Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors

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Colorectal carcinoma represents a heterogeneous entity, with only a fraction of the tumours responding to available therapies, requiring a better molecular understanding of the disease in precision oncology. To address this challenge, the OncoTrack consortium recruited 106 CRC patients (stages I-IV) and developed a pre-clinical platform generating a compendium of drug sensitivity data totalling >4,000 assays testing 16 clinical drugs on patient-derived in vivo and in vitro models. This large biobank of 106 tumours, 35 organoids and 59 xenografts, with extensive omics data comparing donor tumours and derived models provides a resource for advancing our understanding of CRC. Models recapitulate many of the genetic and transcriptomic features of the donors, but defined less complex molecular sub-groups because of the loss of human stroma. Linking molecular profiles with drug sensitivity patterns identifies novel biomarkers, including a signature outperforming RAS/RAF mutations in predicting sensitivity to the EGFR inhibitor cetuximab.
C olorectal cancer (CRC) is a clinically challenging, heterogeneous, disease representing the third most frequent cancer worldwide. CRCs can be classified within distinct molecular groups, although the clinical utility of this classification has not been demonstrated so far\(^1^–^5\). Only a fraction of advanced CRCs respond to the chemotherapeutic agents 5-fluorouracil (5-FU), irinotecan or oxaliplatin. Antibodies targeting the epidermal growth factor receptor (EGFR) offer therapeutic options, but have failed in the adjuvant setting\(^6\). BRAF, KRAS and NRAS (ref. 7) mutations are routinely used as predictive markers of resistance to the EGFR blockade. However a significant fraction of wild-type tumours remain unresponsive to cetuximab targeting EGFR (ref. 8,9) thus requiring novel biomarkers predicting treatment outcomes.

Several pre-clinical studies based on in vivo or in vitro models of CRC have been reported\(^10^–^16\), but without investigating their complex molecular landscapes nor comparing directly the different model systems.

Here we report an integrative pre-clinical approach based on the establishment and extensive molecular characterization of a large CRC biobank consisting of organoids and xenografts derived from a cohort of 106 patients representative of all CRC subtypes. Analysis of the responses of in vitro and in vivo models to a panel of clinically relevant therapeutic agents identifies gene signatures associated with objective drug response patterns.

Results

Establishment of the OncoTrack CRC pre-clinical platform.

The workflow of the OncoTrack (OT) study is summarized in Fig. 1. We collected from a prospective CRC cohort of 106 patients a total of 116 resected tissue samples with matched blood samples, comprising 89 primary tumours (ranging from stage I to IV) and 27 metastases as donors for generating a biobank of pre-clinical experimental models. We established in vitro and in vivo models with a success rate of approximately 60% in both systems. BRAF-mutated tumours engrafted with a higher efficiency (10/11 cases Fisher’s exact test \(P = 0.04\)), likely reflecting their aggressive behaviour\(^17\). Here, we report the analysis of 46 patient-derived organoid cultures (PDO) and 59 xenografts (PDX) (Supplementary Data 1). Nineteen tumours were modelled in both systems, out of which five PDOs were derived from a PDX and two PDXs were established from a PDO. The topological expression of selected CRC markers was similar between models and their matched donor specimens (Supplementary Fig. 1a). PDO cultures formed organized structures featuring a cell-free lumen and proliferating Ki67-positive cells, maintained after transfer into 384-well microtiter plates (Supplementary Fig. 1b; Supplementary Movie 1). We sequenced the genomes, exomes and transcriptomes of the donor cohort and of their matched untreated models, and established a drug-screening platform testing mechanistic compounds and chemotherapeutics, used in clinical standard of care.

Molecular landscapes of the OT tumours and derived models.

We compared the genomic and transcriptome landscapes of the OT tumours with their derived pre-clinical models by integrating whole genome (WGS), whole exome (WES) and RNA sequencing data. We inferred the tumour purity from WGS data (Supplementary Data 1) and excluded samples with <20% tumour content for the mutation scoring (final \(n = 101\) samples/96 patients) (Methods and Supplementary Data 2–4). We called copy number variants (CNVs) and somatic mutations using matched patient germline DNAs as reference. Relevant mutations were sieved based on their expression and predicted damaging effects (methods and Supplementary Data 3). Microsatellite-instable (MSI) samples were near-diploid, whereas microsatellite-stable (MSS) samples were either hyperdiploid (40 cases) or hypodiploid (43 cases) with pervasive loss of heterozygosity (LOH) (Supplementary Fig. 2a). Deletions and focal amplifications were maintained in the models (Supplementary Data 3–5), while chromosomal instability was further accentuated (Supplementary Fig. 2b). We detected a total of 145 gene fusions, including the known driver event PTPRK-RSP03 (ref. 18) (Supplementary Data 4). Novel fusions impacting CRC-relevant pathways inactivated APC or SMAD4, or were activating fusions such as a TRIM24-BRAF (in 196_T MSI) (Fig. 2a) predicted to trigger the conformational activation of the BRAF serine/threonine kinase domain, as observed in pilocytic astrocytoma\(^19,20\) and melanoma\(^21\).

FDFT1-FZD3 (Fig. 2a) and truncating fusions in the negative regulators of Wnt ZNRF3 and DACH1, were predicted to activate the Wnt pathway. Recurrent fusions truncated the haploinsufficient chromatin organizer TCF7 and the solute carrier SLC12A2 (NKCC1) regulating the Cl\(^–\) flux in the intestinal crypt, but their contribution to CRC pathogenesis remains to be demonstrated. One xenograft harboured an ALK fusion as sole driver event (Supplementary Data 4), however we lacked the corresponding patient tumour.

The mutational profiles of the OT cohort and of the TCGA study\(^22\) were very similar (Fig. 2b), demonstrating that our cohort represented the breadth of the CRC genetic landscape and that metastatic tumours did not show a biased mutation pattern. Nonetheless, the OT cohort displayed higher frequency of mutations in SOX9, maintaining the intestinal cell progenitors pool, (13 versus 4% in TCGA), and in TP53 (71 versus 51% in TCGA) (Supplementary Fig. 3a,b) (Fisher’s exact test, Benjamini-Hochberg (BH) adjusted \(P = 0.04\) and 0.02, respectively) overrepresented in stages III and IV (Fisher’s exact test, \(P = 0.012\)) and often homozygous in hyperdiploid tumours (Student’s t-test, \(P = 0.0008\)).

The genetic profiles of the models were generally concordant with their matched donor tumours. However, a number of models displayed clonal and sub-clonal differences irrespective of the tumour stage, with cases of extreme divergences such as for 118_T1 and 165_T and the sibling models derived from 227_T (Fig. 2c; Supplementary Data 6). These differences were more likely reflecting the intra-tumour heterogeneity (ITH) with different mutations found in distant regions of the tumour sampled during model establishment than a genetic drift after serial passages. Early and late passage cell cultures showed virtually identical mutation patterns (Supplementary Fig. 4a,b), as well as five PDXs derived from tumour 150_MET1 (Supplementary Data 7), supporting this hypothesis. Along those lines, a comparable molecular analysis of breast cancer models showed only minimal clonal selection after serial transplantations\(^23\). Interestingly, we observed no discordances for driver mutations in BRAF and KRAS, although 118_MET1 and 227_T donor had KRAS homozygous mutations, whereas their respective models were heterozygous, reflecting ITH for CNVs. Clonality analysis with SciClone\(^24\) identified mutation clusters private to either patient or model, or to one of the sibling models (Fig. 3a, Supplementary Fig. 5 and Supplementary Data 8). Only 3% of the divergent mutations impacted cancer relevant genes\(^25,26\) (Fig. 3a,b), similar to previously reported CRC organoids\(^21\). For example, mutations in EGFR and MLL2 were private to 327_T_PDO, whereas tumour 278_T and its models diverged for mutations in FAM123B, M TOR, and for a P TCH1 frameshift mutation predicted to activate oncogenic sonic hedgehog signalling (Fig. 3b; Supplementary Data 6). Several discordant mutations were either redundant or functionally equivalent. Sample 323 displayed four different mutations in PIK3CA, with R88Q common to both models, A775S private to
PDO, whereas G1049R was common to models and donor. Discordant mutations in APC were all deleterious, therefore functionally equivalent. These data indicated that pre-clinical models might capture only part of the genetic heterogeneity of the CRC bulk donor tumours, but retained systematically RAS/RAF mutations.

Transcriptome landscapes of tumours and their derived models. We analysed the global transcriptome profiles of the OT patient tumours and derived models at several levels. Annotation with the CRC consensus molecular group labels (CMS1 to CMS4) (ref. 2) led to unambiguous classification for only 50/90 patient samples. Further, this classification appeared too coarse for the models, in particular for the organoids (Methods, Supplementary Data 9; Supplementary Fig. 6a,b). Given that we aimed at comparing in details the transcriptome profiles between tumours and model, we analysed de novo the RNAseq data from 65 OT primary tumours, excluding at this stage low purity (< 40%) or metastatic samples to minimize confounding contribution of surrounding tissues (see methods). In a first step, we applied non-negative matrix factorization (NMF) and CLICK algorithms. NMF identified...
Figure 2 | Genomic landscape of the OT patient and model cohorts. (a) Examples of gene fusions, either deleterious (ME2-SMAD4 and APC-REEP5, blue) or activating (TRIM24-BRAF and FDFT1-FZD3, red); schematics display chromosomal location, fusion partners, exon structure (blue, red and grey filled boxes), fusion breakpoint (red line), validation by Sanger sequencing and RNA read coverage. (b) Landscape of the most recurrent somatic alterations in 101 OT primary and metastasis samples (tumour purity of ≥20%) from 96 patients compared with the mutation pattern in 228 primary CRC tumours from TCGA (ref. 25) (SNV + Indel). Genes are grouped according to biological pathways. MSI and hypermutated samples are shaded in grey. Tumour stages are coded in grey shades on top of the matrix. Metastasis and primary samples are indicated respectively in purple and green boxes on top of the matrix. Alteration types are colour-coded as indicated. Obtained PDX and PDO models are depicted in blue and yellow, respectively at the bottom of the matrix. Darker shades correspond to the models shown in Fig. 2c. (c) Fraction of damaging and expressed somatic SNVs/Indels found discordant between original patient tumours and matched models, comparing 56 patient samples (tumour purity ≥40%) and their corresponding ex vivo models (37 PDX and 37 PDO), as well as five PDX-PDO siblings without matching patient samples. Dot colours indicate the following: patient versus PDX (blue), patient versus PDO (yellow) or PDX versus PDO (red). Samples described in Fig. 3b are marked in bold. MSI and hypermutated samples are marked with an asterisk. Panels from left to right: patients with an established PDO only; patients with an established PDO only; patients with established PDO and PDX; PDO/PDO siblings without a corresponding sequenced patient tumour. In the cases of tumours 278 and 302, the suffix 'X' represents patient-derived PDX and 'CX' represents PDO-derived PDX.

three main groups, OT_NM1, OT_NM2 and OT_NM3 (Supplementary Data 10; Supplementary Fig. 7a), defined by tumours sharing global biological features (Supplementary Data 11). CLICK identified 13 gene signatures (OT_C1 to OT_C13) (Supplementary Data 10; Supplementary Fig. 7b) corresponding to co-expressed genes in specific tumour sub-
Figure 3 | Molecular discordances between original tumours and derived models in CRC-relevant genes. (a) Examples of clonality analysis based on the sciClone algorithm, for samples 327_T (MSI—40% tumour purity) (upper panel) and 323_T (MSS—60% tumour purity) (lower panel). The plots display the mutations in diploid regions clustered by their allele frequencies: in original tumour versus PDO (left), original tumour versus PDX (middle) and PDX versus PDO (right). Individual mutation clusters were shown by different colours where grey indicated the mutations found in common between the compared samples. The PDO derived from 327_T displayed private EGFR, MLL2 and PIK3CA mutations in cluster 2. The PDO derived from 323_T displayed different clones harbouring private mutations in PIK3CA. (b) Somatic clonal mutations discordant between patients/PDX (left), patients/PDO (middle) and patients/PDX/PDO (right). Genes found mutated in either tumour or model (or in only one model) are marked by red squares. Genes with different mutations in tumours and models are marked by orange squares. Mutations validated by targeted sequencing are indicated with asterisk. MSI and hypermutated samples are shaded in grey: Only cancer-relevant genes are shown, selected from TCGA, PAN or OT recurrently mutated genes resources (see methods). Patients with a purity of ≥40% are shown.

groups, leveraging the depth of functional annotation attributed to tumour groups (Supplementary Data 11). We rationalized the NMF and CLIck clusters in a meta-analysis of 38 gene signatures incorporating previous CRC molecular groups1,2,5. The mean expression values for these 38 signatures were calculated for 90 OT samples, now including metastases (tumour purity ≥40%). Analysis of this matrix with unsupervised hierarchical clustering and mclust76 identified three main molecular groups with high stability referred to herein as ASCL2/MYC, ECM/EMT and Entero/Goblets (Supplementary Fig. 8a–c), respectively related to CMS2, CMS4 and CMS3 (ref. 2), albeit with differences (Supplementary Fig. 8d). MSI samples (CMS1) clustered within Entero/Goblets. ASCL2/MYC (OT_NMF2) featured higher expression of ASCL2, the master regulator of colonic crypt stem cells29, MYC and AURKA potentially contributing to stem cell phenotypes30, and CDX2 promoting Wnt signalling (Supplementary Fig. 9). This group showed a trend for wild-type BRAF and MACROD2 deletions (Fisher’s exact test, \(P = 0.01\) and 0.02, respectively) and was associated with a specific DNA methylation pattern, namely cluster_4 (\(P = 0.0057\), Chi-square test) (Supplementary Fig. 10). ECM/EMT (OT_NMF1) was characterized by TGFβ and sonic hedgehog signalling, extracellular matrix (ECM), epithelial to mesenchymal transition (EMT), inflammation, enteric neurons and smooth muscle cell markers reflecting tumour invasion in the intestinal myoenteric layer31,32. Entero/Goblets (OT_NMF3), associated with the CIMP-High methylation pattern (Supplementary Fig. 10), had strong features of epithelial enterocytes, entero-endocrine and goblets cells, carbonate dehydratase activity, and higher levels of AGR2, known to promote adenocarcinomas growth33 (Supplementary Fig. 9). Entero/Goblets were mainly early stage tumours whereas ASCL2/MYC and ECM/EMT were rather late stages (Supplementary Fig. 8e). Data highlighted different stromal and immune environments among tumours of the same group (Supplementary Fig. 8b–8f). Entero/Goblets tumours were associated with Th17 cytokines, where MSI cases were unique in expressing innate immunity signatures (for example, OT_C8) (Supplementary Fig. 11a,b). ASCL2/MYC tumours displayed the lowest immune infiltration, in contrast to the highly inflamed tumours of the ECM/EMT group showing a maximal tumour purity of 50% (Methods, Supplementary Fig. 11a,b and Supplementary Data 12).

By comparison, models defined only two main molecular groups, either NMFA strongly enriched for Wnt and stemness processes pointing to ASCL2/MYC, or NMFB corresponding to colonic epithelial cells and entero/goblets (Fig. 4a, Supplementary Fig. 12a). Both in vitro and in vivo model systems lacked ECM, stromal components and human immune-related signatures (Fig. 4a, Supplementary Data 12), whereas showing prominent aurora kinase pathway/crypt progenitors, lipid metabolism and oxidative respiration signatures (OT_C2, OT_C4 and OT_C10), in part because of their higher tumour content. However, the two model types exhibited significant differences (Fig. 4b). Within the limits of the missing human stroma, the main features of the donor tissues were preserved in PDXs where ASCL2/MYC- and ECM/EMT-derived xenografts grouped in NMFA, whereas those engrafted from Entero/Goblets were NMFB. PDOs showed a more complex picture (Fig. 4c), where ASCL2/MYC-derived cells mostly featured the NMFB pattern. Despite the limited number of sibling models with this phenotype, data suggested heterogeneity/plasticity in cell cultures. As a proof of principle, we combined a Wnt reporter assay with RNaseq on PDOs derived from 151_MET1 (ECM/EMT). Data demonstrated the co-existence of two genetically identical cell sub-populations, respectively...
presenting either high-Wnt signalling or low Wnt with epithelial features (Fig. 4d; Supplementary Data 13).

Sibling pairs of PDX/PDO differentially expressed signatures for hypoxia, EMT, G2M checkpoints and proliferation (Fig. 5a), as well as stemness markers (Supplementary Fig. 13a), without a trend for a given model type. However, PDOs had significantly higher levels of the stem cell marker ALDH1A1 (Supplementary Fig. 13b) and of components of carbohydrate, steroid, retinoid and fatty acid metabolism (Fig. 5b). SCD encoding Stearoyl-CoA desaturase-1, a key enzyme in fatty acid metabolism involved in cancer cell survival and UGTs (UDP-glycosyltransferases) triggering glucuronidation activating lipid metabolism and mediating drug resistance were more active in organoids than in 2D CRC cell lines (Fig. 5c). This might be because of metabolic adaptation to the culture conditions or to intrinsic features of organoids.
**Patterns of drug response in CRC-derived models.** We monitored the sensitivity of 94 models to 16 drugs, chemotherapeutics and drugs targeting MEK, mTOR, VEGFR, or EGFR pathways (Figs 1 and 6). In PDOs, we considered efficacy ($E_{\text{max}}$), the maximum growth inhibition reached at the highest drug concentration, as well as potency, the half-maximum inhibitory concentration ($IC_{50}$) relative to the reference compound staurosporine, which exhibited low $IC_{50}$/high $E_{\text{max}}$ (Fig. 6a,b). Potency was used to define four response categories, strong-, moderate-, minor response or resistant (Supplementary Fig. 14c), as shown for AZD8931 (Supplementary Fig. 14d). PDOs exhibited a wide range of sensitivities upon treatment with the different drugs (Supplementary Figs 14 and 15a). Chemotherapeutics achieved only poor responses, particularly oxaliplatin which was ineffective. Irinotecan was used here instead of its active derivative SN-38, given that the cells expressed CES2, essential for metabolizing the pro-drug. Responses to irinotecan were marginal, as previously observed in organoids, potentially because of glucuronidation inactivating SN-38 in organoids. Multi-kinase inhibitors (MKIs) triggered minor responses, (Fig. 6a,b). The sensitivity profiles to linifanib, nintedanib and sunitinib were strongly correlated, but less so to sorafenib and regorafenib, a recently approved MKI for metastatic CRC (ref. 36), structurally similar to each other and found together in a different cluster (Fig. 6c). Vandetanib, targeting both VEGFR and EGFR, was the most potent MKI but correlated strongly with EGFR blockade, indicating that the anti-proliferative effects were likely mediated by the EGFR pathway inhibition. Responders to EGFR blockade were mostly KRA/S/BRF wild-type or carried BRAF-G466V showing reduced kinase activity (Supplementary Fig. 16a). EGFR blockade displayed the widest range of effects across samples, as previously observed (Fig. 6a,b; Supplementary Fig. 15a). Cetuximab was ineffective in cells at clinically relevant concentration (Supplementary Fig. 15b; Supplementary Data 14), corroborating earlier reports and data streaming from an independent CRC organoid cohort estimating IC50 in the mmolar range, although biologically meaningful concentrations of cetuximab remain in the mmolar range. Further, only few KRA/S/BRF wild-type CRC 2D cell lines respond to cetuximab. In contrast, PDOs were sensitive to tyrosine kinase inhibitors (TKIs), in particular to atabinib, and their response to AZD8931 was similar to earlier studies. The model 330_T_CELL with ERBB2 amplification was sensitive to AZD8931 (targeting EGFR, ERBB2, ERBB3) and atabinib (targeting EGFR, ERBB2), but less so to gefitinib targeting only EGFR (Supplementary Fig. 16a). PDO 327_T_CELL had a rare

**Figure 5 | Comparative gene expression profiling between PDO and PDX models.** (a) Differentially expressed canonical pathway gene signatures. Positive or negative normalized enrichment scores (NES) calculated for PDX versus PDO for each given pathway are indicated in purple or green shades, respectively. Asterisks indicate significant enrichment with FDR $< 0.005$. The gene set enrichment was determined using a pre-ranked GSEA. Genes were ranked according to their expression fold change and expression difference between the corresponding model pairs. (b) Differential expression of gene signatures for canonical pathways and GO terms in PDO versus tumour, PDX versus tumour and PDO versus PDX. Lines mark the 25%, 50% and 75% quantiles.
Figure 6 | Overview of drug response in PDX and PDO models. (a) Box plot showing the maximum inhibition ($E_{max}$) values for 14 drugs and for the staurosporine control in 35 PDO models. Irinotecan was tested on 21 PDOs. Range of drug efficiency ($E_{max} \geq 50\%$) or inactivity ($E_{max} < 50\%$) is indicated in blue and grey, respectively. (b) Box plot showing the potency of 14 drugs and of the staurosporine control based on log10(IC50) in 35 PDO models. The four response categories are coloured as indicated in the caption. (c) Hierarchical clustering representing the correlation of drug sensitivity patterns based on T/C values in PDO models. (d) Box plot showing the sensitivity of 57 PDX models to 11 drugs based on tumour/control volumes (T/C in %) values: response categories are labelled with the same symbol above each sample name. The vertical dash line shows the cut-off between responders and non-responders. (e) Box plot showing the sensitivity of 53 PDX models to cetuximab based on tumour/control volumes (T/C in %) values: response categories are labelled with the same symbol above each sample name. The vertical dash line shows the cut-off between responders and non-responders. (f) Waterfall plot showing cetuximab sensitivity for 53 PDX and indicating mutations and fusions in RAS/RAF and RTK pathways. Both T/C and RECIST criteria are indicated; models derived from the same patient are labelled with the same symbol above each sample name. The vertical dash line shows the cut-off between responders and non-responders. (g) Analysis of the activating ALK gene fusion in 353_T_XEN; upper part: schematics displaying chromosomal location, fusion partners, exon structure (grey and red boxes), fusion breakpoint (red line), validation by Sanger sequencing and RNA read coverage. Lower part: treatment response of OT PDX 353_T_XEN to crizotinib, cetuximab, AZD8931 and afatinib: mean tumour volume of treated and corresponding controls measured between day 8 and day 36 after treatment.
EGFR mutation (A864V) and was highly sensitive to both AZD8931 and afatinib. Treatment with MEK or mTOR inhibitors achieved objective responses but PDOs displayed different drug response patterns (Fig. 6a–c).

For xenografts, drug response was assessed by the relative tumour volume of the treated PDX versus its matched untreated control (T/C), and classified into four categories strong-, moderate-, minor response or resistant (Fig. 6d, Supplementary Data 14). Among chemotherapeutic agents, the outstanding response to irinotecan was uninformative given the intense metabolic activation of the pro-drug in the mouse. In contrast, the anti-metabolite drug 5-FU, that has comparable serum pharmacokinetics in human patients and nudes, was the most efficient chemotherapy in PDXs (Fig. 6d; Supplementary Fig. 15c). The three compounds targeting angiogenesis triggered objective responses (Fig. 6d). Mechanistically, VEGFα was expressed in PDXs, but not the human VEGF receptors (FLT1, FLT3, KDR), which were however replaced by the murine compartment (Supplementary Fig. 17), supporting active VEGF signalling via murine endothelial cells. The response pattern to regorafenib43 correlated strongly with nintedanib targeting VEGFR, but only weakly with Avastin, a monoclonal antibody inhibiting human VEGFα (Fig. 6e). Interestingly, targeted inhibition of mTOR (mTORC1/C2) correlated with nintedanib and regorafenib response profiles (Fig. 6e), which might be mechanistically related to the contribution of mTORC2 as a critical signalling node for VEGF-mediated angiogenesis. The MEK inhibitor selumetinib displayed a response profile different to that of all other drugs.

The EGFR blockade achieved tumour growth inhibition >50% for one third of the xenografts (Fig. 6d, Supplementary Fig. 15c, Supplementary Data 14). Responses to TKIs targeting the intracellular domain of EGFR and other ERBB family members were comparable with each other but less so with the selective EGFR antibody cetuximab, performing best in achieving a substantial growth delay in 10% of the xenografts (Fig. 6f–i). BRAF V600E was the most stringent independent predictor of resistance, as previously reported45. RAS mutations appeared less specific based on the T/C criteria, with four PDXs carrying KRAS or NRAS mutations among the moderate responders. However, when applying the RECIST clinical criteria, some of the responders were considered progressive (Fig. 6f, Supplementary Fig 16b). This apparent discrepancy reflects the fact that RECIST records whether progression exceeds 20% of the initial tumour volume after treatment, whereas T/C compares the relative tumour volume of the treated tumour versus its untreated matched control, thus permitting to detect objective tumour growth delay, even minor (Fig. 6f). Similar cases of non-progressive KRAS mutated tumours were already reported for patients treated with cetuximab46,47. In some cases, we could mechanistically link the response profile to EGFR blockade to specific mutations. 106_T_XEN carrying ERBB3-E928G and the activating mutation ERBB2-L755S conferring sensitivity to ERBB2-targeted drugs but resistance to EGFR inhibitors48 showed indeed objective response to afatinib and AZD8931 but resistance to cetuximab (Fig. 6f; Supplementary Fig. 16b). PDX 353_T_XEN carrying an EML4-ALK fusion as sole putative driver was resistant to all EGFR inhibitors (Fig. 6f, Supplementary Fig. 16b), but showed exquisite sensitivity to crizotinib (Fig. 6g), approved for targeting ALK fusions in lung cancer49. A similar fusion responding to crizotinib was described for one CRC cell line50. ALK fusions are found in ~1% of the CRCs50. We present the first in vivo pre-clinical data suggesting that crizotinib might represent a therapeutic option for CRC patients with rare ALK fusions.

Comparative drug responses between PDX/PDO sibling pairs. We compared the treatment outcomes of eight drugs for 19 pairs of PDO/PDX siblings (Fig. 7; Supplementary Fig. 15d), although inherent differences in the microenvironment (matrigel versus vascularized tissues), biology of the models, oxygen levels, catalbism/anabolism of the compounds in vitro and in vivo, and criteria assessing drug sensitivity (IC50/Emax versus T/C) are challenging this exercise. Irinotecan and cetuximab were excluded since they were uninformative in PDXs or in PDOs. Relying on the four response categories defined above, we called concordant response between PDX/ PDO siblings if their responses did not differ by more than one rank (for example, minor versus moderate) (Fig. 7, blue and white areas). Despite the aforementioned contingencies, response patterns between the two systems were fairly concordant excepted for AZD8931 and 5-FU. For the EGFR blockade, afatinib responses were in reasonable agreement but less so for AZD8931 more potent in PDOs than in PDXs, despite its low efficacy in cells (Fig. 7). This is likely to be because of differences in the pharmacokinetic behaviour of this compound, to biological differences between models, or to genetic heterogeneity. Models derived from 327_T differed for EGFR-A864V private to PDOs and absent in PDXs, which showed high-sensitivity or resistance to EGFR inhibitors, respectively (Supplementary Fig. 15d). 5-FU was the second most discrepant drug (Fig. 7) possibly because of its complex metabolism. 5-FU is catalyzed in vivo via the dihydrothymine dehydrogenase (DPYD/DPD), so that only 1–3% of the initial 5-FU concentration leads to active metabolites in plasma, generating modified nucleotides that are incorporated into the RNA and DNA via different enzymatic reactions51. Despite the fact that PDOs do not express DPD, the Emax was <50%, suggesting that anabolic routes might be less efficient in vitro.

Molecular classifiers of drug response. Exploiting the high dynamic range of RNAseq, we performed differential gene expression analyses for identifying molecular profiles associated with drug response profiles (see Methods). This approach was successful for 5-FU, Avastin and EGFR inhibitors (Supplementary Data 15), but failed for the other drugs in part because of their low efficacy precluding the assessment of true responder groups. However, we were also unable to identify common features characterizing responders to the efficient compound BI 860585, inhibiting both mTORC1 and mTORC2, suggesting that this drug might trigger complex cascade effects, difficult to capture in our present analysis.

For 5-FU, we focused on PDXs more comparable to the patient situation42. Responding xenografts tended to belong to NMfb (Fisher’s exact test P = 0.03) and included the MSIs (Fig. 8a). We identified a discriminative gene signature characterized by intestine epithelial lineage (P = 1 × 10-8) and HIF-1 transcription network (P = 1 × 10-5). Combined with recursive feature elimination and support vector machine (SVM) algorithms, this signature led to build a mini-classifier of 14 genes able to segregate responders from non-responders (Methods, and Fig. 8b). The stability of the classifier was estimated by cross-validation on the PDX OT-cohort (Supplementary Data 16). On the OT-patient cohort, the 5-FU classifier predicted that the best responders would be from the Enterob/Goblet molecular group, including 7/8 MSI-high samples (Fig. 8c; Supplementary Data 16). However, the predictive power of the classifier will require further assessments on additional cohorts treated exclusively with 5-FU. Considering inhibitors of angiogenesis, only the response to the antibody Avastin blocking VEGFα could be associated with a clear molecular signature, although the limited number of responders impeded statistical assessments. Sensitive tumours exhibited higher activity of genes involved in energy-coupled mitochondrial transport (P = 1 × 10-4), whereas resistant tumours exhibited strong colonic cell features and higher expression of ERF (Supplementary Fig. 18a).
Response patterns to EGFR inhibitors were associated with characteristic molecular profiles, albeit different ones in PDOs and i nP D X s ( F i g . 9 , Su p p l e m e n t a r y F i g . 1 8 b , Su p p l e m e n t a r y D a t a 1 5 ) . PDOs sensitive to afatinib featured enterocyte differentiation factors \((P = 1 \times 10^{-6})\) such as \(A T O H 1\) and higher expression of \(E G F\), and \(G R E M 1\), which promotes stem cell properties in colonic cells outside of the stem cell niche\(^{52}\). Response to AZD8931 defined a similar profile to afatinib, in contrast to gefitinib and vandetanib, which were less efficient (Figs 6b and 9a,b; Supplementary Fig. 18b). Fourteen genes were associated with resistance to all EGFR inhibitors (Supplementary Fig. 18c–e), including \(R G S 4\), \(B A S P 1\) and \(I G F 2\) known as marker of EGRF blockade insensitivity\(^{53}\).

In PDXs, strong responders to the EGFR blockade were mostly from molecular group NMFa (stem/Wnt) whereas resistant ones

Figure 7 | Bubble plot comparison of drug sensitivity profiles in 19 sibling pairs of PDX and PDO models. Drug response categories are displayed on the axes (\(x = \text{PDX}, y = \text{PDO}\)). The size of each bubble is proportional to the number of model pairs in a given response category, where this number is indicated in the bubbles. The colours represent the eight drugs tested in both model systems, as indicated in the caption. The background colour in each square indicates the degree of concordance between the two models where grey indicate discordant drug response.

Figure 8 | Molecular classification of response to 5-FU in PDX models. (a) Hierarchical clustering of PDX samples based on the 5-FU response gene signature (\(n = 251\) genes). The drug sensitivity is given by T/C continuous values coloured as indicated in the caption (dark green: strong response, red: resistance). MSI samples, OT-NMF-groups and CMS classifications are coloured as indicated in the caption. (b) Hierarchical clustering of PDX samples based on the mini-classifier (\(n = 14\) genes) obtained by SVM-based machine learning on 49 PDX. (see Supplementary Data 14 for cross-validation). (c) Distribution of the predicted responders and resistant patient tumours (tumour purity \(\geq 40\%\)) in the three molecular groups (ECM/EMT, ASCL2/MYC, Entero/Goblets).
were epithelial (Fig. 9c–e). Resistance to AZD8931 and to afatinib was associated to a molecular signature enriched for developmental genes ($P = 1.03 \times 10^{-6}$ and $1 \times 10^{-9}$), including the oncogene $ETV5$ (Supplementary Data 15) and for AZD8931, $REG4$, a multifunctional mitogenic protein known as a potent activator of the EGFR pathway in CRC (refs 54,55). Response profiles to cetuximab showed distinctive signatures segregating strong responders from resistant tumours, showing also a third group of PDXs displaying moderate/minor responders that included KRAS mutants (Fig. 9e). Resistance to cetuximab was also associated with expression of $REG4$, and featured strong epithelial ($P = 2 \times 10^{-9}$), as well as developmental ($P = 1 \times 10^{-7}$) signatures whereas sensitive tumours featured expression of $MYC$, $JUN$ and $E2F5$, and of previously reported markers of cetuximab sensitivity $CEACAM7$ and $EREG^{46,56}$. Sensitivity was correlated with hypoploidy (Fisher’s exact test $P = 0.003$), and with CNV gain of $MYC$ and $AURKA$ (Fig. 9e), suggesting that tumours sensitive to EGFR blockade might rely on the MYC-AURKA axis, a relevant pathway in CRC (ref. 57).

The EGFR response signatures of PDX and PDOs shared only a few genes, highlighting their biological differences and the difficulties in comparing directly these systems (Supplementary Fig. 18f,g).

Classifier of cetuximab sensitivity in KRAS-WT tumours. Given that a fraction of patients with $BRAF/KRAS/NRAS$ wild-type (WT) CRCs do not benefit from cetuximab, we aimed at identifying an independent classifier predicting cetuximab sensitivity for addressing this unmet clinical need. On the basis of the
**Figure 10 | Validation of the mini-classifier of cetuximab response in PDX.** The drug sensitivity is given by T/C continuous values coloured as indicated in the caption (dark green: strong response, red: resistance). MSI samples, OT-NMF-groups, CMS classification and mutations are coloured as indicated in the caption (dark green: strong response, red: resistance). MS1 samples, OT-NMF-groups, CMS classification and mutations are coloured as indicated in the caption (dark green: strong response, red: resistance). Figure 10 | Validation of the mini-classifier of cetuximab response in PDX. (a) Hierarchical clustering of 48 PDX samples based on the mini-classifier (n = 16 genes) obtained by SVM-based machine learning. (b) Principal component analysis (PCA) showing the classifier’s ability to separate responders from non-responders. (c) Respective performances of the OT mini-classifier and of the RAS/RAF mutation status in predicting cetuximab sensitivity in the following cohorts OT-PDX (OT) (cross-validation), EPO PDX, the Gao et al.12 PDX (NV) and Kambata-Ford et al.46 primary tumours (KF). The mutation status was defined by mutations in codon 12 and 13 of KRAS or detected activating mutations in KRAS, BRAF or NRAS (BRAF mutations: V600E; KRAS/NRAS mutations: G12, G13, Q22, Q61, A146). In an additional setup, stable disease (SD) samples were excluded. The dot plot shows the specificity, sensitivity and balanced accuracy of the predictions. The 0.8 value is highlighted with a black dotted line. (d) Same as in (c) but taking as validation cohort 164 samples merged from OT, EPO, NV and KF. (e) Distribution of the predicted responders and resistant patient tumours (tumour purity ≥ 40%) in the three molecular groups (ECM/EMT, ASCL2/MYC, Entero/Goblets).
signature shown in Fig. 9e, we built a mini-classifier of 16 genes able to segregate responders from non-responders, using a combination of recursive feature elimination and SVM algorithms (see Methods, Supplementary Data 17, Fig. 10a,b). We tested the stability of this mini-classifier by cross-validation on the OT xenografts, showing an overall improved performance over the RAR/RAS mutation status alone in predicting the outcome of cetuximab treatment, in terms of both sensitivity and specificity (Fig. 10c). In addition, we validated the classifier’s predictive power on two independent CRC xenograft cohorts and one independent human cohort. We generated RNAseq data on a previously reported collection from EPO (n = 60)38 and downloaded the informative data from Novartis PDOs (NV, n = 36)39, and from a human cohort of 68 patients KF (n = 68)40, all treated with cetuximab (Fig. 10c; Supplementary Table 1). On the xenograft cohorts, the OT mini-classifier outperformed the mutation status-based predictor for predicting responders and achieved a sensitivity of 0.92 and 1 for KRAS/NRAS/BRAF wild type samples on the NV and EPO xenografts, respectively.

In the Khambata–Ford (KF) cohort, the KRAS mutation status was highly sensitive but not very specific, because of a number of WT-KRAS tumours that did not respond to cetuximab (of note, the BRAF status was not tested in this retrospective study), whereas the OT mini-classifier exhibited higher sensitivity (0.83), and specificity (0.86) (without stable disease). Overall, the OT mini-classifier outperformed the BRAF/KRAS/NRAS status in predicting cetuximab response, despite inherent differences between datasets including patients from different populations and ethnic origins (US, European and Chinese), tumour stages and methodologies (microarrays versus RNAseq). We then merged the EPO, NV and KF cohorts in a superset of 164 samples for testing the OT mini-classifier, showing a sensitivity of 0.94 and a specificity of 0.79 for the KRAS/NRAS/BRAF wild type samples (Fig. 10d; Supplementary Table 2). On the basis of this performance, we applied the OT mini-classifier to the original OT-patient cohort (Fig. 10e), predicting that sensitive cases were mainly found in the ASCL2/MYC group, in particular early stage tumours (Fisher’s exact test \(P = 0.0068\)). Although a few sensitive tumours belonged to the ECM/EMT group, those were showing similarities with the ASCL2/MYC samples (Supplementary Fig. 8f, subgroup A).

Discussion

We report here a unique CRC biobank comprising 59 xenografts and 35 organoids tested on a panel of clinically relevant compounds. This is the first large study providing extensive molecular characterization of a patient donor cohort, encompassing all the disease stages, and representative of the typical CRC molecular groups, with matched in vivo and in vitro models.

A key issue for evaluating the potential of pre-clinical models relies on their capacity to retain the complex molecular and biological characteristics of the parental tumours. We showed that most models recapitulated the genetic landscapes of donor tumours, whereas clonal discords found at early passages were attributed to ITH, reflecting the initial sampling of the bulk tumour. We identified few discordant mutations potentially impacting treatment outcome, (for example, EGFR, PTCH1), suggesting that heterogeneous parts of the tumours could respond differently to targeted drugs. This issue needs to be taken into consideration in CRC personalized settings, requiring ideally the establishment of multiple models recapitulating ITH, challenging the 1 × 1 × 1 scheme12. Transcriptome landscapes indicated that cellular and biological pathways were reasonably well conserved in both model types, except for immune and stromal environment. Xenografts are currently considered as gold standard tools for precision medicine, whereas the potential of organoid technologies is in early evaluation19. We found that PDOs appeared closer to the CRC molecular groups than PDOs, in particular those derived from the ASCL2/MYC group, but follow-up studies are required for confirming this observation. PDOs featured higher expression of genes involved in xenobiotic and fatty acid processes, possibly impacting cancer cell survival34, and may exhibit higher plasticity, displaying heterogeneous stem/differentiated enterocyte cell populations. These differences have a crucial impact on the choice of a suitable ex vivo model system for compound screening, and were investigated by comparative analyses of drug response in genetically identical PDO/PDOX sibling pairs. Striking sensitivity differences between the model systems were seen for AZD8931 more potent in PDOs than in PDOX. Evaluation of drug response depends on pharmacokinetic parameters such as the concentration of active compounds at the tumour site. Assessing the clinical relevance of potency levels in vitro should consider clinical achievable drug plasma concentration (C_max or C_m), maximum or steady-state concentrations, providing these values are known from clinical studies. Drugs exhibiting low efficacy in cell models, yet having high tolerable C_max values in patients might trigger a response although PDOs were scored insensitive. For instance, regorafenib reaches a C_max value of 2.5 mg · ml⁻¹ following a single 160 mg dose in patients with advanced solid tumours39.

Here, we tested 11 drugs in 59 PDO models recapitulating most of the molecular features of the donor human tumours, providing the equivalent of phase II/III-like response data. Dissecting the stromal human components in models, leading to more specific signatures of the tumour cells, contributed to identify novel classifiers of response to the clinical compounds 5-FU and cetuximab, correlating responders with specific CRC molecular sub-groups. We derived a novel classifier predicting cetuximab response with high sensitivity and specificity, outperforming current genetic biomarkers and able to predict the drug sensitivity of RAS/RAF wild-type tumours, a group of tumours, which up to now lacked efficient biomarkers associated with EGFR blockade sensitivity.

We demonstrated here the power of integrative analyses for capturing the complexity of the CRC biology. Understanding the molecular make-up of each tumour and model is a paradigm becoming realizable with increasingly affordable NGS protocols and availability of powerful analysis tools. We identified clinically relevant gene fusions, and provided the first CRC in vivo model of ALK fusion responding to crizotinib. The OncoTrack study provides a large biobank and data repository based on patients and pre-clinical models with unprecedented breadth and depth, and a compendium of drug sensitivity data, a resource that can be exploited further for improved drug discovery and understanding of CRC biology.

Methods

Patients. In this study we included patients with colorectal cancer aged between 18 and 100 years. The only exclusion criterion has been infectious diseases. Samples were stored according to the current GCP guidelines. Informed consent was obtained from all human subjects included in the study. The study was approved by the local Institutional Review Board of Charité University Medicine (Charité Ethics Cie: Charitéplatz 1, 10117 Berlin, Germany) (EA 1/0699/11) and the ethics committee of the Medical University of Graz (Ethic commission of the Medical University of Graz, Auenbruggerplatz 2, 8036 Graz, Austria), confirmed by the ethics committee of the St John of God Hospital Graz (23-015 ex 10/11).

Nucleic acid preparations. Nucleic acid preparations were performed either using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, 80004) or the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, 80224). DNA extraction from blood was carried out using the QIAamp DNA Blood Maxi Kit 10 (Qiagen, 51192). Concentrations were determined on Qubit Fluorometer. RNA integrity was evaluated with Bioanalyzer 2100 (Agilent, Palo Alto, CA).
Targeted sequencing. Targeted sequencing libraries were prepared with the TruSeq Custom Amplicon Kit (Illumina, FC-130-1001) and Index Kit (Illumina, FC-130-1003) following the True Seq Custom Amplicon Low Input Library Prep protocol (October 2015). TruSeq Custom Amplicon panels were designed with Illuminus DesignStudio. Paired-end (PE) libraries were sequenced on MiSeq PE 151 dual Index.

Mutation validation with Sanger sequencing. PCR primers are listed in Supplementary Table 3. PCR products were purified and processed by Sanger sequencing (Eurofins MWG Operon).

Whole genome sequencing. High coverage WGS libraries were prepared with the TruSeq DNA Sample Prep v2 kit (Illumina, set A: FC-121-2001; set B: FC-121-2002) following the Illumina Low Throughput (LT) Protocol (August 2011). Paired-end libraries were sequenced on HiSeq 2,000/2,500 instruments with v3 chemistry using 2 × 101 bp reads to ≥ 50 coverage. For low coverage, WGS libraries were prepared using Nextera Rapid Capture Exome and Expanded Exome Kit (Illumina, FC-140-1006), but omitting the exome enrichment step. Paired-end libraries were sequenced (2 × 51 bp) on HiSeq 2,000/2,500 instruments with v3 chemistry to ≥1 coverage.

Whole exome sequencing. WES was carried out on either SOLiD or Illumina platforms. SOLiD libraries were prepared either with Sure Select XT Human All Exon 50 Mb (Agilent Technologies, 5190-0407) or with SureSelect Human All Exon V4 (Agilent Technologies, 5190-4631). Sequencing was carried out either on SOLiD 5400 in Frags75/ECC mode or on SOLiD Wildfire in Frags50/ECC runs using single-end mode. For Illumina, libraries were prepared with Nextera Rapid Capture Exome and Expanded Exome Kits (Illumina, FC-140-1006). Paired-end libraries (2 × 51 bp) were sequenced on HiSeq 2000/2500 instruments with v3 chemistry.

Whole transcriptome sequencing. RNAseq libraries were prepared using either TruSeq RNA Sample Prep Kit v2 (Illumina, set A: RS-122-2009; set B: RS-122-2010). For ten total RNA samples we used the Ribo-Zero Magnetic Gold Kit (Epicentre, MRZG12324). Sequencing (2 × 51 bp) was performed on HiSeq 2000/2500 instruments with v3 chemistry.

DNA data processing. DNA reads were aligned to the human reference genome hg19 using BWA (bwa0.7.7-r441-mem for 75/101 bp, bwa0.5.9-r16-aln for 51 bp single-end mode. For Illumina, DNA reads were aligned to the human reference genome using BWA and SAMtools. RPKM values were log2 transformed and centred by the trimmed mean of normalized expression.

Copy number variants. Copy number variants were estimated as described above and compared between corresponding samples. Cancer relevant gene selection. Cancer relevant gene selection was done by taking the overlap with 31 significantly mutated genes in the CRC TCGA study and 86 genes recurrently mutated in CRC from the TCGA pan-cancer analysis.

Identification of molecular tumour groups. RPKM values of 65 primary CRC samples (purity ≥40%) were analysed with non-negative matrix factorization (NMF) and the CLICK algorithm, respectively. For both methods, we selected the most variable genes (5,329 genes, cut-off >0.5 RPKM in more than three samples) in the 65 primary tumour samples using quantile absolute deviation (QAD = |X̅ − median(X)|), calculated on raw and log transformed RPKMs.

CLICK clusters. RPKM values were log, transformed and centred by the trimmed mean. For gene co-expression clusters, best results were achieved with an expected homogeneity of 0.60 leading to a separation of −0.085, to an overall homogeneity of 0.753 and 13 clusters (named OT_C1 - OT_C13). A 14th cluster was excluded since it mainly contained uncharacterized genes. On the basis of the mean pattern obtained for each CLICK cluster, tumour samples were divided into two groups with partitioning around medoids (pam) implemented in R63. Among these, genes up-regulated in one or more of the NMF groups were selected in 961, 20 and 146 specifying OT_NMF1, OT_NMF2 and OT_C1-13, respectively (Supplementary Data 10).

Mean pattern matrix of primary CRCs. In addition to the OT 16 gene signatures (OT_NMF1, OT_NMF2, OT_NMF3 and OT_C1-13), we collected signatures from external resources: (1) 286 inflammation genes extracted from expression arrays, (2) 18 Cancer related gene signatures, we used the provided single list of classifier genes and assigned them to the different subtypes using centroids and PAM. From Schlücker et al. we used the provided gene signatures. For Maruta et al. we assigned the probes to the 6 subtypes by taking a logFC >0.5 and P<0.0001 as cutoff on their list of 1,108 probes. We performed the conversion mapping of Affy-133-plus-2-probeset IDs to Ensembl IDs (v70) using BioMart (Ensembl),
obtaining a total of 38 signatures that we combined in a so-called ‘mean pattern matrix’ integrating 70 primary CRCs and 20 metastatic samples (tumour purity ≥80%). We calculated the mean patterns for each specific signature in each of the CRC samples (trimmed mean of log2 and Z-score transformed RPKM values) resulting in a matrix of 90 × 38 entries (the mean pattern values are shown in Supplementary Data 12). To test the stability of hierarchical clustering the approach previout (R package, v 2.0) was applied with 5,000 iterations and a sample size of 80, 85, 90, 95, 100, 105, 110, 115 and 120% (ref. 64).

Clustering of the mean pattern matrix. On the basis of the values of the mean pattern matrix, patient tumours were clustered into subgroups using the mclust package in R, which tested for 2 up to 15 expected clusters25. On the basis of the Bayesian information criterion (BIC), 3 and 6 clusters were the best solutions.

Mean pattern matrix comparing CRCs with experimental models. Mean RPKM values for each model and gene signature were calculated as described above. To compare the models directly to the initial patient samples, we used the mean and standard deviation of a gene in the patient tumour cohort and calculated the mean of the Z-transformed RPKM values.

NMF groups in models. NMF was applied to the most variable expressed genes based on the QAD (see above) resulting in 5,130 and 4,629 genes for PDX and PDO models, respectively. On the basis of cophenetic scores we obtained two clusters for the PDX and PDO samples, respectively. Differentially expressed genes between the two NMF groups were identified using edgeR (Supplementary Data 10). The attribution of NMF groups for each of the PDX and PDO samples is given in Supplementary Data 9.

Single sample Gene Set Enrichment Analysis. Single sample Gene Set Enrichment Analysis (ssGSEA) was performed using GSEA package in R65. We used as input log2-transformed RPKM values and gene sets of immune66-68 and 16 OT signatures as referred in the test (OT_NMF1, OT_NMF2, OT_NMF3 and Glik1-13). The homogeneity of variance was fulfilled for OT_C2, OT_C7, OT_C12, inflammation, OT_C5, OT_C13, OT_C8. The three main CRC groups were compared using one-way analysis of variance and Tukey’s range test. For the pairwise comparison of the sub-group and the main group against MSI samples a Welch’s t-test was applied. The P values were adjusted for multiple testing using the Benjamini–Hochberg procedure (false discovery rate—FDR). Differences between groups of patients were considered significant with a FDR <0.01 for the 16 OT signatures and FDR <0.05 for immune signatures.

Functional annotations. Functional annotations were achieved by gene set over-representation analysis using the GeP Genomatrix software (v.3.10124 and v.3.5116).

Global gene expression profile comparison. Global gene expression profile comparison of tumours and model systems was carried out using 90 original patient samples, as well as untreated controls of 54 PDX and 33 PDO (Fig. 5b). From the five xenografts that derived from 150_MET1 only the sample 150_MET1_XEN1 was included into the analysis, since all of them shared a similar expression profile. We performed a DGEA using edgeR and compared PDX versus patients, PDX versus patients and PDO versus PDX. To identify global differences in gene expression we applied strict cutoffs: FDR ≤0.005, log2(FC)≥log2(2.5) and difference of mean expression ≥2. Functional analyses of the up- and down-regulated genes were performed using the GeP Genomatrix software.

Pairwise gene expression profile comparison of models. Pairwise gene expression profile comparison of models was applied on 17 corresponding PDO-PDX pairs using a pre-ranked gene set enrichment analysis (GSEA, Broad Institute) (Fig. 5a). To establish the gene ranking based on the expression values for a matching PDX X and PDO Y, the ranking score τ was calculated by taking the geometric mean of the fold change and difference of expression, 

\[ \tau = \log_2 \left( \frac{x_{\text{PG}} + 1}{y_{\text{PG}} + 1} \right) \]

where g indicates a specific protein coding gene and p a specific pair of correspond-

Differences between groups were considered significant with a FDR <0.05, |log2(FC)|≥40% were classified into the consensus molecular subtypes (CMS) reported by Guinney et al. and the provided single-sample predictor (SSP) and random forest (RF) CMS classifier to the 90 OT CRC samples (R package downloaded from www.synapse.org/#!Synapse:syn26327306/wiki/). With the default parameters, 25/90 and 24/90 of the samples (28%) were unclassified using RF and SSP, respectively. Thus, we lowered thresholds for subgroup assignments (RF: minPosterior = 0.4; SSP: minPosteriorCor = 0.1, minDeltaCor = 0.03). For RF we used a minimum probability of 0.4 since it shows just a slightly lower specificity compared with the default value 0.5 (Guinney et al.; Supplementary Fig. 3). Applying RF and SSP with lowered thresholds 8 and 17 samples remained unclassified, respectively (Supplementary Data 9). The lowered thresholds were also applied on the PDX and PDO OT cohorts.

C性EL 2D cell lines. BAM-files for 41 COAD CCLE cell lines (April 2014) were downloaded from https://cgphub.ucsc.edu/index. Gene expression values were normalized as RPKM (see methods RNA data processing) using gene models from Ensembl Release 73.

Drug response analysis preprocessing. Five xenografts that derived from one CRC (150_MET1) shared highly similar global expression profiles and were merged into one artificial single sample by taking an average of the RPKM and of the T/C values, respectively. Four PDX (234, 272, 283 and 128) had no RNAseq data. For cetuximab one PDX (261) was considered as outlier and excluded from the analysis, since it was the only sample that showed a T/C value far above 100%. Two PDDos (159, 161) had no RNAseq data.

Correlation of drug sensitivity values. Drugs were pairwise and per model system compared based on log10 normalized IC50 values for 35 PDDos and T/C values for 53 PDDos and by calculating Spearman’s rank correlation coefficient.

Drug response gene signatures in PDX and PDO. We performed differential gene expression (DGE) analysis using the R package edgeR per drug and model system to identify signatures associated with drug response results based on the four response categories: strong, moderate, minor, resistant (see below drug response). DGE analysis was applied in different setups as follows: (a) combined strong + moderate versus combined minor + resistant, (b) combined strong + moderate versus minor versus resistant and (c): 20 most sensitive PDX or 10 most sensitive versus 10 least sensitive PDO. Genes were filtered by FDR ≤0.01, |log2(FC)|≥1 and RPKM difference ≥1. In addition, (setup d), we used the IC50 or T/C values as phenotype vector in a general linear model (GLM) provided by the edgeR package. Genes were filtered by FDR ≤0.01 and dis-

Drug response classifier the parameter set with the third highest F1-score was taken as optimal solution, since it showed the highest sensitivity among the top three: C = 0.03, feature size = 16. The described procedure resulted for the 5-FU gene signature into a mini-classifier with 14 genes.

CMS classification. 90 OT CRC samples (tumour purity ≥40%) were classified into the consensus molecular subtypes (CMS) reported by Guinney et al.2 We applied the provided single-sample predictor (SSP) and random forest (RF) CMS classifier to the 90 OT CRC samples (R package downloaded from www.synapse.org/#!Synapse:syn26327306/wiki/). With the default parameters, 25/90 and 24/90 of the samples (28%) were unclassified using RF and SSP, respectively. Thus, we lowered thresholds for subgroup assignments (RF: minPosterior = 0.4; SSP: minPosteriorCor = 0.1, minDeltaCor = 0.03). For RF we used a minimum probability of 0.4 since it shows just a slightly lower specificity compared with the default value 0.5 (Guinney et al.; Supplementary Fig. 3). Applying RF and SSP with lowered thresholds 8 and 17 samples remained unclassified, respectively (Supplementary Data 9). The lowered thresholds were also applied on the PDX and PDO OT cohorts.

Building drug response classifiers for cetuximab and 5-FU. Of the 241 genes making the cetuximab signature only 179 genes could be mapped to external datasets11,46,58. The following procedure was applied to the 179 mapped genes. RPKM values were log2 transformed and z-score normalized. Eleven genes that showed lower mean expression between highly correlated gene pairs (Pearson correlation ≥0.8) were excluded in two iterations. For the drug response classifier a linear support vector machine (SVM) implemented in the R package e1071 (v1.6-7) was trained on 48 PDX of the OT cohort (14 responding and 34 resistant PDX). To address the imbalance of the training set, a class weighted SVM was used for each of classes resistance and response (C_{res}, C_{resp}). The feature (gene) selection included feature ranking and feature size selection. To avoid overfitting, a model validated on the OT PDO cohort, on one external human external human cohort for 80 metastatic CRC patients with array expression (Affymetrix U133A v2.0 GeneChips; Khambara-Ford et al.49) and two external PDX cohorts with 59
and 60 models with RNASeq data (Gao et al., Pechnak et al.) all treated with cetuximab. For 68 samples of the Kambha-Ford and for 36 samples of the Gao dataset expression and cetuximab response data were available. Ensembl gene identifiers were mapped to u133av2 probeset IDs (Kambha-Ford) and to gene symbols (Gao). The expression values of the external PDX cohorts were log2-normalized and of all three external data sets were compared (CR), partial response (PR), stable disease (SD) and progressive disease (PD). PDX of the EPO were divided in four response categories based on given T/C values as described in this paper. The performance of the classifier was estimated from the number of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) predictions as well as their sensitivity, specificity and balanced accuracy. Cross-validation on the OT PDX cohort was achieved via a 100 times repeated 10-fold cross-validation. Performance values were averaged over the repeats. In a second analysis, SD samples were excluded to determine their influence on the classifier’s performance (XF: 49 samples, Gao: 32 samples). Additionally, we tested the performance of the classifier on KRAS wild type. The true all-RAS/RAF wild type samples. For the KRAS wild type, in codon 12 and 13 of KRAS were considered. For the all-RAS/RAF wild type, KRAS and NRAS mutations (G12, G13, Q22, Q61, A146), as well as BRAF mutations (V600E) were checked. The Kambha-Ford data set provided only mutations in codon 12 and 13 of KRAS. Cross-validation of 5-FU response. The 5-FU mini-classifier (14 genes) was cross-validated on the OT PDX cohort via a 100 times repeated 10-fold cross-validation. The performance values were averaged over the repeats. The performance of the classifier was estimated from the number of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) predictions as well as the sensitivity, specificity and balanced accuracy.

Establishment of PDO cell cultures. Upon received sample receipt, fatty and necrotic tissues were removed macroscopically. Remaining tissue was rinsed with HBSS ( Gibco), minced, and digested with Collagenase IV (Sigma-Aldrich), DNaseI (AppliChem, Germany) and Dispase (StemCell Technologies, Germany) at 37 °C for 66 min, followed by pelleting the suspension at 300 g for 3 min, re-suspension in medium and filtration steps as in Konno et al.7. The 40–100 μm aggregates were centrifuged at 300 g for 3 min. After depletion of red blood cells using Red Blood Cell Lysis Solution (Miltenyi, Germany), cells were mixed with phenol-red free growth factor-reduced Matrigel (Corning) and seeded into 24-well plates. Solidified droplets were carefully overlaid with 500 μl of culture medium as in Sato et al.7. During the first week 2.5% and 10 μM of ROCK-II inhibitor Y27632 (Sigma-Aldrich) were added to cultures. The cultures were passaged when the aggregates reached a diameter of approximately 800 μm. Cellular aggregates were released from Matrigel by adding 5 ml Advanced DMEM/F12 followed by centrifugation. Pellets were digested with TrypLE (Gibco). Trypsinization was stopped with 5 ml Advanced DMEM/F12 and cell clusters were re-plated on a 24-well plate. PDO cell cultures were generated from 41 patient tumours and 5 xenografts (Supplementary Data 1). The cell cultures were routinely tested for Mycoplasma contamination and found to be negative.

For immunohistochemistry, 2 μm de-paraffinized FFPE tissue sections of donor tumours were used to establish PDOs and was grown for five days. We stained the primary antibodies anti-Ck7 (clone OV-TL12/30, Dako, Germany), anti-Ck20 (clone KS20, Dako), anti-Cdx2 (clone Cdx2-88, BioGenex, USA) and anti-Kl6 (clone MB-1, Dako) for 32 min at 37 °C, ultraView DAB detection kit (Ventana, USA) on the BenchMark XT instrument (Ventana). Counterstaining was performed with Hematoxylin (Sigma-Aldrich) II and Bluing (Sigma-Aldrich) for nuclear immunofluorescence imaging. PDO cell aggregates were fixed and permeabilized with 4% PFA/1% Triton X for 30 min, followed by treatment with 1% Triton X overnight at 4 °C. PDO aggregates were then washed in PBS with 10% FCS. Secondary antibody (Alexa Fluor488, Invitrogen) was added at 4 °C overnight and removed by washing in PBS. Nuclei were stained with DAPI (Sigma-Aldrich) for 30 min. F-actin was stained accordingly with TRITC-labelled Phalloidin (Sigma-Aldrich). Microscopy was performed with a Zeiss Axiosview 400 microscope (Zeiss, Germany).

Semi-automated high-throughput drug sensitivity assays. PDO cultures were digested with TrypLE (Gibco) to single cell suspension. Trypsinization was stopped with Advanced DMEM/F12. We seeded 5000–50 000 cells/well in 96-well plates in 100 μl Advanced DMEM/F12 medium. The cultured Matrigel into 384-well plates using a robotic platform (Tecan, Spain). Cells were cultured for four days before compound treatment. Growth curves were determined by assessing the cell viability by luminescence (CellTiter-Glo, Promega), using the EnVision plate reader (PerkinElmer) 24 h after the addition of the reagents. The 384-well plate layout included appropriate Min (minimum signal, 5 μM staurosorine) and Max (maximum signal, vehicle, 0.25% DMSO) controls to determine signal intensity cutoffs. PDO cultures were screened in two replicates with the test compounds ranging from 60 μM to 3.05 μM with 1:3 serial dilution steps, and cetuximab ranging from 5 μg per ml to 0.25 mg per ml with 1:3 serial dilution steps. Cetuximab signal was continued throughout medium exchange. The treatment duration covered two population doubling times for each cell culture strain. Plate uniformity was validated as previously described7 and in accordance with published Eli Lilly-NIH Chemical Genomics Center Guidelines for assay enablement and statistical validation3. Emax values were calculated as the percentage of inhibition at the top concentration. The linear relationship between the 50% IC50 values were calculated with the four-parameter nonlinear logistic equation and were classified into four response categories based on the tested concentration range: resistant (IC50 > 5.0656 μM), moderate (0.4277 μM to 0.0361 μM) or strong responders (IC50 < 0.0361 μM). For the response evaluation included the four response categories and are defined as: resistant (IC50 > 1.483 μM) and minor (IC50 < 1.483 μM to 3.63 μM), moderate (IC50 < 3.63 μM to 4.703 μM) or strong responders (IC50 < 5.777 μM).

Live imaging and confocal microscopy. For the time-lapse analysis, the organoid cultures in 384-well plate was monitored using a HC PL APO × 10/0.40 AN (× 10) objective, a Hamamatsu ORCA-AG CCD camera and an inverted motorized microscope (Leica DM 4500B) coupled with an incubation system to control the temperature and CO2 levels during the experiments. Images were taken every 15 min for 72 h using the Leica LAS AF software (Version 2.4.1). Confocal microscopy was carried out using a Leica TCS SP5 X confocal microscope equipped with a resonant scanner, a dry × 20 Plan Apochromatic, 0.7 AN objective, and Leica LAS AF software (Version 2.4.1) for image capturing, and the Imaris software (Bitplane) for image analysis.

WNT reporter assay. Wnt pathway activity was assessed by lentiviral transduction with the Cignal Lenti TCF/LEF Reporter (GFP) Kit CLS-018 G (QiAGEN, Hilden, Germany). Organoids were released from Matrigel, plated into 96well round bottom ultra-low attachment plates (Corning) with 20 μl virus suspension. Following transduction, organoids were re-transplanted in Matrigel to be continuously cultured with puromycin. After selection, organoids were released from Matrigel, digested to a single cell suspension and sorted into GFP-high/GFP-low/GFP-negative fractions by FACS. Ribonuclease acid was isolated from the sorted cells using the AllPrep DNA/RNA Mini Kit (QiAGEN) and provided for RNA-Seq.

Development and characterization of PDxs. Resected tumour tissues were transplanted to immunocompetent mice (NMRU nude or NOG, Tacomb, Böhm- holderg, DK- Tac-NMRU-Foxn1nu, females, 6–8 weeks at start of transplantation) using previously described methods by Fichtner et al.7. Animal experiments were carried out in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals and of the German Animal Protection Law and approved by the local responsible authorities. EPO strictly follows the EU guideline European convention for the protection of vertebrate animals used for experimental and other scientific purposes. (EST 123) and ‘German Animal Protection Law -Version July 2014’ (Tierschutzgesetz: zuletzt geändert durch Art. 3 G. v. 28.7.2014 I 1308). Further we handle our animals according to Regulation on the protection of experimental scientific purposes or other purposes used animals (Tierschutz-Versuchstierverordnung- TierSchVeraV). Geändert durch Art. 6 V. v. 12.12.2013 I 4145). Compliance with the above rules and regulations is monitored by the Landesamt für Gesundheit und Soziales (LAGeSo) which is the responsible regulatory authority monitoring the animal husbandry based on the German Animal Welfare Act, last revised in 2009. Animal use was given after careful consideration of the species feeding & water, ventilation, temperature & humidity, cleaning and hygiene concepts. Mice were monitored three times weekly for tumour engraftment up to 3 months. Engrafted tumours at a size of about 1 cm3 were surgically excised and smaller fragments (transplants) up to 0.5 g for further passage. Within passage 1–3 numerous samples were cryo-conserved (DMSO-medium) for further experiments. Tumours were passaged not more than 6 times. For confirmation of tumour histology, tissue samples was formalin fixed and parafin embedded (FFPE) and 5 μm sections were prepared. Samples were stained according to a standard protocol for hematoxlin, eosin and K676 to ensure xenograft comparability to the original specimen. Cases with changed histological pattern were sent for pathological review and outgrowth of lymphoproliferative disorders was excluded. In this study, no blinding was done.

In vivo drug response testing of the xenografts. Response to the selected compounds was evaluated in early passages using the design of a preclinical phase II study. Tumour fragments of similar size were transplanted subcutaneously to a group of equal size (20 plants). The compound treatment was divided over a period of four weeks (4 cycles) till tumour size exceeded 1 cm3 or animals showed loss of > 15% body weight. From the first treatment day onwards the tumour volumes and body weights were recorded twice weekly. At the end of the treatment period animals were sacrificed, blood and tumour samples collected, and stored in liquid nitrogen immediately. Tumours samples were homogenized using a motor-driven dispersing medium. Values were calculated from the length and width of subcutaneous tumours (V = (length × width3)/2). Sensitivity to the tested compounds was determined as tumour growth inhibition %.
DNA methylation profiling. 500 ng genomic DNA was bisulfite converted (EZ DNA Methylation Gold, Zymo Research) in accordance with the manufacturer’s protocol with alternative incubation conditions (that is, 16 cycles (95°C for 30 s, 50°C for 60 min)). Following bisulfite conversion, 14 randomly selected samples were quality controlled with qPCR using primers designed to anneal to genomic and bisulfite converted DNA (Primer are listed in Supplementary Table 6). Bisulfite converted DNA was amplified using primers having genomic and bisulfite converted primer pairs were hybridized to Infinium 450 K BeadChips (Illumina) and scanned with iScan (Illumina). Raw data (IDAT files) were pre-processed using minfi implemented in R. DNA extracts with more than 5% low detection metrics were excluded. Following DNA extract- and probe-based data normalization by using functional normalization, a sum of diameters of target lesions was used to assess the size of the tumour volume at the start of treatment.

Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions (RTV <0.7)—Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions. (RTV > 1.2)

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. (RTV 0.7–1.2). As T/C and RTV are condensed summary parameter, no standard deviation values for replicate measurements are given in the Supplementary Data 14—these values have been determined and are available in the raw data.

Data availability. The complete set of NGS data for patient tumours, matching reference blood samples, xenografts and cell models are available upon request in the European Genome-phenome Archive (EGA) of the EBI data repository under reference blood samples, xenografts and cell models are available upon request in the European Genome-phenome Archive (EGA) of the EBI data repository under

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Author contributions
The clinical cohort was managed, recruited and tissue samples were distributed to other partners with the help of C.S., N.G.S., S. Lax, S.U., I.K., A.F., S. Liebs under the leadership of J. Haybäck and U.K. NGS data generation and analysis: M. Sultan, M.-L.Y. and T.B. supervised the operations in the sequencing platforms and analyses were performed under the leadership of M.L.Y. Data processing and genome analyses were carried out by M. Schütte, N.A.A., C.L.W., V.A. Transcriptome analyses were carried out by T.R. and C.J. and of the Alacris Theranostics group for its support. We acknowledge Mathieu Boniol, Hannes-Friedrich Ulbrich and Tom Goddard for their expertise and discussions on statistical analysis, Andreas Dühl, David Meierhofer, Niko Hildebrandt, Ivonne Marondel, Anthony Rowe, Ben Sidders, for useful discussions, Carl Steinbeisser for project management support. We also thank Laura Álvaro, Ester Arrobo, Eduarda Soicoceia and Julia Gutiérrez from Eli Lilly and Company for technical assistance. We thank Amin El-Heibri, Tabea Hohbense, Andreas Punzchart, Philipp Steigler from the Graz Hospital for helping in collecting clinical samples. Development and characterization of the PDO models were supported by DTÖK (RSC) and Berliner Krebsgesellschaft (CRAR; Grant# 201402). This work was supported in part by the Max Planck Society, by the Swedish Research Council, and VINNOVA.

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