CYP3A5 mediates basal and acquired therapy resistance in different subtypes of pancreatic ductal adenocarcinoma

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Although subtypes of pancreatic ductal adenocarcinoma (PDAC) have been described, this malignancy is clinically still treated as a single disease. Here we present patient-derived models representing the full spectrum of previously identified quasi-mesenchymal (QM-PDA), classical and exocrine-like PDAC subtypes, and identify two markers—HNF1A and KRT81—that enable stratification of tumors into different subtypes by using immunohistochemistry. Individuals with tumors of these subtypes showed substantial differences in overall survival, and their tumors differed in drug sensitivity, with the exocrine-like subtype being resistant to tyrosine kinase inhibitors and paclitaxel. Cytochrome P450 3A5 (CYP3A5) metabolizes these compounds in tumors of the exocrine-like subtype, and pharmacological or short hairpin RNA (shRNA)-mediated CYP3A5 inhibition sensitizes tumor cells to these drugs. Whereas hepatocyte nuclear factor 4, alpha (HNF4A) controls basal expression of CYP3A5, drug-induced CYP3A5 upregulation is mediated by the nuclear receptor NR1I2. CYP3A5 also contributes to acquired drug resistance in QM-PDA and classical PDAC, and it is highly expressed in several additional malignancies. These findings designate CYP3A5 as a predictor of therapy response and as a tumor cell–autonomous detoxification mechanism that must be overcome to prevent drug resistance.

PDAC is a very aggressive disease with poor prognosis. In both Europe and the USA, pancreatic cancer is the fourth leading cause of cancer deaths. Treatment with gemcitabine, the FOLFIRINOX scheme (a combination of oxaliplatin, irinotecan, fluorouracil and leucovorin) or the nanoparticle albumin-bound paclitaxel (nab-paclitaxel) offers only a modest increase in overall survival. Despite extensive testing of targeted therapies in clinical trials, all of the examined compounds confer little or no survival benefit in unselected cohorts of individuals with PDAC.

Although patient stratification according to molecular characteristics has not yet been performed in clinical trials for PDAC, transcriptional profiling of whole-tumor tissues suggest the existence of subtypes of PDAC that differ in patient survival and tumor metastasis. Additionally, three PDAC subtypes (classical, QM-PDA and exocrine-like) were described on the basis of gene expression profiling analyses of laser capture–microdissected epithelial tumors. However, in a larger panel of human and mouse PDAC cell lines, only the classical and the QM-PDA subtypes were identified, suggesting that currently used PDAC cell lines inadequately represent the heterogeneity of human PDAC. In addition, the classical and QM-PDA subtypes were suggested to differ in their responses to a range of chemotherapeutics, and the drug sensitivity of the exocrine-like subtype has yet to be determined.

Although resistance of PDAC to therapy is well described, little is known about the molecular mechanisms mediating it. Members of the cytochrome P450 (CYP) enzyme family have been previously investigated only with regards to a role in systemic drug metabolism or their up- or downregulation in solid tumors as compared to normal tissues. Thus, the functional role and impact of CYPs on tumor cell–autonomous drug resistance remains mostly unknown.

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Here we show that the exocrine-like PDAC subtype is resistant toward the small-molecule drugs dasatinib, erlotinib and paclitaxel, and that this resistance is mediated by a cell-autonomous CYP3A5-dependent drug detoxification mechanism. CYP3A5 also contributes to acquired drug resistance in other subtypes of PDAC and in other malignancies.

RESULTS
Establishment of PDAC models
First, we established patient-derived PDAC models to provide an in vitro and in vivo platform for functional studies. Patient-derived PDAC specimens were surgically grafted onto the pancreas of immune-deficient NOD.Cg-PrkdcscidIl2rgtm1Wjl (NSG) mice, which allow efficient human cell engraftment. Tumors from primary xenografts (PT) were then used to propagate primary PDAC cell lines referred to as PACO (pancreatic adenocarcinoma) lines (Fig. 1a and Supplementary Table 1). Comparison of the resulting PACO cell–derived tumors (DT) with the original xenografts (PT) showed conservation of histomorphological characteristics (Fig. 1a) and RNA expression profiles (Supplementary Table 2). By matching recent genomic profiling data16-18, we found that all eight analyzed PACO lines harbored mutations in KRS and that this resistance is mediated by a cell-autonomous CYP3A5-dependent drug detoxification mechanism. CYP3A5 also contributes to acquired drug resistance in other subtypes of PDAC and in other malignancies.

Next we determined which PDAC subtypes were represented in our PDAC models by using the PDAssigner genes11—a gene signature developed for classifying classical, QM-PDA and exocrine-like PDAC—and gene-set enrichment analysis (GSEA) to subtype eight PACO lines, as well as their respective PT and DT xenografts. All three subtypes, including the exocrine-like subtype, were represented (Supplementary Fig. 1a,b). Specifically, the gene expression profiles of two of our PACO lines and xenografts were enriched for the classical subtype (PACO2 and PACO17), whereas three were enriched for the exocrine-like subtype (PACO10, PACO14 and PACO18) and three for the QM-PDA subtype (PACO7, PACO9 and PACO19). Taken together, these results demonstrate that our models faithfully preserve the histomorphological characteristics of the originating tumors and, for the first time, enable the study of functional differences between all three PDAC subtypes.

Prognostic value of HNF1A and KRT81 expression
Because histopathology supplemented by immunohistochemistry is still currently the standard method for tumor subtyping, we sought to identify surrogate protein markers for each of these three PDAC subtypes to facilitate clinical patient stratification. We subjected PACO cell lines to array-based gene expression profiling. To generate a candidate biomarker list, we filtered a list of genes that were strongly (>5-fold; P < 0.05) differentially expressed between the different PACO subtypes for candidates that showed heterogeneous expression across PDAC specimens in the Protein Atlas database19. Additionally, GSEA of transcription factor activity gene sets on the original expression profiles revealed an enrichment of transcripts with binding sites for the transcription factor HNF1A in the exocrine-like subtype, suggesting HNF1A as a putative marker for this subtype (data not shown). We stained PACO lines and xenografts for all marker candidates, and

Figure 1 Subtype stratification of PDAC models and patients by using two markers.
(a) Schematic overview of the experimental workflow used to generate orthotopic xenografts and PACO cell lines, and images showing H&E staining of a human PDAC tumor sample and the corresponding first-passage xenograft (PT), phase-contrast imaging of the derived cell line (PACO10) and H&E staining of the respective derived xenograft (DT). Scale bars, 100 µm.
(b) Immunofluorescence staining for KRT81 (left, red) and HNF1A (right, green) in PACO lines from the QM-PDA (top), exocrine-like (exocrine; middle) and classical (bottom) PDAC subtypes (n = 3 per group). Nuclei are represented in blue. Scale bars, 50 µm.
(c) KRT81- (left) and HNF1A-immunostained (right) paraffin sections from a TMA of individuals with PDAC (n = 241). Scale bars, 100 µm. (d) Kaplan-Meier analysis of overall survival of subjects with PDAC (n = 217). Tumor sections on the TMA were retrospectively subtyped into three groups on the basis of KRT81 and HNF1A expression, as determined by immunostaining (HNF1A+ (exocrine-like), n = 46; DN (classical), n = 92; KRT81+ (QM-PDA), n = 79). P < 0.001 by log-rank test.
Figure 2  Exocrine-like PDAC cells, which express CYP3A5, are resistant to TKIs. (a) PACO line–specific drug sensitivities to 1 µM erlotinib (left) or dasatinib (right) after 48 h of treatment. Each symbol represents an individual PACO line (n = 2 biological replicates per PACO line). Error bars depict mean ± s.e.m. ***P < 0.001; by grouped one-way analysis of variance (ANOVA). (b) GSEA of the PACO exocrine-like PDAC cell lines (left) or xenografts (PT + DT; right) compared to the cell lines or xenografts, respectively, of the classical and QM-PDA PDAC subtypes (REST), using the indicated gene signatures. Statistical significance was assessed using 10,000 permutations. ES, enrichment score; NES, normalized enrichment score; FDR, false-discovery rate. (c) Sensitivity of QM-PDA (top), exocrine-like (middle) and classical (bottom) PACO lines treated with erlotinib (left) or dasatinib (right) for 48 h after pretreatment with ketoconazole (100 nM; open diamonds) or vehicle (colored triangles) for 2 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (d,e) CYP3A5 expression, as measured by qRT-PCR, in PACO lines (d) and PACO-derived xenografts (DT) (e), as compared to that in pancreas and liver (n = 3 per cell type). Values are relative to those for CYP3A5 expression in PAC018 cells. Error bars depict mean ± s.e.m. **P < 0.05; by grouped one-way ANOVA. (f) Immunoblot analysis for CYP3A5 expression in PACO cell lines. Vinculin was used as a loading control. L, liver protein lysate. (g) Representative immunohistochemical images showing immunostaining for HNF1A (left) and CYP3A5 (right) in sections from individuals with HNF1A* PDAC (n = 217). Scale bars, 100 µm. (h) qRT-PCR (top, graphs) and immunoblot (bottom) analyses for CYP3A5 expression before (control) and after treatment with 10 µM dasatinib or erlotinib in representative QM-PDA (PAC09), exocrine-like (PAC010 and PAC014) and classical (PAC02) PACO lines (n = 3 per group under condition). Actin was used as a loading control for immunoblot. For qRT-PCR data, values are relative to those of untreated controls. Error bars depict mean ± s.e.m. *P < 0.05, **P < 0.01; n.s., not significant; by Student's t-test.
Figure 3 CYP3A5 mediates drug resistance and is regulated by HNF4A and NR1I2 expression in exocrine-like PDAC cells in vitro. (a) Top, immunoblot analysis of CYP3A5 expression in untreated PACO14 exocrine-like PDAC cells or those transfected with a non-targeting (siNT-control) or CYP3A5-specific (siCYP3A5) siRNA. Vinculin was used as a loading control. Bottom, concentrations, as determined by LC-MS/MS analysis, of erlotinib or dasatinib in the supernatants of PACO14 cell cultures after transfection with siCYP3A5 or siNT-control and treatment with erlotinib or dasatinib (10 µM) (n = 3 per condition). Error bars depict mean ± s.e.m. **P < 0.01; by grouped one-way ANOVA. (b) Sensitivity of PACO14 exocrine-like PDAC cells that were transfected with siNT-control (green) or siCYP3A5 (black) to treatment with erlotinib (left) or dasatinib (right) for 48 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (c) PACO line–specific sensitivities to 10 µM paclitaxel after 48 h of treatment. Each symbol represents an individual PACO line (n = 2 biological replicates per PACO line). Error bars depict mean ± s.e.m. of technical replicates. (d) Immunoblot analysis for CYP3A5 in two shCYP3A5- or shScr-expressing exocrine-like PDAC lines. Vinculin was used as a loading control. (e) Sensitivity of shCYP3A5- or shScr-expressing PACO10 (left) and PACO14 (right) cells to treatment with paclitaxel for 48 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (f,g) qRT-PCR analysis, normalized to mRNA levels in PACO18 cells, of HNF4A (f) and NR1I2 (g) in PACO lines, pancreas and liver (n = 3 per cell type). Error bars depict mean ± s.e.m. *P < 0.05; by grouped one-way ANOVA. (h)–(j) Immunoblot analysis for CYP3A5 expression (left, n = 3 per group) and drug sensitivity (right; n = 3 per group, one representative example is shown) of siNT-control–transfected (h–j), siHNF4A-transfected (h), siNR1I2-transfected (i), or siHNF4A- and siNR1I2-transfected (j) PACO14 cells after treatment with 10 µM (left) or serial dilutions (right) of paclitaxel or DMSO (control) for 48 h. mRNA expression values are relative to those from DMSO-treated, siNT-control–transfected cells. Error bars depict mean ± s.e.m. of biological replicates (left) and of technical replicates (right). *P < 0.05, **P < 0.01; n.s., not significant; by Student’s t-test.

evaluated signal intensity and subtype specificity (Supplementary Table 4). We excluded markers that stained only weakly or that were not subtype specific. This analysis identified positive nuclear staining for HNF1A specifically in exocrine-like PDAC cells, whereas staining for cytokeratin 81 (KRT81) was specific for cells of the QM-PDA PDAC subtype (Fig. 1b). Additionally, in The Cancer Genome Atlas (TCGA) pancreatic adenocarcinoma (PaAD) cohort, KRT81 expression also inversely correlated with that of HNF1A (Supplementary Fig. 1c). None of the candidate markers for the classical PDAC subtype showed a reliable and exclusive staining pattern in cells of this subtype. Nevertheless, the specificity of KRT81 and HNF1A staining allowed us to define classical subtype specimens as being double negative (DN) for these markers. Hence, we defined surrogate markers for the three subtypes as KRT81+HNF1A− for the QM-PDA subtype, KRT81–HNF1A+ for the exocrine-like subtype and KRT81+HNF1A− for the classical subtype.

We verified the association of our marker-defined subtypes with the PDAssigner signatures in an independent validation cohort of primary PDAC xenografts (Supplementary Table 5). Principal component analysis (PCA) demonstrated that transcriptional profiles clustered according to KRT81- and HNF1A-defined subtypes (Supplementary Fig. 1d). Hierarchical clustering using the PDAssigner genes further showed a separation into three groups, revealing a high concordance with our marker-defined groups (Supplementary Fig. 1e). GSEA
of the marker-defined groups revealed enrichment of the QM-PDA signature in the KRT81+ tumors and of the exocrine-like signature in the HNF1A+ tumors. The DN tumors were enriched for all three signatures and could not be unequivocally assigned (Supplementary Fig. 1f), suggesting that the PDAssigner signature would need to be improved for a more robust classification. Collectively, our surrogate markers separate the validation cohort into three distinct groups, of which the KRT81+ and HNF1A+ cases are enriched in the respective PDAssigner-defined subtypes11.

Next we tested whether subtype stratification of a cohort of 231 individuals with PDAC, by using these two markers is associated with clinical outcome. In a retrospective study using immunohistochemistry, we designated these PDAC tumors as 45% DN, 35% KRT81+ and 20% HNF1A+ (Fig. 1c,d and Supplementary Table 6). We also identified 14 KRT81+HNF1A+ double-positive specimens and excluded them from the analysis. Log-rank analysis revealed significant differences in overall survival of individuals with PDAC of different subtypes (P < 0.001) (Fig. 1d). Subjects with HNF1A+ tumors had the best mean survival (43.5 months), followed by those with the DN subtype (26.3 months) and the KRT81+ subtype (16.5 months). Moreover, Cox proportional-hazards multivariate regression analysis revealed that the survival impact of the subtype classification is independent of established conventional prognostic factors such as stage, grade and age20,21 (Supplementary Table 7). Subtype was associated with grade, as the HNF1A+ cases were more differentiated (24% grade 3), the KRT81+ samples tended to be less differentiated (50.6% grade 3), and the DN cases ranged in between (41.5% grade 3) (Supplementary Table 8). Although this association was significant (P = 0.01), grade alone was insufficient to predict subtype. Hence, HNF1A and KRT81 can be used to stratify patients into subtypes of PDAC that are associated with differences in overall patient survival.

Exocrine-like PDAC cells are resistant to tyrosine kinase inhibitors

To address whether the subtypes differ in drug sensitivity, PACO lines were treated with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) erlotinib and the SRC and ABL1 tyrosine kinase–selective TKI dasatinib, which are approved or under investigation for the treatment of PDAC, respectively22–24. We treated PACO lines of each subtype with 1 µM (Fig. 2a) or 10 µM (Supplementary Fig. 2a) erlotinib and dasatinib, which corresponds to 0.3- to 3-fold and 6- to 60-fold peak plasma concentrations reported in humans, respectively25,26. Analysis after 48 h of treatment revealed that the classical and QM-PDA cells were drug sensitive, whereas the exocrine-like cells were almost completely drug resistant. To exclude the possibility that the observed resistance was due to varying proliferation rates, we treated the PACO lines as described above for 7 d and confirmed the difference in drug response (Supplementary Fig. 2b,c). To identify the mechanisms underlying the observed drug resistance, we used GSEA to compare the exocrine-like PACO lines and xenografts with the classical and the QM-PDA PACO lines and xenografts. This analysis revealed an enrichment of signatures comprising genes involved in xenobiotic biotransformation in the exocrine-like PDAC models (Fig. 2b and Supplementary Fig. 2d,e). For validation we analyzed an independent data set generated from laser-microdissected PDAC11 and confirmed the upregulation of similar gene sets in exocrine-like PDAC samples (Supplementary Fig. 2f). Thus, xenobiotic biotransformation might contribute to the observed drug resistance in cells of the exocrine-like PDAC subtype.

CYP family enzymes systemically metabolize small-molecule drugs by oxidation, resulting in potential inactivation of the drugs27,28. To test whether xenobiotic biotransformation is indeed involved in the observed drug resistance, we pretreated cells of each subtype with the pan-CYP inhibitor ketoconazole14. We pretreated one PACO line of each subtype with 100 nM ketoconazole or vehicle for 2 h, added serial dilutions of erlotinib or dasatinib and determined relative cell viability after 48 h. To compare TKI effects across independent experiments, we calculated activity areas as previously described29. Ketoconazole pretreatment significantly increased TKI sensitivity exclusively in the exocrine-like PDAC cells, rendering their drug response comparable to that in the other subtypes (Fig. 2c and Supplementary Fig. 2g). These results suggest that CYPs contribute to drug resistance in PDACs of the exocrine-like subtype.

CYP3A5 is expressed and inducible in exocrine-like PDAC cells

Members of the CYP3A subfamily are major contributors to xenobiotic biotransformation of small-molecule drugs in the liver12.

Figure 4 CYP3A5 mediates drug resistance in exocrine-like PDAC cells in vivo. (a) Growth curves of PDAC xenografts from mice with shScr- or shCYP3A5-expressing PACO14 exocrine-like cells that were treated for two cycles of 5 d with erlotinib (100 mg per kg body weight (mg/kg)) and 2 d of recovery, followed by 4 d of treatment with paclitaxel (left, round 1). Cells from one xenograft per treatment group were re-injected into previously untreated mice, which were then treated for two cycles of 5 d with paclitaxel and 2 d of recovery (right, round 2) (n = 6 mice per treatment group). Tumor volume was measured with a digital caliper and normalized to that at baseline (day 0, dashed line). Error bars depict mean ± s.e.m. P values were determined at the end point using one-sided Mann-Whitney U test. *P < 0.05; **P < 0.01; n.s., not significant.
We thus tested expression of all three CYP3A family members—CYP3A4, CYP3A5, and CYP3A7—in PACO cell lines and DTs. qRT-PCR analysis revealed that CYP3A5 is exclusively expressed in the exocrine-like subtype at comparable or even higher levels than in normal liver and pancreas (Fig. 2d,e). In contrast, expression of CYP3A4 and CYP3A7 was low or absent (Supplementary Fig. 2h–k). The specific expression of CYP3A5 in exocrine-like PDAC cells was also confirmed at the protein level in PACO lines (Fig. 2f and Supplementary Fig. 2i) and in specimens of individuals with HNF1A+ tumors (Fig. 2g). The marker-defined exocrine-like

Figure 5 CYP3A5 contributes to acquired resistance in QM-PDA and classical PDAC cells. (a) Left, growth curves of PDAC xenografts derived from PACO17 classical PDAC cells treated as described for Figure 4b round 1. Right, growth curves of tumors using cells from one xenograft per group that were re-injected into previously untreated mice, which were then treated as described for Figure 4b round 2 (n = 6 mice per treatment group). Error bars depict mean ± s.e.m. P values were determined at the end point using one-sided Mann-Whitney U test. **P < 0.01; n.s., not significant. (b) qRT-PCR analysis of CYP3A5 expression in PACO17-derived round one (R1) and round two (R2) tumors after treatment with paclitaxel or vehicle (n = 3 mice per group). Values are relative to those of R1 tumors from vehicle-treated control mice. Error bars depict mean ± s.e.m. **P < 0.01; n.s., not significant, by Student's t-test. (c) Representative images (n = 3) for CYP3A5 immunostaining in sections from PACO17-derived R1 (top) and R2 (bottom) xenografts after treatment of mice with vehicle (left) or paclitaxel (right). Scale bars, 100 µm. (d) Drug-sensitivity curves for parental (PACO2Ctrl and PACO7Ctrl) and paclitaxel-resistant (PACO2PR and PACO7PR, respectively) classical (PACO2) and QM-PDA (PACO7) cell lines treated with paclitaxel for 48 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (e) qRT-PCR analysis comparing CYP3A5 expression in PACO2Ctrl and PACO7Ctrl cells to that in PACO2PR and PACO7PR cells (n = 3 per group). Values are relative to those derived from liver mRNA. Error bars depict mean ± s.e.m. **P < 0.01; by Student's t-test. (f) Drug-sensitivity curves for PACO2PR (left) and PACO7PR (right) cells treated with paclitaxel for 48 h after pretreatment with ketoconazole (100 nM) or vehicle for 2 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (g) Anti-CYP3A5 immunoblot of sNT-control or siCYP3A5 transfected PACO2PR and PACO7PR cells. Vinculin was used as a loading control. (h) Drug-sensitivity curves for sNT-control- or siCYP3A5-transfected PACO2PR (left) and PACO7PR (right) cells treated with paclitaxel for 48 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates. (i) Representative immunoblot analysis (n = 3) for CYP3A5 in classical (PACO2) and QM-PDA (PACO19) cell lines transduced with either an empty control vector (Ctrl) or one that overexpresses CYP3A5 (CYP3A5OE). Vinculin was used as a loading control. (j) Sensitivity of PACO2 (left) and PACO19 (right) cells transduced with a control or a CYP3A5-overexpressing vector to treatment with paclitaxel for 48 h (n = 3 per group; one representative example of three biological replicates is shown). Error bars depict mean ± s.e.m. of technical replicates.
Enzymes involved in xenobiotic biotransformation can be induced in response to their substrates. To test whether this regulatory mechanism is also functional in PDAC cells, we measured CYP3A5 mRNA and CYP3A5 protein expression in PACO lines at steady state and in response to treatment with 10 µM dasatinib or erlotinib. Exposure to either drug boosted CYP3A5 expression in the exocrine-like, but not in the classical and QM-PDA, PACO lines (Fig. 2h). No increase in expression of CYP3A4 or CYP3A7 was observed (Supplementary Fig. 2o). Taken together, these data reveal that CYP3A5 is highly expressed, and that its expression can be further induced, in cells of the exocrine-like PDAC subtype in vitro.

CYP3A5 mediates drug resistance in exocrine-like PDAC cells

To test whether CYP3A5 metabolizes erlotinib and dasatinib in exocrine-like PDAC cells, we measured the chemical modification of these drugs in two different exocrine-like PACO lines that were transfected with either a CYP3A5-specific (siCYP3A5) or a non-targeting (siNT-control) short interfering RNA (siRNA). Quantitative mass spectrometric analysis (using liquid chromatography–coupled tandem mass spectrometry (LC-MS/MS)) revealed a rapid conversion of erlotinib and dasatinib in siNT-control–transfected cells, as illustrated by loss of their unmodified forms from the supernatant; this did not occur in cells transfected with the siCYP3A5 (Fig. 3a and Supplementary Fig. 3a,b). Chemical modifications by CYP enzymes can have a neutral effect, or they can activate or inactivate small-molecule inhibitors. If CYP3A5 inactivates these compounds, its expression would explain the observed resistance to erlotinib and dasatinib in exocrine-like PDAC cells. Indeed, siRNA-mediated knockdown of CYP3A5 significantly and exclusively sensitized the exocrine-like PACO cells to these drugs (Fig. 3b and Supplementary Fig. 3c,d). As microtubule-targeting taxanes are also substrates for CYP3A family members, we next asked whether CYP3A5 expression affects the recently introduced paclitaxel-based treatment for PDAC. Treatment of PACO lines with paclitaxel indeed revealed that the exocrine-like PDAC subtype was significantly more resistant than the other two subtypes (Fig. 3c and Supplementary Fig. 3e–g). To verify the role of CYP3A5 in this context, we established exocrine-like PACO lines that stably expressed control (shScr) or CYP3A5–specific (shCYP3A5) shRNAs (Fig. 3d). Similarly to that of erlotinib and dasatinib (Supplementary Fig. 3h,i), knockdown of CYP3A5 sensitized the exocrine-like PACO cells to paclitaxel (Fig. 3e).

The strong upregulation of CYP3A5 in response to erlotinib, dasatinib and paclitaxel (Fig. 2h and Supplementary Fig. 3j) suggests a major contribution of CYP3A5 induction to the observed drug resistance. Of the genes that encode transcription factors known to regulate the expression of CYP3A family members, HNF4A and NR1I2 (also called PXR) are selectively expressed in exocrine-like PACO cells at levels comparable to those in normal liver (Fig. 3f). Whereas HNF4A–dependent transcription is activated by its ubiquitous ligand, linoleic acid, NR1I2 initiates transcription in response to xenobiotics such as erlotinib, dasatinib and paclitaxel.

We performed individual or combined siRNA knockdowns for HNF4A (using siHNF4A) and NR1I2 (using siNR1I2) to test their contribution to basal and induced expression of CYP3A5 (Supplementary Fig. 3k). Basal expression of CYP3A5 was significantly reduced by knockdown of HNF4A but not NR1I2 (Fig. 3h,i and Supplementary Fig. 3l,m). In contrast, induction of CYP3A5 expression after treatment with erlotinib, dasatinib and paclitaxel was significantly impaired by knockdown of NR1I2 but not HNF4A (Fig. 3h,i and Supplementary Fig. 3l,m). Combined knockdown of HNF4A and NR1I2 significantly decreased both basal and drug-induced CYP3A5 expression (Fig. 3j and Supplementary Fig. 3n,o). Next, we tested the contribution of both factors to drug resistance. Knockdown of either HNF4A or NR1I2 rendered exocrine-like PACO cells susceptible to all of the drugs tested (Fig. 3i,h and Supplementary Fig. 3l,m). The combined knockdown of HNF4A and NR1I2 rendered the cells even more sensitive to drug treatment than that achieved by the knockdown of HNF4A or NR1I2 alone (Fig. 3j and Supplementary Fig. 3n).

We next asked whether ablation of CYP3A5 expression could sensitize established tumors to small-molecule drugs in vivo. We thus established subcutaneous tumors from shScr- or shCYP3A5–expressing exocrine-like PACO cells in NSG mice. After the tumors reached an average volume of 200 mm³, the mice were treated with...
erlotinib or vehicle by oral gavage for five consecutive days followed by 2 d of rest, for a total of 14 d. Whereas treatment with erlotinib had no significant effect on the growth rate of the shScr-expressing tumors, growth of the shCYP3A5-expressing tumors was significantly inhibited (Fig. 4a and Supplementary Fig. 4a). Additionally, CYP3A5 expression was significantly (PAC010: P < 0.05; PACO14: P < 0.01) increased in shScr-expressing tumors after treatment of mice with erlotinib but not vehicle (Supplementary Fig. 4b). Knockdown of CYP3A5 also significantly enhanced the response to paclitaxel treatment (Fig. 4b and Supplementary Fig. 4c). To extend the in vivo treatment period, we re-injected cells recovered after treatment round one into secondary mice (round two). Even in round two, paclitaxel treatment significantly suppressed the growth of shCYP3A5-expressing tumors (Fig. 4b and Supplementary Fig. 4c), and CYP3A5 expression was significantly (P < 0.01) higher in shScr-expressing tumors from paclitaxel-treated mice as compared to those from vehicle-treated mice (Supplementary Fig. 4d). We conclude that long-term suppression of CYP3A5 expression in exocrine-like xenografts does not lead to induction of alternative resistance pathways.

CYP3A5 contributes to acquired resistance in QM-PDA and classical PDAC cells

The development of secondary resistance limits the efficacy of drug treatment in PDAC1. We thus asked whether CYP3A5 also contributes to acquired resistance. To this end, tumors of the classical subtype were treated with paclitaxel for two rounds for a total of 32 d. Paclitaxel treatment significantly inhibited tumor growth of classical xenografts during the first round of treatment, whereas longer-term treatment led to a marked development of paclitaxel resistance (Fig. 5d). CYP3A5 knockdown (Supplementary Fig. 5a) restored drug sensitivity in the paclitaxel-resistant sublines as compared to those from vehicle-treated mice (Supplementary Fig. 4d). We conclude that long-term suppression of CYP3A5 expression in exocrine-like xenografts does not lead to induction of alternative resistance pathways.

CYP3A5 contributes to acquired resistance in other malignancies

Expression of CYP family members has been described in a range of tumors14,41. To address whether CYP3A5 mediates resistance in tumor types other than PDAC, we stained a tissue microarray (TMA) comprising 438 individual tissue samples from 33 distinct tumor types for CYP3A5 (Fig. 6a and Supplementary Fig. 6a). Samples from 10 of 33 tumor types expressed detectable amounts of CYP3A5. We found particularly high expression of CYP3A5 in the majority of hepatocellular carcinoma, gastric carcinoma, cervical carcinoma, adrenal gland cortical carcinoma and biliary tract cancer tissue samples, indicating that CYP3A5 may mediate drug resistance in a considerable fraction of solid tumors (Supplementary Table 9). To begin to test this hypothesis, we screened a number of gastric and hepatocellular carcinoma cell lines for CYP3A5 expression. The gastric cancer cell line SNU 5 and the hepatocellular carcinoma cell line HepG2 expressed CYP3A5 at levels comparable to those in normal liver and were selected for further experiments (Fig. 6b and Supplementary Fig. 6b). We found that paclitaxel exposure resulted in the induction of CYP3A5 expression in these cell lines (Supplementary Fig. 6b) and that pretreatment with ketoconazole sensitized both cell lines to paclitaxel treatment (Fig. 6d). We observed a similar sensitization after CYP3A5 knockdown in HepG2 cells (Fig. 6c,e and Supplementary Fig. 6d), suggesting that CYP3A5 expression contributes to drug resistance in tumor types other than PDAC.

DISCUSSION

Here we confirm the existence of three reported PDAC subtypes11 and identify two surrogate markers, HNF1A and KRT81, for tumor stratification by using immunohistochemistry. Our finding—that individuals with resectable HNF1A+ exocrine-like PDAC have the best survival rates—might be perceived contradictory at first. However, patient survival is not only determined by drug response; growth rate of the primary tumor as well as the propensity for, and the pattern of, metastasis also influence survival28. In fact, exocrine-like PACO cells are slower to expand in culture and have a delayed onset in xenograft formation as compared to PACO cells of the classical and QM-PDA subtypes (data not shown). This suggests that tumors originating from exocrine-like PDAC cells are the least aggressive PDAC subtype, despite their resistance to drug treatments.

Drug response in cancer patients is influenced by hepatic CYPs that mediate systemic drug metabolism, whereas only minor amounts of these enzymes are expressed in other tissues22,43. Although a role for CYPs in tumor cell–autonomous drug detoxification has been postulated14,44,55, this concept has never been functionally demonstrated. We now demonstrate that CYP3A5 contributes to both basal and acquired resistance to small-molecule drugs in PDAC. Because CYP3A5 is dispensable for normal physiology26,57, its inhibition in cancers is a promising therapeutic option. It may be challenging to design a CYP3A5-specific inhibitor owing to the structural similarities between CYP3A family members, although a highly selective CYP3A4 inhibitor has been reported58. Expression of CYP family enzymes are frequently induced by their substrates12,37. We show that basal and substrate-induced expression of CYP3A5 is differentially regulated by HNF4A and NR1I2. Interfering with these regulatory mechanisms may provide an alternative approach to suppress the CYP3A5 pathway, thus overcoming basal and acquired drug resistance in PDAC.

The described CYP3A5-mediated resistance mechanism is not limited to PDAC, as expression and functional analyses suggest that subsets of other cancer types may use the same resistance strategy. Consequently, CYP3A5 expression should be taken into consideration in the interpretation of results from drug trials, as targets of this enzyme probably have decreased efficacy in CYP3A5-expressing tumors.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4029.
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AUTHOR CONTRIBUTIONS

E.M.N. and C.E. share first authorship, and A.S. and E.E. share second authorship of this paper. E.M.N. and C.E. established, conducted and analyzed the experiments; A.S., A.M. and W.W. performed immunohistochemical analyses of all of the tissue specimens presented and performed respective data analyses; E.E. did the immunofluorescence staining experiments and analyses on publicly available data sets; B.K., W.N. and C.R. performed RNA expression analyses on the PDAC validation cohort; C.K., V.V., J.E., F.M.Z., O.E., M.S. and R.E. provided technical and experimental support; C.L. and M.K. conducted and analyzed LC-MS/MS experiments; X.J. and A.K.-S. performed activity area calculations; P.N., M.B. and B.V.S. provided PDAC tissue microarray characterization; N.A.G., T.H., O.S., J.W. and M.W.B. provided samples of individuals with PDAC; A.T. and M.R.S. supervised the project; E.N., C.E., A.T. and M.R.S. developed the concept, designed experimental studies, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
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ONLINE METHODS

Human tissue specimens. Tissue samples were obtained from patients admitted to the Department of General, Visceral and Transplantation Surgery, University of Heidelberg (by M.W.B.). The study was approved by the ethical committee of the University of Heidelberg (case number 301/2001) and conducted in accordance with the Helsinki Declaration; written informed consent was obtained from all patients. Patient and tumor characteristics are summarized in Supplementary Table 1. The PDAC validation cohort consists of a subset of the HIPO-015 study, for which xenografts were readily available. The xenografts were also derived from surgically removed specimens from individuals with PDAC that received partial pancreateoduodenectomy at the University Hospital Heidelberg. The study was approved by the ethical committee of the University of Heidelberg (case number 5-206/2011) and conducted in accordance with the Helsinki Declaration; written informed consent was obtained from all patients. Patient and tumor characteristics of the PDAC xenograft validation cohort are summarized in Supplementary Table 5.

Xenografts of primary tumor specimens and PACO cell lines. To establish primary xenografts, tumors were cut into 1–2 mm³ pieces and implanted onto the pancreas of 8- to 12-week-old female NOD.Cg-Prkd<sup>−/−</sup>Il2rg<sup>−/−</sup>Il19<sup>−/−</sup> (NSG) mice, which were bred in the animal facility of the German Cancer Research Center. For the generation of xenografts from the PACO lines, a suspension of 10<sup>5</sup>–10<sup>6</sup> cultured cells in Matrigel (2 mg/ml) (BD) was injected into the pancreas of NSG mice. Engraftment of tumors and subsequent growth was monitored by regular palpation of the implantation site. Orthotopically grafted tumors were surgically removed and subsequently analyzed by immunohistochemistry and gene expression profiling, or used for the generation of PACO cultures. Animal care and all procedures followed the German legal regulations and were previously approved by the governmental review board of the state of Baden-Wuerttemberg, Regierungspräsidium Karlsruhe authorization numbers G64-10, G39-13, G105-14 and G80-15.

Cell culture. For the generation of PACO cultures, primary xenografts were resected after attaining a volume of approximately 1 cm³. Tumor pieces were first minced using sterile scalpels and then dissociated into single cells by incubation with 1 µg/ml collagenase IV (Sigma) for 2 h at 37 °C. The resulting suspension was filtered through a 100 µm mesh, and cell debris and dead cells were removed by density centrifugation (Ficoll Paque Plus, Amersham). The remaining erythrocytes were removed using ACK Buffer (Lonza). To establish PACO cultures, single cells (5 × 10<sup>5</sup>) were seeded in T75 flasks (Primaria, BD) in serum-free medium (referred to as PACO medium) as described before<sup>39</sup>. PACO medium contains advanced Dulbecco’s modified Eagle’s medium–glucose mixture F-12 (DMEM/F12; Life Technologies) with N2 supplement (Life Technologies), 50 ng/ml basic fibroblast growth factor (bFGF; Peprotech), 20 ng/ml epidermal growth factor (EGF; Peprotech), 10 ng/ml LONG R<sup>3</sup> insulin-like growth factor–I (IGF-I) (Sigma), 100 µM β-mercaptoethanol (Life Technologies), 2 µg/ml heparin (Sigma). Adherent monolayer cultures were maintained at 37 °C and 5% CO<sub>2</sub>. After the outgrowth of tumor cells, contaminating fibroblasts were removed by trypsinization. We obtained SNU 5, SNU 16, KATO III, NCI N87, SK Hep1 and HepG2 cells from the American Type Culture Collection (ATCCC).

Erlotinib-, dasatinib- and paclitaxel-resistant PACO cells were generated by continuously exposing cells to the individual compounds (10 nM or 20 nM) for 2 months. In brief, medium supplemented with the respective drugs was replaced every 4 d. At a confluency of 70%, cells were passaged and allowed to recover for 24 h until treatment was continued. Erlotinib, dasatinib or paclitaxel resistances were confirmed by dose-response studies as described below.

All cell lines used were authenticated monthly by single-nucleotide polymorphism profiling and tested for mycoplasma contamination (both by Multiplexion).

Sanger sequencing. Genomic DNA was prepared from PACO cells using DNAeasy Blood and Tissue Kit (Qiagen). Genomic DNA regions containing KRAS and TP53 mutations were amplified by PCR using Q5 hot start high-fidelity master mix (New England Biolabs Inc.) according to the manufacturer’s instructions. PCR primer pairs used are summarized in Supplementary Table 10. PCR products were purified using the High Pure PCR Product Purification Kit (Roche). Sanger sequencing was subsequently performed (GATC Biotech) and analyzed using ApE software, Version 2.0.49 (M. Wayne Davis; available for free at http://biologylabs.utah.edu/wayne/apE/). Variant positions are relative to the reference sequences NM_004985 (KRAS) and NM_000546 (TP53).

Gene expression analysis. Total RNA was isolated from different PACO lines at early and late passages at 80% confluence, or from 50 mg of tumor tissue using the miRNeasy kit (Qiagen) according to the manufacturer’s instructions. Gene expression analysis was performed using Illumina HumanHT12v4 BeadChips at the Genomics and Proteomics Core Facility of the German Cancer Research Center (GDPF DFKZ, Heidelberg). Correlation plots, Spearman’s rank correlation coefficients and significance (two-tailed) of log<sub>2</sub> gene expression from the PACO data sets (PACO lines, primary (PT) and secondary (DT) xenografts) were generated using GraphPad Prism 6.0b software.

Gene set enrichment analysis (GSEA) was conducted on quantile-normalized data from the PACO and validation cohort data sets. In order to assign the corresponding PDAC subtype to the individual PACO samples, the previously described PDAssigner signature was used to derive gene sets for each individual subtype<sup>11</sup>. We computed P values using 1,000 or 10,000 permutations for each gene set and corrected them with the false-discovery rate (FDR) method<sup>20</sup>. We performed subtype assignment for the initial eight samples by comparing each individual sample against the remaining seven samples (designated REST) for each gene set. Samples were assigned to the signature and the respective subtype with the lowest FDR value (QM-PDA: PACO7, PACO9, PACO19; exocrine-like: PACO10, PACO14, PACO18; classical: PACO2, PACO17). GSEA was then repeated using the stratified groups for comparison. This initial cohort was used to identify surrogate protein markers for each subtype, and subsequent PDAC subtype classifications were marker based using immunohistochemistry.

RNA-seq by expectation maximization (RSEM<sup>42</sup>)-normalized RNA-seq expression data of 183 primary PDAC tumors were obtained from The Cancer Genome Atlas (TCGA) PAAD data sets available online (Broad Institute). Spearman’s rank correlation coefficients and significance (two-tailed) were calculated using GraphPad Prism 6.0b software for each pair of genes.

Gene expression data of the HIPO-015 xenograft validation cohort were quantile-normalized and corrected for unwanted variation using the tsva function of the surrogate variable analysis (SVA) package<sup>62,63</sup>. Specifically, first, the number of latent factors (‘surrogate variables’) was determined by the function n.sv(). Then, the surrogate variables were estimated using the function sv(a) (with the subtypes as known covariates), and finally the function fsv(a) was used to regress out the surrogate variables and to obtain the corrected gene expression data. Probes mapping to multiple genes were excluded, and the first probe per gene was retained from the remaining set. Unsupervised principal component analysis (PCA) was then computed from the log<sub>2</sub>-transformed data using the 500 genes with highest variability across samples<sup>64</sup>. Calculations were performed by R Version 3.2.2.

The significance analysis of microarray (SAM)<sup>65</sup> was used to identify differentially regulated genes at an FDR < 0.05 with a fold change of >2. Additionally, differential expression of CYP3A5 was validated based on log<sub>2</sub>-transformed mRNA expression data of the annotated xenograft samples from the validation cohort. Intensities of the probe with the highest average intensity per gene were retrieved from quantile-normalized microarray data. Unpaired t-test (two-tailed) was used to compute statistical significance (P < 0.05). Calculations were performed by R Version 3.2.2.

Hierarchical clustering analysis was performed on quantile-normalized, SVA-corrected, log<sub>2</sub>-transformed gene expression data of the validation cohort using R Version 3.2.2. Probes mapping to multiple genes were excluded, and the first probe per gene was retained from the remaining set. The intersection between the set of gene expression symbols and the previously determined PDAssigner gene set contained the variables used for hierarchical clustering with Manhattan distances and single linkage.

Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4029. The results shown...
in Supplementary Figures 1c and 2n are, in part, based upon data generated by TCGA Research Network (http://cancergenome.nih.gov/).

LC-MS/MS analysis. (S)-(−)-propranolol hydrochloride (internal standard) was purchased from Sigma. Acetonitrile was from Merck; ammonium acetate and formic acid from Merck; methanol from VWR International; and dimethylsulfoxide from Applichem. 500 µl of reaction medium was quenched with 1,000 µl of acetonitrile at each time point and mixed.

After centrifugation, clear supernatants were prediluted with PACO medium and acetonitrile at a ratio of 1:2.5. 100 µl of the sample was transferred into a new vial, 10 µl of (S)-(−)-propranolol hydrochloride solution (105 µg/µl) was added, and the solution was vigorously mixed. Calibration and quality control samples were prepared by spiking PACO medium with either dasatinib or erlotinib. 10 µl of the samples was injected onto a PerfectSil Target ODS-3 HPLC column (3 µm, 100 × 2.1 mm, MZ-Analysetechnik), using an Agilent 1100 (Agilent) binary pump and degasser, with a CTC PAL sampler (CTC Analytics). Chromatographic separation was performed by gradient elution at a constant flow rate of 250 µl/min for 15 min. The gradient consisted of 20 mM NH₄OAc plus 0.1% formic acid (mobile phase A) and 400 mM NH₄OAc/methanol/acetonitrile (5:5:90) plus 0.1% formic acid (mobile phase B). The gradient applied was 0.0 min, 70% A–30% B; 1.5 min, 70% A–30% B; 3.0 min, 5% A–95% B; 11.0 min, 5% A–95% B; 11.5 min, 70% A–30% B; and 15 min, 70% A–30% B. From 4- to 8-run time minute, the eluate was directed to a QTrap 5500 mass spectrometer (SCIEX) equipped with an electrospray ion source. Mass transitions of 488.1 to 401.1 for dasatinib, 394.0 to 278.1 for erlotinib and 260.1 to 116.1 for (−)-(−)-propranolol were monitored. Ionization was achieved at 5.5 kV and a temperature of 400 °C. Nitrogen was applied as curtain, collision and drying gas. Declustering potentials, collision energy and collision exit potential was as follows: 26 V, 39 V and 12 V for dasatinib; 16 V, 43 V and 26 V for erlotinib; and 26 V, 39 V and 12 V for (S)-(−)-propranolol.

Tissue microarray. The tissue microarray (TMA) was constructed from individuals that received partial pancreatectoduodenectomy for PDAC between 1991 and 2006 at the Charité University Hospital Berlin. The use of this tumor cohort for biomarker analysis has been approved by the Charité University ethics committee (EA1/06/2004). Formalin-fixed and paraffin-embedded tissue samples were used to generate tissue microarrays as previously described.

Briefly, each PDAC sample included was represented by three different tissue cores, each measuring 1 mm in diameter and chosen by a board of certified pathologists as being representative for the respective tumor. From the defined regions, tissue cylinders of 1.5-mm diameter were punched from each donor sample and arrayed into a new ‘recipient’ paraffin block using a semiautomatic tissue microarrayer (Beecher Instruments). The human various cancers high-density TMA, which is composed of VA2-SBC, VB2-SBC and VC2-SBC samples were used to generate tissue microarrays as previously described.

Primary antibodies and dilutions are described in Supplementary Table 11 and HNF1A if the tumor cells in the respective tissue-microarray spots showed a detectable staining regardless of the strength of the signal or the number of positive cells. However, in those instances in which staining of tumor cells was detectable for any of the markers, the respective staining was usually strong.

Hence, if any tumor cell was found positive for KRT81 or HNF1A expression in any of the cores, the tumor was defined as QM-PDA or exocrine-like, respectively. Stromal cells were negative in all instances; normal acinar pancreatic cells (when present) expressed HNF1A homogenously to a moderate degree but were consistently negative for the other two markers.

As whole-tissue slides were used in the validation cohort of xenografts, the scoring system was adapted to account for a higher level of heterogeneity, as observed in the samples stained for KRT81 and HNF1A. Specifically, a cutoff of at least 10% KRT81-positive tumor cells was introduced to consider a sample to be QM-PDA. Positive staining of a single tumor cell did not justify an allocation to a specific biological subtype. Additionally, the evaluation of HNF1A staining was adapted by only considering samples with moderate or strong nuclear staining reactions to be exocrine-like. A few cases with an extremely light nuclear staining reaction of HNF1A were observed, and these were not considered to represent biologically relevant HNF1A expression.

Immunofluorescence. PACO cells were seeded on T75 flasks (Primaria, BD) and grown to 60–70% confluency. Cells were fixed in 4% freshly depolymerized formaldehyde for 15 min, permeabilized with 0.25% (vol/vol) Triton X-100 (Sigma) in PBS for 15 min and blocked with 1% BSA in PBS for 1 h. Primary antibodies (Supplementary Table 11) were incubated overnight (O/N) at 4 °C, and detected by fluorescence using secondary antibodies coupled to fluorochromes (Life Technologies) that were diluted 1:1,000 and incubated for 1 h in the dark. Isotype-matched secondary antibodies conjugated with Alexa Fluor 488 or phycoerythrin (PE) were incubated for 1 h at room temperature (RT). Slides were mounted using ProLong Antifade GOLD with DAPI (Life Technologies), as described by the manufacturer.

Western blot analysis. Whole-cell lysates of PACO cells were prepared using RIPA buffer (Cell Signaling Technology), 1 mM PMSF (Sigma), 1 mM EDTA and Halt Protease-Phosphatase Inhibitor Cocktail (Pierce). Protein lysates were resolved on 4–12% Bis-Tris NuPage gels with MOPS running buffer (Life Technologies) and blotted on nitrocellulose membranes (Amersham International). Membranes were blocked for 1 h in TBS containing 0.1% (vol/vol) Tween-20 with 20% (wt/vol) nonfat dry milk powder (blocking solution). Primary antibodies (Supplementary Table 11) were incubated O/N at 4 °C in blocking solution. Secondary HRP-coupled antibodies (Southern Biotech) were diluted 1:10,000 in blocking solution and incubated for 1 h at RT. Membranes were washed in 0.1% TBS–TWEEN-20, and immunocomplexes were detected using the ECL kit (Amersham International). As positive controls, recombinant CYP3A5, CYP3A4, CYP3A7 (Abnova) and human liver lysates (Novus) were used.

Real-time quantitative PCR. Total RNA was extracted using the miRNeasy mini kit (Qiagen) and reverse-transcribed using the high capacity cDNA reverse-transcription kit (Applied Biosystems). cDNA corresponding to 10 ng of starting RNA was used for relative RNA quantification (qRT-PCR). TaqMan probes (Applied Biosystems) for CYP3A5 (Hs00241417_m1), CYP3A4 (Hs00600406_m1), CYP3A7 (Hs00426361_m1), HNF4A (Hs00230853_m1), NR1I2 (Hs01114267_m1), PPIA (Hs04194521_s1) and GAPDH (Hs99999905_m1) were used to acquire expression data with the Viia 7 Real-Time PCR System (Applied Biosystems). The Viia 7 software 1.1 was used for data acquisition and analysis. As positive controls, RNA samples from total normal liver and pancreas were used (Novus).

siRNA transfection of PACO cells. PACO cells were grown to 80% confluency. The transfection reagent Dharmafect 4 (Thermo Scientific), non-targeting (siNT-control) and siRNAs targeting CYP3A5, HNF4A or NR1I2 (On-Target plus SMARTpool Thermo Scientific; Supplementary Table 12) were pre-incubated at RT for 5 min at a ratio of 1:4 in IMDM culture medium ( Gibco). For the HNF4A and NR1I2 double-knockdown cells, the individual siRNAs were pre-incubated together at a ratio of 1:8 in IMDM culture medium (Gibco). Dharmafect 4
was then combined with the siRNAs and incubated for another 20 min at RT. The mixture was then added to the PACO culture medium. The culture medium was aspirated, and the transfection agent–RNA complex mixture was added to the monolayer. Flasks were incubated at 37 °C for 72 h until further analysis.

Generation of stable knockdown cells. Stable shRNA-mediated knockdown of CYP3A5 was achieved by targeting the sequence 5′-TTGATTTCAGGATCTTTCT-3′ in a pGIPZ vector (shCYP3A5) (GE Healthcare, Thermo Scientific; Supplementary Table 12). In addition, the non-silencing control pGIPZ vector (shScr) was used as negative control (GE Healthcare, Thermo Scientific). Lentiviral particles were produced in HEK 293T cells (ATCC). Viral particles were concentrated, and PACO cells were transduced at a multiplicity of infection of 1:5. Successfully transduced cells were selected by cell sorting for GFP and western blotting analyses confirmed knockdown efficiency.

Stable expression of CYP3A5. PACO cells were stably transduced with either the expression vector pLenti-GIIJ-CMV-RFP-2A-Puro (Applied Biological Materials Inc.) containing the full CYP3A5 open reading frame (CYP3A5OE) or an empty control vector (Ctrl). Lentiviral particles were produced in HEK 293T cells. Viral particles were concentrated, and PACO cells were transduced at a multiplicity of infection of 1:5. Successfully transduced cells were selected by cell sorting for RFP and western blotting confirmed CYP3A5 expression.

Drug-treatment assays. Dasatinib, erlotinib and paclitaxel (LC Laboratories) were dissolved in water-free DMSO. For the determination of relative cell viability, 10 µM and 1 µM, or serial dilutions, of the three drugs were screened in quadruplicate. In brief, 8,000 cells/well were seeded in 96-well plates 24 h before the addition of the individual compounds. For the co-treatment experiments, either siRNA transfection was carried out as described or cells were pretreated with 100 nM ketoconazole for 2 h and then treated in the presence of ketoconazole. After incubation for 48 h or 7 d, cell viability was assessed using CellTitertBlue (Promega) following the manufacturer's instructions. Treatment with vehicle (DMSO) was used as a negative control. Treatment with 20 µM staurosporine (LC Laboratories) was used as a positive control. Responses were normalized to DMSO- and staurosporine-treated controls. Relative cell viability curves were plotted using GraphPad Prism 6.0b software.

In vivo drug treatment. Tumors were established by subcutaneously injecting 5 × 10³ shCYP3A5– or shScr-expressing exocrine-like (PACO10, PACO14) and classical (PACO17) cells into 8- to 12-week-old female NSG mice, using Matrigel (2 mg/ml) in a total injection volume of 100 µl. After the tumors reached a size of approximately 200 mm³, mice were randomly assigned to the respective treatment groups (n = 6 per group) for drug administration. Erlotinib was prepared in 0.5% methylcellulose, 0.1% Tween 80 and 99.4% water for injection (WFI). Erlotinib (100 mg/kg) or vehicle was administrated by oral gavage for five consecutive days followed by 2 d of rest, for a duration of 14 d. Paclitaxel was prepared in 50% Cremophor EL (Sigma) and 50% absolute ethanol (Sigma) to a concentration of 6 mg/ml. Before administration, paclitaxel was further diluted in 0.9% NaCl (Braun) to a final concentration of 0.4 mg/ml. Paclitaxel (2 mg/kg) or vehicle was then administrated by intraperitoneal injection for two cycles of 5 d with paclitaxel and 2 d of recovery followed by 4 d with paclitaxel. Cells from one xenograft per treatment group were re-injected subcutaneously, and the mice were treated for two cycles of 5 d with paclitaxel and 2 d of recovery. Tumor volumes were determined (blinded; twice weekly by caliper measurements) and calculated according to the formula (length × height × width) × (π/6) at the end of the experiments. Tumor growth was calculated for each individual tumor by normalizing to the tumor volume at day 0. After 14 d or 18 d of treatment, mice were sacrificed and tumors were resected for further analyses. Mice were excluded if they had to be sacrificed before the treatment started or if tumor size exceeded the maximum allowable volume during the treatment experiment.

Statistical analysis. For all in vitro experiments, at least three biological replicates were used or grouped analyses were carried out. For all in vivo experiments, at least six mice per treatment group were used. Hence, for the reported differences, the sample size used gave sufficient power to call them reliable. Quantitative results were analyzed by one-way analysis of variance (one-way ANOVA), one-sided Mann-Whitney U test and Student's t-test using GraphPad Prism 6.0b software. Survival analysis was performed by using the Mantel-Cox log-rank test as well as the Cox proportional-hazards multivariate regression analysis using the Statistical Package for the Social Sciences (IBM SPSS software). Additionally, the Pearson chi-squared test was used for comparative data analysis, using SPSS. We considered P < 0.05 (two-sided) as statistically significant. For GSEA, a false discovery rate (FDR) of <0.2 was considered statistically significant. In vitro treatment data were evaluated by determining the activity area² from each dose-response curve by adding ‘max’ (100 – mean response, 0) for every concentration. Activity areas were compared by paired t-test. Calculations were performed in R Version 3.1.0 (ref. 67). Estimation of variation within each group was determined by s.e.m. or s.d.

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