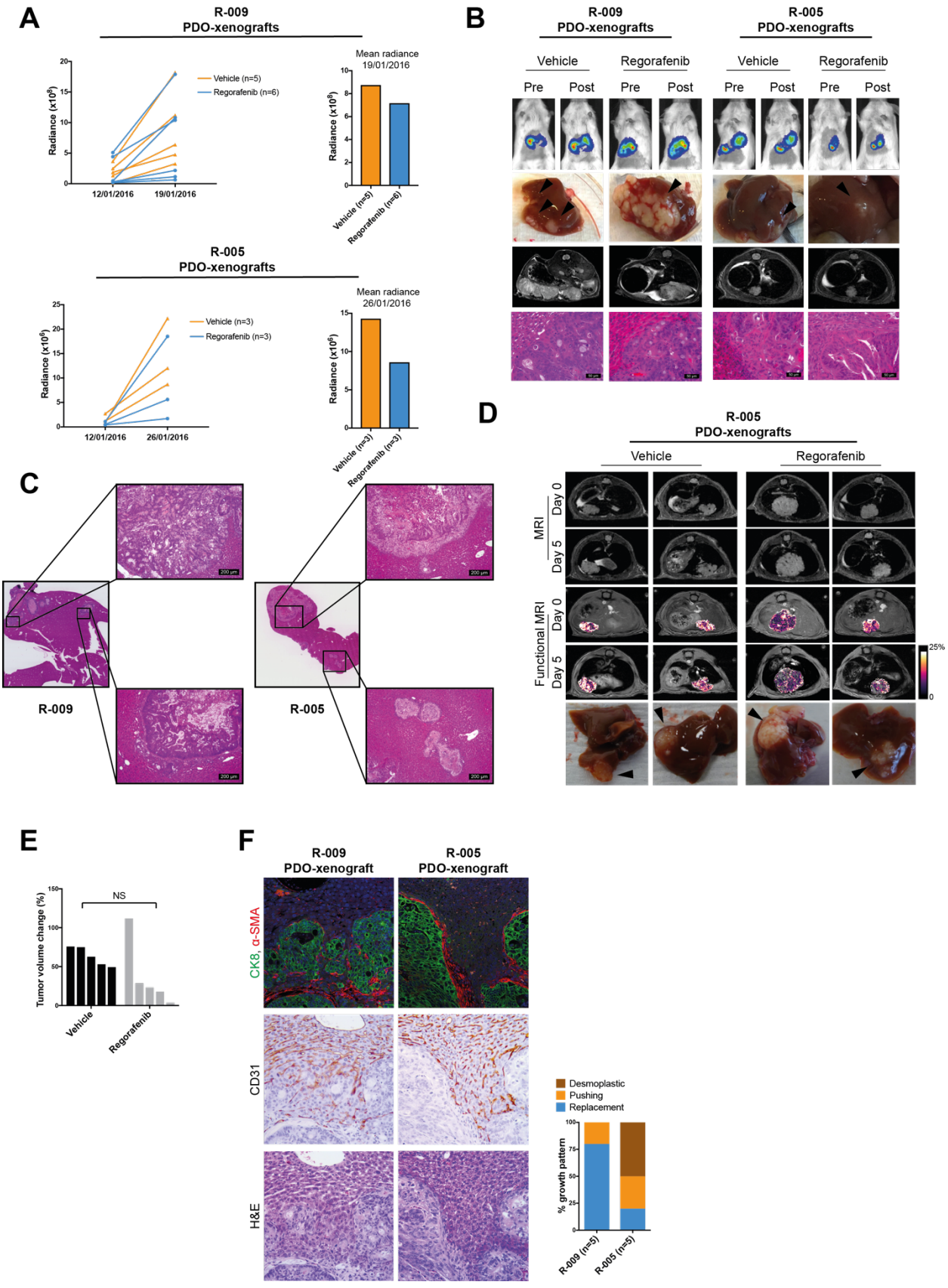


Fig. S12. PDO-based liver orthotopic xenograft tumor mouse models. (**A**&**B**) Luc+ PDOs from a regorafenib-resistant (R-009) and a long-term responder (R-005) patient were implanted orthotopically in the liver of NSG mice. Mice were monitored by longitudinal bioluminescence imaging, randomized in two groups with similar median IVIS radiance, and treated with regorafenib or vehicle for 5 days. n= number of animals analyzed. (**C**) PDOs maintained their ability to metastasize within the liver, even after several *in vitro* passages. (**D**) Anatomical and susceptibility-contrast MRI were performed prior to and after regorafenib treatment in a second cohort of mice carrying long-term responder tumor (R-005) PDO-xenografts. Representative examples of changes in tumor fractional blood volume in response to regorafenib are indicated in the functional MRI panel. (**E**) In line with clinical data, no significant changes in tumor volume were observed in regorafenib-treated mice carrying R-005 PDO-xenografts compared to vehicle-treated animals. Statistical significance was determined using Student's unpaired t-test. (**F**) Analysis of histopathological growth patterns (HGP) in mice carrying regorafenib-resistant (R-009) and responsive (R-005) PDO-xenografts revealed a predominance of replacement HGP in the resistant group, suggesting vessel co-option as a potential mechanism of primary resistance to regorafenib.

PDOs= patient-derived organoids; NSG= NOD *scid* gamma; IVIS= *in vivo* imaging system; BL= baseline; PD= progressive disease; HGP= histopathological growth pattern; α-SMA= mouse alpha smooth muscle actin (fibroblast marker); CK8= human cytokeratin 8 (epithelial tumor marker).

Suppl. fig. S13

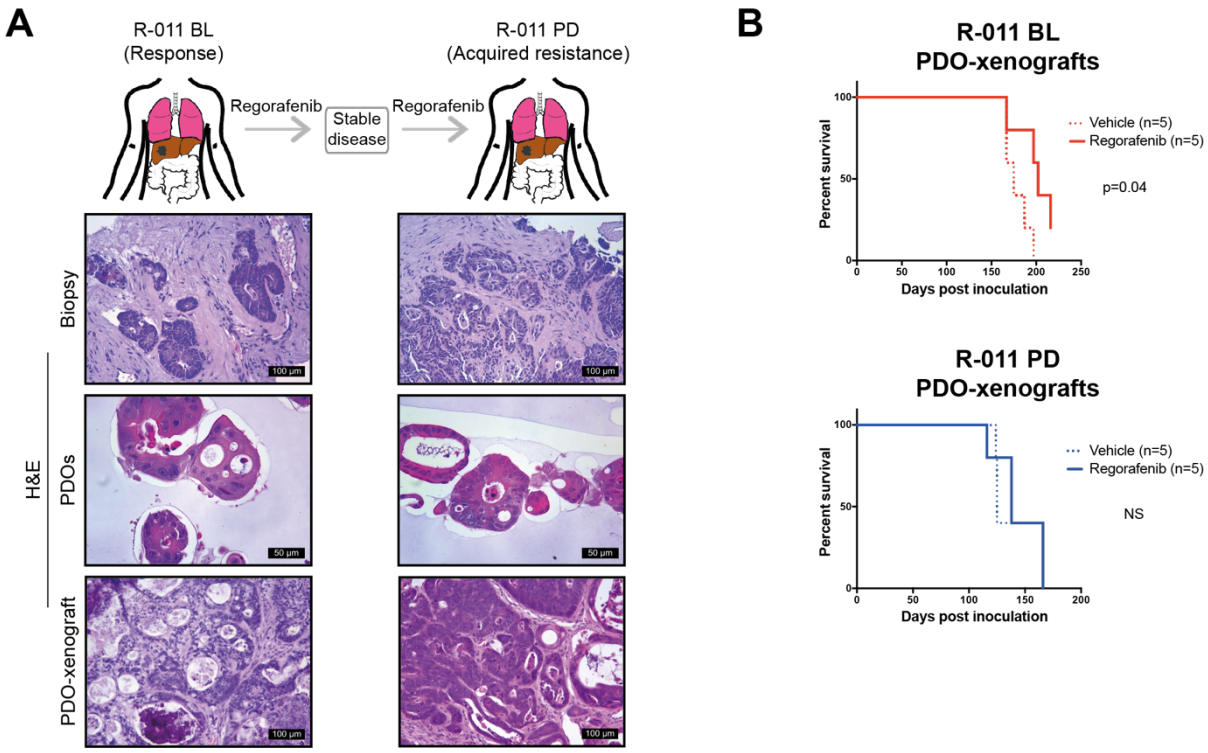


Fig. S13. Survival study using PDO-based liver orthotopic xenograft tumor mouse models. (**A**) A chemo-refractory mCRC patient (R-011) was treated with regorafenib for 4 months; two months post treatment initiation the patient's scan showed stable disease (SD), and then disease progressed on the subsequent scan. Liver biopsies were performed pre- (BL) and post (PD) treatment as well as at stable disease (SD), and PDOs were established at each time point. (**B**) Kaplan-Mayer curves of regorafenib- or vehicle-treated mice bearing BL and PD R-011 PDO-xenografts calculated from the date of tumor inoculation; regorafenib treatment was associated with a selective survival benefit in mice carrying R-011 BL PDO-xenografts. Statistical significance was determined using the Mantel-Cox log-rank test; n= number of mice analyzed.

PDOs= patient-derived organoids; BL= baseline; SD= stable disease; PD= progressive disease.