Selective multi-kinase inhibition sensitizes mesenchymal pancreatic cancer to immune checkpoint blockade by remodeling the tumor microenvironment

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KRAS-mutant pancreatic ductal adenocarcinoma (PDAC) is highly immunosuppressive and resistant to targeted and immunotherapies. Among the different PDAC subtypes, basal-like mesenchymal PDAC, which is driven by allelic imbalance, increased gene dosage and subsequent high expression levels of oncogenic KRAS, shows the most aggressive phenotype and strongest therapy resistance. In the present study, we performed a systematic high-throughput combination drug screen and identified a synergistic interaction between the MEK inhibitor trametinib and the multi-kinase inhibitor nintedanib, which targets KRAS-directed oncogenic signaling in mesenchymal PDAC. This combination treatment induces cell-cycle arrest and cell death, and initiates a context-dependent remodeling of the immunosuppressive cancer cell secretome. Using a combination of single-cell RNA-sequencing, CRISPR screens and immunophenotyping, we show that this combination therapy promotes intratumor infiltration of cytotoxic and effector T cells, which sensitizes mesenchymal PDAC to PD-L1 immune checkpoint inhibition. Overall, our results open new avenues to target this aggressive and therapy-refractory mesenchymal PDAC subtype.
PDAC tumor microenvironment (TME), this leads to scarcity of tumor-infiltrating lymphocytes (TILs). Recent studies reported rare cases of PDAC patients presenting tumors with high T-cell infiltration, a feature associated with prolonged overall survival10–12. These reports highlighted the potential to treat PDAC more effectively by targeting the immunosuppressive TME and recruiting TILs via rational combination therapies.

**KRAS** is mutationally activated in >90% of PDAC patients. However, to date no clinically applicable strategy has been developed to effectively treat KRAS-mutant PDAC. Downstream of oncopgenic KRAS, the RAF–MEK–ERK pathway plays a central role in tumor initiation13. Although MEK inhibitors yielded therapeutic value in RAS-mutant melanoma and lung cancer14,15, unstratified trials failed in PDAC patients. Recently, we and others showed that an increased gene dosage (IGD) and expression of mutant KRAS (KRAS-mut) drives the disease, with the mesenchymal, nonglandular, basal-like subtype displaying the highest IGD and expression of mutant KRAS (KRAS-mut)28 (Fig. 1a) and expression of mutant KRAS (KRAS-mut) drives the disease, with the mesenchymal, nonglandular, basal-like subtype displaying the highest IGD and expression of mutant KRAS (KRAS-mut)28 (Fig. 1a). In line with these findings, we validated our findings in long-term clonogenic assays in a larger cohort and observed synergistic interactions of trametinib and nintedanib in 11 of 15 hPDAC cultures, with the strongest effect in the mesenchymal subtypes (Fig. 2c–e, P=0.0099). We extended the combination screen to 30 additional mPDAC cultures. A high synergism was achieved in most of the models, with the mesenchymal KRAS-mut-overexpressing cells predominantly benefitting from the treatment (Fig. 2f–h). Antagonism was observed in 3 of 15 human and 6 of 30 mouse cell cultures, respectively, which were characterized almost exclusively by a classical epithelial morphology. In line with the heterogeneity of responses observed in the clinical setting, even within molecularly stratified cohorts, we detected synergism in some classical PDAC cultures, demonstrating heterogeneity within subtypes (Fig. 2c–h and Extended Data Fig. 3a,b). Trametinib and nintedanib (T/N)-treated epithelial and mesenchymal PDAC cells revealed similar changes in ERK phosphorylation, indicating that primary sensitivity and resistance to T/N is not due to differences in canonical pathway blockade (Extended Data Fig. 3c). Increasing the dosage of both inhibitors did not alter the antagonistic action of the drug combination substantially, arguing for fundamental biological differences between the tumors (Extended Data Fig. 3d). Strikingly, we observed cell death on T/N treatment, with the strongest effects in mesenchymal PDAC (Fig. 2i).

**Drug screening identifies new therapies for mesenchymal PDAC.** We performed a systematic, high-throughput, combination compound screen to identify drugs synergizing with MEKi. We screened two hPDAC and two mPDAC cultures, representing both classical and mesenchymal KRAS subtypes, with trametinib in combination with 418 drugs in preclinical and clinical investigations (Fig. 2a and Supplementary Table 2). One of the top hits in mesenchymal PDAC was the clinically approved RTK inhibitor nintedanib (Fig. 2b). We validated our findings in long-term clonogenic assays in a larger cohort and observed synergistic interactions of trametinib and nintedanib in 11 of 15 hPDAC cultures, with the strongest effect in the mesenchymal subtypes (Fig. 2c–e, P=0.0099). We extended the combination screen to 30 additional mPDAC cultures. A high synergism was achieved in most of the models, with the mesenchymal KRAS-mut-overexpressing cells predominantly benefitting from the treatment (Fig. 2f–h). Antagonism was observed in 3 of 15 human and 6 of 30 mouse cell cultures, respectively, which were characterized almost exclusively by a classical epithelial morphology. In line with the heterogeneity of responses observed in the clinical setting, even within molecularly stratified cohorts, we detected synergism in some classical PDAC cultures, demonstrating heterogeneity within subtypes (Fig. 2c–h and Extended Data Fig. 3a,b). Trametinib and nintedanib (T/N)-treated epithelial and mesenchymal PDAC cells revealed similar changes in ERK phosphorylation, indicating that primary sensitivity and resistance to T/N is not due to differences in canonical pathway blockade (Extended Data Fig. 3c). Increasing the dosage of both inhibitors did not alter the antagonistic action of the drug combination substantially, arguing for fundamental biological differences between the tumors (Extended Data Fig. 3d). Strikingly, we observed cell death on T/N treatment, with the strongest effects in mesenchymal PDAC (Fig. 2i).

We assessed the consequences of oncopgenic KRAS-mut expression on cell morphology and treatment response using classical mPDAC cells transduced with a doxycycline-inducible KRAS vector or GFP as control. KRAS-mut overexpression led to increased ERK1/2 phosphorylation, decreased E-cadherin expression (Cdh1) and morphological changes of the epithelial PDAC cells toward a mesenchymal spindle-shaped morphology, which increased from day 1 to day 14 (Extended Data Fig. 3e–h). T/N treatment revealed a remarkable change in drug sensitivity. Although the controls
Fig. 1 | Resistance to MEK inhibition in vitro and in vivo. a, Kaplan–Meier analysis comparing survival of surgically resected patients having either G1–G2 or G3–G4 tumor grading. We combined data from refs. 6–8. b, Left: percentage of cell viability at 10 nM trametinib in hPDAC cell lines. Cell morphology and ssGSEA EMT signature are integrated below. Cells showing a classical phenotype are marked in yellow, and those presenting a mesenchymal phenotype in blue. The hPDAC17 and hPDAC7 are primary human patient-derived PDAC cell cultures generated from PDX models in our laboratory4. Right: violin plots comparing the percentage of cell viability at 10 nM trametinib between classical and mesenchymal hPDAC cell cultures. c, Allele-specific KrasG12D mRNA expression in classical (n = 21 mice) and mesenchymal (n = 9 mice) tumors by combining amplicon-based RNA-seq and reverse transcription-quantitative PCR. AU, arbitrary units. d, Left: percentage of cell viability at 10 nM trametinib in mPDAC cell cultures. Cell morphology and ssGSEA EMT signature are integrated below. Right: comparison of the percentage of cell viability at 10 nM trametinib between classical and mesenchymal mPDAC cell cultures. e, Schematic representation of the dual-recombinase system to inducibly delete floxed Mek1 and Mek2 in established tumors using a tamoxifen-activatable CreERT2 allele. f, Viability assay of 4-hydroxytamoxifen (4-OHT)-treated Mek1lox/lox;Mek2lox/lox (mPDAC1), Mek1lox/lox;Mek2lox/lox (mPDAC2) and Mek1lox/lox;Mek2lox/lox (mPDAC3) cell cultures compared with vehicle (ethanol, ETOH). Data are shown as mean ± s.d. (n = 3 independent experiments). g, Schematic representation of the experimental set-up to test treatment efficacy of trametinib in vivo using subtype-specific models. Mesenchymal (9091) and classical (8661) mPDAC cells were used for the orthotopic transplantation experiments. h, Representative MRI of trametinib-treated mice before (week 2) and after 1 week of treatment (week 3). Scale bars, 5 mm. i, Waterfall plot of the fold-change in tumor volume compared with baseline (determined by MRI-based volumetric measurements) of orthotopically transplanted classical and mesenchymal tumors after 1 week of trametinib treatment. P values in b (right), c (right) and i were calculated using a two-tailed, unpaired Student’s t-test. The P value in a was calculated using the log(rank) (Mantel–Cox) test.
showed antagonism, this was reverted to synergy in the KRAS\textsuperscript{G12D} overexpression system (Extended Data Fig. 3i,j), supporting the notion that KRAS levels impact on cellular differentiation states\textsuperscript{16}, are the main drivers of mesenchymal PDAC and provide therapeutic vulnerabilities.

To uncover the direct targets of trametinib and nintedanib, and to elucidate the drivers of treatment response and resistance, we performed kinobead pulldown assays on six mPDAC cultures of both subtypes. Although trametinib showed selectivity for binding MEK1/2, nintedanib displayed a broad range of targets, enriched in RTKs and cell-surface receptors (Extended Data Fig. 4a–c). Importantly, expression of the nintedanib targets was subtype dependent. Four were selective for mesenchymal PDAC, including PDGF receptor B (PDGFRB), fibroblast growth factor receptor 1 (FGFR1) and discoidin domain tyrosine kinase receptor 2 (DDR2), and 24 were shared by both subtypes. Furthermore, expression of these targets remained largely unchanged after drug perturbation (Extended Data Fig. 4d,e). Therefore, differences in the basal gene expression program underlie synergistic drug action in PDAC subtypes.

To identify the functional relevant pathways mediating response to T/N, we analyzed changes in the phosphoproteome. In mesenchymal PDAC, we observed a decreased activity of a broad range of important cancer-relevant pathways, such as the cell-cycle regulators cyclin-dependent kinase 2 (CDK2), cyclin D and cyclin E, PP2A and immediate early response 3 (IER3) that regulate phosphoinositide 3-kinase/AKT signaling, and ERBB2, mTOR and KIT downstream signaling, as well as RAF-dependent and -independent ERK1/2 activation (Extended Data Fig. 4f and Supplementary Table 3). These findings support the view that mesenchymal PDAC cells depend on broad RTK-driven signaling input. In contrast, we found...
a more limited spectrum of altered pathways in classical tumors, including distinct downregulation of the VEGFA/VEGFR pathway and Rho GTPases, as well as decreased mitogen-activated protein kinase activation (Extended Data Fig. 4g and Supplementary Table 3). This confirmed and extended our initial observation of fundamental differences in signaling between classical and mesenchymal tumors under basal conditions (Extended Data Fig. 1), reinforcing the notion that multiple kinases need to be targeted to achieve meaningful responses in the mesenchymal subtype.

Next, we assessed other drugs sharing targets with nintedanib. Strikingly, in mesenchymal PDAC several top hits of our combination drug screen are multi-kinase inhibitors that display an overlapping target spectrum with nintedanib (Extended Data Fig. 5a,b and Supplementary Tables 2 and 4). In addition, we tested new compounds with overlapping targets (Extended Data Fig. 5c; https://www.proteomicsdb.org). AZD4547, which shares with nintedanib, among others, the targets PDGFRB, FGFR1, DDR1 and DDR2, mimics the synergistic responses observed with T/N and could potentially be used in combination with trametinib. In contrast, other drugs that share targets with nintedanib, including imatinib, display highly heterogeneous responses, showing cell-type- and context-specific synergisms (Extended Data Fig. 5c–e).

To functionalize the targets of nintedanib and decipher key genes synergizing with MEKI, we employed pooled genome-wide, as well as focused, CRISPR–Cas9-based negative selection (viability) screens in three mesenchymal mPDAC cell cultures. We performed a genome-scale screen in 9091 cells and an in-house-developed, nintedanib-target-focused screen in 8248 and 8570 cells, with and without trametinib (Fig. 3a–e, Extended Data Fig. 6a–f and Supplementary Tables 5 and 6). To identify genes altering trametinib sensitivity, we calculated differential sensitivity scores, as the difference in β-score between the trametinib- and dimethylsulfoxide (DMSO)-treated arms. We focused on genes displaying a negative differential sensitivity score, indicating enhanced depletion in the presence of trametinib (Fig. 3b and Extended Data Fig. 6f).

In the genome-scale CRISPR screen we identified 8 nintedanib targets out of a total of 758 hits with statistically significant β-scores, the inactivation of which resulted in the specific depletion of trametinib-treated cells (Fig. 3b–d). In the focused screens in 8248 and 8570 cells, nine and four nintedanib targets cooperated with trametinib, respectively (Fig. 3d). Altogether, of 53 nintedanib targets identified in kinobead pulldown experiments, 15 showed functional relevance in the presence of trametinib in mesenchymal PDAC cells (Fig. 3d). Importantly, the three different mesenchymal cell cultures showed some degree of heterogeneity across relevant nintedanib targets, indicating potential differences in underlying genetic, epigenetic and phenotypic characteristics of this subtype. Further analysis of the targets and their interaction using the STRING database (http://string-db.org) revealed a high degree of interconnectivity and convergence on FGFR, MEK/ERK family members and PDGFR-regulated networks, with different members of these pathways observed in the three screens (Fig. 3d). Unexpectedly, gene expression profiling revealed no clear correlation between messenger RNA abundance of nintedanib targets and trametinib sensitization on depletion (Extended Data Fig. 6g). Taken together, these data indicate that no single kinase, but rather a specific spectrum of targets, such as PDGFR, FGFR and MEK/ERK family members, act in concert to mediate therapeutic efficacy in a context-specific manner.

Analysis of the 758 hits of the genome-scale CRISPR screen allowed us to identify pathways in mesenchymal PDAC, which globally cooperated with MEKI, such as ERBB, PDGFRB, KIT and the retinoblastoma gene (RBI) (Fig. 3e). These pathways correlated to a high degree with the phosphoproteomic analysis of the T/N-treated mesenchymal PDAC cells, thereby crossvalidating these results functionally (Extended Data Fig. 4).

To extend and validate the top-scoring nintedanib targets of our negative selection screens, we exploited single and combination CRISPR–Cas9 single-guide (sg)RNA-based approaches. First, we individually depleted Acrv1, Grb2, Map2k5, Map3k3, Prkaa1 and Fgfr1, and assessed cell growth using clonogenic assays with and without trametinib. In line with our negative selection screens, we observed heterogeneity in the cooperation of these nintedanib targets with MEKI across the different models (Fig. 3f,g and Extended Data Fig. 6h–j). To probe the cooperation among the identified targets in mediating trametinib sensitization, we next depleted Acrv1, Grb2, Map2k5, Map3k3, Prkaa1 and Fgfr1 in triple combinations with and without trametinib, using a transfection-based Cas9–sgRNA RNP approach (Fig. 3h–k and Extended Data Fig. 6k,l). In addition, we correlated the relative indel frequency of each combination with the loss of viability in the presence of trametinib (Fig. 3h,k). Consistently, we observed a heterogeneous response across the three cellular models, with the combination depletion of Prkaa1, Fgfr1 and Map2k5 being most efficient in two out of three mesenchymal cell cultures in the presence of trametinib. This confirmed our hypothesis that the depletion not of one, but of a combination of, multiple targets is important to sensitize mesenchymal PDAC toward MEKI. Thus, broad targeting is needed to efficiently and comprehensively treat mesenchymal PDAC.

**T/N triggers a T cell-dependent anti-tumor immune response.** Our in vitro findings prompted us to explore the combination treatment in vivo in syngeneic orthotopic transplantation models of
classical and mesenchymal PDAC (Fig. 1g). These models resemble the clinical course of PDAC patients, with the mesenchymal subtype model being more aggressive, displaying rapid and uniform tumor progression to death (Fig. 4a).

We randomized tumor-bearing mice and observed that the T/N combination led to a remarkable response of mesenchymal PDAC with a significant reduction in tumor volume of up to ~40% and doubled survival (Fig. 4b–d). Moreover, we observed not only a decrease in tumor-cell proliferation, but also vascular remodeling as evidenced by an increased amount of CD31+ vessels (Fig. 4e–h), as well as markers of angiogenesis and endothelial cell activation (Extended Data Fig. 7a–c).
Unexpectedly, also the classical subtype responded to the combination therapy; however, this effect was mainly mediated by trametinib as evidenced by similar tumor volumes of trametinib- and T/N-treated cancers ($P=0.786$; Fig. 4b). In addition, mesenchymal tumors showed a superior overall response with two partial remissions and stable disease in 12 of 21 mice treated with T/N, according to the response evaluation criteria in solid tumors (RECIST) (Fig. 4b,c). In contrast, classical tumors showed no partial remission and only 3 of 18 mice displayed stable disease. Accordingly, the difference in T/N-induced tumor regression between both subtypes is statistically significant ($P=0.0162$; Fig. 4b). This translated into improved overall survival, which was doubled in the mesenchymal subtype (36 d for T/N versus 16 d for controls), whereas classical tumors displayed only a 50% increase (27 d for T/N versus 20 d for controls; Fig. 4d). This difference in survival benefit is statistically significant ($P=0.0007$; Fig. 4d, right panel). Thus, the new combination is, to our knowledge, the first preclinical therapy that induces tumor regression and an increase in overall survival in Kras-mut IGD-driven mesenchymal PDAC. In line with the differential response, we observed a subtype-specific decrease in PDGFRB–Tyr1021 and AMPKα–Thr172 (PRKAA1) phosphorylation of T/N-treated mesenchymal tumors in vivo (Fig. 4i). Both kinases are important nintedanib targets identified in the proteomic kinobead-based pulldown approach (Extended Data Fig. 4).

PDAC is considered to be immunologically ‘cold’: its microenvironment is characterized by a lack of cytotoxic T cells and infiltration of immunosuppressive immune cells. T/N treatment substantially increased T-cell infiltration into mesenchymal tumors. In contrast, classical tumors displayed features of immune exclusion—showing only a moderate enrichment of T cells at the tumor margins (Fig. 5a–d and Extended Data Fig. 7d–f). Immunophenotyping of mesenchymal tumors revealed increased infiltration of CD8+ T cells, localized preferentially around vessels, arguing that vascular remodeling on drug perturbation contributes to cytotoxic T-cell infiltration (Extended Data Fig. 7a–c).

To investigate the role of T cells in therapy response, we employed CD3ε-knockout mice on a C57BL/6 background, lacking all T cells (Extended Data Fig. 7g,h). T-cell deficiency blunted the T/N response and diminished the survival benefits of mesenchymal tumors (Fig. 5e–h and Extended Data Fig. 7b). The tumor volume decreased significantly in wild-type (WT) versus CD3ε-knockout mice ($P=0.0124$; Fig. 5f). In addition, T/N treatment prolonged survival compared with controls by 20 d in WT mice, but only marginally by 5 d in mice lacking T cells ($P=4.9 \times 10^{-2}$; Fig. 5g,h). These data support the notion that T cells contribute significantly to the in vivo efficacy of the T/N combination in the mesenchymal subtype. However, treatment response is not mediated by T cells alone, but depends on TME reprogramming and tumor-cell intrinsic drug action.

In contrast to mesenchymal PDAC, the classical subtype showed a mixed response in T-cell-deficient animals. There was no difference in tumor volume ($P=0.563$, WT versus CD3ε-knockout mice; Fig. 5e), but a slight effect of T-cell deficiency on survival, which was reduced by 5 d in T/N-treated CD3ε-knockout mice ($P=0.028$; Fig. 5g,h), indicating immune surveillance also in this model on therapy, but to a significantly smaller extent ($P=0.0014$; Fig. 5h).

Cancer therapies can affect macrophage functions and alter their recruitment or polarization states. The combination treatment did not change the overall number of macrophages substantially (Extended Data Fig. 8a,b). However, their polarization changed from the pro-tumorigenic M2- to an anti-tumorigenic M1-like state (Extended Data Fig. 8c–e), suggesting that they might contribute to anti-tumor responses. However, this effect was present in both subtypes. Therefore, these changes do not explain the observed subtype-specific differences.

Distinctively, epithelial tumors displayed an increase of neutrophils on treatment (Extended Data Fig. 8a,f). The role of tumor-associated neutrophils is controversial; some studies have shown their ability to block anti-tumor immune responses and mediate CD8+ T-cell suppression, and others have highlighted their various anti-tumor properties, including direct cytotoxicity and inhibition of metastasis. Therefore, distinct immune cell types and mechanisms seem to contribute to the anti-tumor effects of the drug combination in both subtypes, with T cells being the key contributor to the strong effects observed in mesenchymal PDAC.

The T/N combination sensitizes mesenchymal PDAC toward ICB. Our in vivo findings prompted us to investigate whether the combination therapy could sensitize the highly aggressive mesenchymal subtype toward ICB. Compared with controls, anti-programmed death-ligand 1 (PD-L1) and T/N-treated groups, T/N + anti-PD-L1 therapy-induced tumor regression up to ~80% and increased survival selectively in mesenchymal PDAC ($P=0.016$, T/N + anti-PD-L1 versus T/N; Fig. 6a–c). The median survival benefit by adding anti-PD-L1 to the combination was 10.5 d compared with T/N alone and 30.5 d compared with vehicle, representing an almost threefold increase in survival (Fig. 6c). Six of sixteen mice showed objective tumor regression with a partial response according to RECIST. Of the remaining ten mice, eight displayed stable and only two progressive disease ($P=0.078$ T/N + anti-PD-L1 versus T/N; Fig. 6a). In contrast, no tumor regression, survival benefit or increased response rate was observed in classical PDAC, and both subtypes did not respond to ICB with PD-L1 alone (Fig. 6a,c).

In summary, the triple treatment with ICB markedly improves anti-tumor responses, offering a clear survival benefit selectively in mesenchymal PDAC.

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Fig 4 | The combination treatment prolongs survival and reprograms the TME in vivo. a, Kaplan–Meier curve comparing the survival of classic and mesenchymal orthotopic PDAC models. The number of mice per treatment condition is indicated. b, Quantification of tumor volume changes of the classical and mesenchymal subtype after 1 week of indicated treatment assessed by MRI. Each column represents one mouse, in comparison with baseline MRI measurements before treatment. Control, C57BL/6 mice treated with vehicle (control) or T/N for 1 week. Scale bars, 50 µm. c, Representative MRI of vehicle (control) and T/N-treated mice before (week 2) and after 1 week of treatment (week 3). Scale bars, 5 mm. d, Left and middle panels: Kaplan–Meier survival curve of classical and mesenchymal orthotopic models. The number of mice per treatment condition is indicated. Right: comparison of the ratio between the survival of mice treated with T/N and the median survival of their respective vehicle-treated controls. e, Representative H&E and IHC staining for Ki67 and CD31 of tumor sections from orthotopically transplanted classical and mesenchymal PDAC subtype models treated with vehicle (control) or T/N for 1 week. Scale bars, 100 µm. P values in b,d (right), f–h were calculated using a two-tailed, unpaired Student’s t-test. P values in a and d (left and middle) were calculated using log(rank) (Mantel–Cox) test. Note that the classical and mesenchymal cohorts in a are the same as those shown in d. Vehicle- and trametinib-treated cohorts shown in b are the same as shown in Fig. 1.
ScRNA-seq reveals context-specific T/N-driven changes. To investigate therapy-induced TME changes in a global and unbiased fashion, and to mechanistically decipher the drug action on epithelial and mesenchymal tumor cells and their environment in vivo, we performed single-cell RNA sequencing (scRNA-seq) of whole tumors. One to two PDAC per model and treatment condition were dissociated, sorted into mesenchymal/fibroblast and epithelial/immune-enriched cell fractions and sequenced (Fig. 7a). To define cell populations, we combined the data from both subtypes and all treatment groups, representing a total of 30,677 cells (1,677–13,169 cells per model and treatment condition; Fig. 7b). In both subtypes we identified tumor cells, acinar cells, T cells, natural killer cells, B cells, myeloid populations, such as macrophages and neutrophils, and fibroblasts (Extended Data Fig. 9a). In the classical tumors we
additionaly identified a cluster of endothelial cells (Fig. 7b and Extended Data Fig. 9a,b).

Treatment-induced cancer cell transcriptional changes. We assessed the impact of the combination therapies on gene expression of cancer cells and observed no evidence of epithelial-to-mesenchymal transition (EMT) or the reverse process (MET) in T/N-treated tumors with and without ICB (Extended Data Fig. 9c). Gene set enrichment analysis (GSEA) uncovered widespread and, in part, context-specific, treatment-induced alterations of immune-related pathways in tumor cells (Fig. 7c–e). For both subtypes, we observed an induction of antigen processing and crosspresentation (Fig. 7d). In addition, mesenchymal PDAC showed an exclusive and striking enrichment for interferon signaling signatures, including an interferon-γ response as well as interferon regulatory factor 2 (IRF2) induction in the context of triple therapy (Fig. 7d,e and Extended Data Fig. 9d).

A growing body of evidence connects immune responses to errors in DNA replication and genomic instability37,38. GSEA revealed treatment-induced DNA damage in both subtypes. We validated this observation by γH2AX IHC, a marker for DNA damage. It is interesting that induction of DNA damage was stronger in the classical subtype, indicating that the remarkable treatment response of mesenchymal PDAC is not mediated by DNA damage alone (Extended Data Fig. 9e–g). Several studies link DNA damage to the senescence-associated secretory phenotype (SASP) as well as anti-tumor immune responses39. GSEA showed that SASP was strongly enriched exclusively in classical PDAC and this was maintained across treatments (Fig. 7f). Senescence-associated β-galactosidase (SA-β-Gal) staining confirmed this phenotype (Fig. 7g). This indicates that the therapeutic effects observed in the classical subtype are mediated by a complex combination of mechanisms, which include induction of DNA damage, SASP and a reduction in cell proliferation.

Drug treatment-induced immune responses. Our in vivo immune profiling shows a robust anti-tumor immune program in mesenchymal PDAC centered on T cells on T/N treatment. Analysis of the T cells (3,260 cells) of our scRNA-seq dataset (Fig. 8) revealed 6 subpopulations for both PDAC subtypes (clusters 1–6) (Fig. 8a and Methods).

T/N-treated mesenchymal tumors showed a substantial decrease of CD4+ and CD8+ T cells with a gene expression signature resembling naïve T cells, and a dramatic increase in T cells with functional cytotoxic, effector and memory gene expression signatures (Fig. 8b,c). Addition of anti-PD-L1 to T/N resulted in a further increase of cytotoxic and effector T cells up to almost 75% of all T cells (Fig. 8b).

In contrast to recent studies indicating that SASP induces vascular remodeling leading to T-cell infiltration and anti-tumor immunity41, the T/N combination reprogrammed the TME of classical PDAC toward a decrease of regulatory and effector T cells and an increase in the naive-like CD4+ compartment (Fig. 8b). Furthermore, the functional gene expression signature was much weaker in the cytotoxic and effector clusters in classical compared with mesenchymal tumors (Fig. 8c).

Together, our findings show that, in mesenchymal tumors, the T/N combination alone leads to a considerable increase of effector-like, activated and cytotoxic T cells, indicating an effective anti-tumor immune response, which was further enhanced by anti-PD-L1 treatment. This contrasts with the classical subtype, which showed a reduction of regulatory and effector T cells on treatment despite displaying an SASP phenotype.

To gain insights into changes that could mediate T-cell infiltration on therapy, we analyzed the secretomes of T/N-treated epithelial and mesenchymal tumor cells with mass spectrometry (MS)-based proteomics42. We observed profound T/N-induced changes in the secretion of immunomodulatory chemokines and cytokines between both subtypes (Extended Data Fig. 10a). To get a comprehensive overview of the resulting intercellular communication networks, we integrated tumor-cell-secreted proteins across treatment conditions with cell populations expressing the corresponding receptors as identified by scRNA-seq. This revealed key differences in the secretion of factors previously shown to modulate immune cell recruitment, differentiation and expansion, as well as tumor immune responses (Fig. 8d and Extended Data Fig. 10b, inner circle)43–45. In mesenchymal PDAC, the T/N combination specifically induced CXCL12, CXCL16 and TNSF5 secretion, whereas CCL2, CSF1 and LGALS9 were downregulated (Fig. 8d). This contrasts with the classical subtype, which showed reduced levels of secreted CXCL12, CXCL16 and CXCL20, as well as an increase in CCL2 and CSF1 on T/N treatment (Extended Data Fig. 10a,b). CXCL16, which is upregulated in mesenchymal PDAC on T/N treatment, is a key chemoeattractor for TILs and high expression levels correlate with a favorable prognosis and increases TILs, for example, in colorectal and breast cancer46–48. Accordingly, PDAC tumors with high CXCL16 mRNA expression levels display an increased overall survival (n = 176 samples, log(rank) test, P = 0.042; https://www.proteinatlas.org/ENSG00000161921-CXCL16/pathology/pancreatic+cancer). CXCL12 increases cytotoxic T-cell infiltration in osteosarcoma49 and CCL2 and CSF1 mediate immunosuppression in various cancer types50,51. Therefore, T/N treatment induces a tumor-cell-derived favorable reprogramming of the immunosuppressive TME, thereby priming mesenchymal PDAC for ICB.

Cancer-associated fibroblast reprogramming. PDAC subtypes differ in cancer-associated fibroblasts (CAFs), desmoplastic stroma and extracellular matrix content, most abundant in classical, although...
**a** Classical vs. Mesenchymal

- Control: n = 5
  - T cells: 2.3%
  - B cells: 1.4%
  - Non T and B cells: 1%

- T/N: n = 6
  - T cells: 1.4%
  - B cells: 5.2%

**b** Classical vs. Mesenchymal

- Control
  - % CD4+ T cells
  - % CD4+ T cells
  - % CD4+ T cells

- T/N
  - % CD4+ T cells
  - % CD4+ T cells
  - % CD4+ T cells

**c** CD3 vs. CD8

- Control
  - CD3
  - CD8

- T/N
  - CD3
  - CD8

**d** Control vs. T/N

- Classical
  - Tumor core
  - Invasive margin

- Mesenchymal
  - Tumor core
  - Invasive margin

**e** Tumor volume - change from baseline (%)

- Classical C57BL/6 WT
- Classical CD3 KO mice

**f** Tumor volume - change from baseline (%)

- Mesenchymal C57BL/6 WT
- Mesenchymal CD3 KO mice

**g** Survival (%)

- Classical C57BL/6 WT
- Classical CD3 KO mice

**h** Ratio T/N to median survival respective control

- Classical C57BL/6 WT
- CD3 KO
- Classical CD3 KO mice
almost absent in mesenchymal, tumors. CAFs are highly heterogeneous, displaying diverse effects on tumor progression and therapeutic response. Recently, at least three different CAF subpopulations, myofibroblast-like (termed myoCAFs), inflammatory (iCAFs) and antigen-presenting CAFs (apCAFs), were characterized in PDAC.

To investigate the dynamics of CAF subtypes in the PDAC TME, we analyzed their number and polarization making use of our scRNA-seq dataset (Extended Data Fig. 10c–h). Fibroblasts comprised only 9% of sorted classical tumors, in line with recent human PDAC datasets, which contain only 2% CAFs out of all cells in tumor samples. Mesenchymal tumors consist of densely packed tumor cells that lack the classical desmoplastic stroma, and almost absent in mesenchymal, tumors. CAFs are highly heterogeneous, displaying diverse effects on tumor progression and therapeutic response. Recently, at least three different CAF subpopulations, myofibroblast-like (termed myoCAFs), inflammatory (iCAFs) and antigen-presenting CAFs (apCAFs), were characterized in PDAC.

In classical PDAC, T/N-treated tumors displayed a substantially reduced number of myoCAFs and a remarkable increase in iCAFs. In addition, myoCAFs presented reduced expression of Tgfβ1 on dual and triple treatment (Extended Data Fig. 10c–h). In line with its well-known role in blocking immune responses within the TME, transforming growth factor (TGF)-β plays a major role in primary therapy resistance. Conversely, the mesenchymal KRAS-mut iGD PDAC subtype shows high tumor cellularity and sparse stroma, and is commonly more aggressive and unresponsive to all, to date, attempted polychemotherapies, targeted therapies and ICB.

In the present study, we identified a new targeted therapy for mesenchymal PDAC by high-throughput combination drug screening. We showed how this therapy remodels the immune landscape in vivo and creates new vulnerabilities toward ICB in mesenchymal tumors. The T/N combination reprogrammed the immunosuppressive mesenchymal cancer cell secretome and downregulated cytokines and chemokines, including CCL2 and CSF1, capable of attracting and inducing expansion of macrophages and myeloid-derived suppressor cells. In parallel, it induced secretion of T-cell modulators, such as CXCL16 and CXCL12, important for TIL recruitment, and provoked upregulation of antigen-processing and presentation pathways in mesenchymal PDAC cells in vivo. Finally, the combination strongly impacts on the TME by increasing blood vessel density, inducing endothelial cell activation and vascular remodeling, and facilitating cytotoxic and effector T-cell infiltration. Thus, we show that reprogramming of the immunologically ‘cold’ to a ‘hot’ tumor stroma of the highly aggressive mesenchymal.
Fig. 7 | scRNA-seq analysis reveals context-specific responses of tumor cells and their microenvironment on combination drug treatment. a, Scheme of the experimental strategy of the scRNA-seq experiment. One or two tumors per model and treatment condition were dissociated and sorted into mesenchymal/fibroblast (a.) and epithelial/immune-enriched (b.) fractions and subjected to scRNA-seq analysis (10x Chromium). b, Left: UMAP plot showing all identified cell populations within the scRNA-seq experiment. Middle: UMAP plot showing classic (yellow) and mesenchymal (blue) tumors from all groups. Right: UMAP plot showing the treatment-induced changes in cell-type composition among the identified cell populations across subtypes. Sorted cell fractions depicted in panel a are indicated with (a.) or (b.). c, Venn diagram showing the overlap of immune-related signatures obtained from GSEA of the tumor-cell cluster across treatment conditions for both subtypes. Only those presenting an FDR value > 0.05 are shown. d, GSEA of the tumor-cell cluster across treatment conditions for both subtypes. Only those presenting an FDR value < 0.05 are shown. e, Normalized enrichment scores (NESs) are plotted in the heatmap. The red dotted line marks the signatures showing an FDR q value > 0.05. e, GSEA of the differentially expressed genes induced by T/N + anti-PD-L1 in classical and mesenchymal tumor cells in vivo. For the mesenchymal subtype, the top immune-related, positively regulated ‘hallmark’ signature is ‘interferon-gamma response’ and is shown for both classical and mesenchymal tumors. The NES and FDR q values are indicated. f, GSEA of the differentially expressed genes induced by T/N in classical and mesenchymal tumor cells in vivo. The ‘reactome’ signature SASP is shown. g, Representative images of three independent experiments of SA-β-Gal staining of tissue sections of classical and mesenchymal control and T/N-treated tumors. Scale bars, 70 μm.

Subtype is possible and can be exploited therapeutically by adding anti-PD-L1 ICB to the T/N combination.

The context-specific impact of the T/N combination on the immune system was not expected. Although a link across the DNA-damage response, induction of antigen processing and presentation, and activation of strong immune responses is well established, the lack of these effects in classical PDAC was surprising. Although the combination induces DNA damage, it also induces immunosuppression by increasing the secretion of CCL2 and CSF1. Differences in oncogenic KRAS signaling outputs might drive immune signaling divergence between subtypes. High levels of KRAS expression, as observed in mesenchymal tumors, were shown to repress interferon-γ signaling in vivo as evidenced by reduction in IRF2 (ref. 53). Blockade of KRAS signaling by MEKi in combination with broad RTK and PD-L1 inhibition might release this important pathway and—in combination with therapy-induced DNA damage—boost neoantigen presentation while inducing anti-tumor immunity in mesenchymal tumors54. In line with this, we observed selective upregulation of IRF2 in PDAC cells on T/N + anti-PD-L1 therapy in this subtype in vivo.

Although highly antagonistic in vitro, the combination also showed substantial anti-tumor effects in classical PDAC in vivo, indicating an impact on the TME54. The failure of the in vitro drug screen to predict in vivo responses of this subtype is remarkable. Therefore, treatment-mediated TME remodeling should be considered for the transition of in vitro screening hits to the (pre) clinical setting.

We observed an antiproliferative effect of the combination in classical PDAC, as well as the selective induction of SASP. SASP is characterized by the secretion of chemokines, cytokines, matrix metalloproteinases and other paracrine factors55,56. In classical KRAS-driven PDAC, it has been shown that the combination of MEKi...
and CDK4/6 inhibitors induces a strong SASP response, with the release of proangiogenic factors that promote tumor vascularization, endothelial cell activation and vascular cell adhesion protein 1 expression. This, in turn, promotes T-cell extravasation into tumors and sensitizes this subtype to ICB21. In contrast, we found no evidence for SASP-induced vascular remodeling in classical PDAC on T/N therapy. In line with this, classical T/N-treated tumors did not show T-cell infiltration into the tumor core. This might be due to insufficient levels of SASP induction or other treatment-induced, context-specific effects on the cancer cells and their environment, counteracting vascular remodeling, T-cell extravasation and reactivity. Of note, our study supports the notion that SASP induction is context specific29 because we were unable to detect therapy-induced senescence in vivo in mesenchymal KRAS-mut-driven PDAC. This demonstrates that the biology, stromal composition and treatment response of PDAC are highly context dependent and differ substantially between PDAC subtypes.

**Fig. 8** | The combination therapy induces a T-cell-mediated anti-tumor immune response in mesenchymal PDAC. a. Left: UMAP plots displaying Cd3g, Cd4 and Cd8a marker gene expression across the whole population of T cells identified by scRNA-seq in classical and mesenchymal tumors. Center: UMAP plots of classical (yellow) and mesenchymal (blue) T cells from all treatment and vehicle groups. Right: UMAP plots showing the six T-cell subpopulations identified by scRNA-seq. b, Proportion of cells divided by treatment condition and PDAC subtype as identified by scRNA-seq analysis of the T-cell clusters annotated in a. c, Heatmap displaying expression of selected genes across the identified T-cell clusters (1–6) for both classical and mesenchymal tumors. The different treatment conditions are shown separately. d, Circos plot showing the key communication signals from tumor cells to T-cell subpopulations, tumor cells and acinar cells in mesenchymal mPDAC. The ligand protein expression fold-change, identified from secretome experiments, between T/N and control is shown in the middle. Normalized receptor expression levels obtained from scRNA-seq data are shown in the outer concentric circles.
Trametinib is used for treatment of patients with unresectable or metastatic BRAF-mutant melanoma, NSCLC and anaplastic thyroid cancer. Nintedanib, in combination with docetaxel, has been approved for the second-line treatment of advanced NSCLC and first-line therapy for idiopathic pulmonary fibrosis (IPF) (www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=BasicSearchprocess). IPF is characterized by the activation of fibroblasts toward a myofibroblast differentiation.9,10 Accordingly, blocking myofibroblast activation by nintedanib, or pirfenidone, which targets the TGF-β pathway, slows down pulmonary disease progression.11 Classical PDAC harbors a dense stroma, which is composed in large parts of activated myoCAFs and TGF-β expression (Extended Data Fig. 10)4,12. We therefore hypothesized that adding nintedanib to trametinib might reprogram myoCAFs, thereby contributing to stromal normalization in classical PDAC. Using scRNA-seq, we observed that the number of myoCAFs decreased on T/N therapy, and TGF-β1 expression, which can contribute to immunosuppression via regulatory T cells, is downregulated. This demonstrates that reprogramming of the fibrotic microenvironment of the classical subtype is feasible and could be exploited for further improved therapeutic responses.

To identify the therapeutically relevant targets of the multi-kinase inhibitor nintedanib, we performed multiscale analyses, ranging from kinobead-based proteomic identification of the nintedanib-bound kinases to phosphoproteomic analyses of drug action, and genome-scale and focused CRISPR–Cas9-based negative selection screens. This revealed a key set of nintedanib targets, including FGFR, kinases belonging to the MEK/ERK family and PDGFR-regulated networks that cooperate with trametinib in mesenchymal PDAC. However, it also revealed heterogeneity of the functionally relevant targets within this subtype. This is remarkable and indicates that, indeed, broad multi-kinase inhibition is needed to target this highly aggressive and therapy-resistant subtype efficiently across the whole spectrum of its phenotypes. In addition, it supports the notion that no single target gene is responsible for the synergistic effect of the T/N combination and challenges the one biomarker per drug paradigm in oncology. Indeed, mesenchymal cancer cell morphology and the underlying genetic program are the strongest predictor of therapeutic response to T/N.

In summary, our work sets the basis for the combination of T/N with immunotherapy in the treatment of mesenchymal PDAC and provides a step toward molecularly stratified combination therapies in the clinic. Considering the widespread occurrence of RAS-driven tumor entities with an increased RAS gene dosage and a mesenchymal differentiation state, our data suggest that combining T/N with ICB or other forms of immunotherapy might trigger anti-tumor immunity and improve therapeutic outcomes across entities.

Methods
Our study complies with all relevant ethical regulations. Animal experiments were approved by the institutional animal care and use committees (IACUCs) of the local authorities of Technische Universität München and Regierung von Oberbayern.

Primary PDAC cell culture, clonogenics and inhibitors. Primary mPDAC cell cultures were isolated from autochthonous PDAC and cultured as described previously13. All cells were cultivated for <30 passages, authenticated by genotyping and tested for Mycoplasma contamination by PCR. Conventional hPDAC cell lines and primary cell cultures were seeded in 24-well plates (density 1–2 × 10^4 cells per well, depending on growth rate). The following day, plates were treated with different concentrations of drugs as indicated. Every 7 d, medium and drug were refreshed. Cells were fixed and stained with 0.2% Crystal Violet in an ethanol/water solution 7–13 d after the start of treatment, according to the confluence reached by the untreated control. Crystal Violet was solubilized with 10% acetic acid and absorbance was quantified at 595 nm. The resulting values were used to calculate the Bliss synergy score with the online software Synergy Finder (v.1.0.9)14. All assays were performed independently at least three times. Trametinib, nintedanib, AZD4547, imatinib and PF-3758369 were obtained from Selleckchem. 4-hydroxytamoxifen (4-OHT) from Sigma-Aldrich, murine anti-PD-L1 monoclonal antibody (Anti–PD-L1-mlgG1e3 ImmunoGlobulin) was purchased from InvivoGen and tamoxifen for in vivo treatment from Sigma-Aldrich.

Caspase-3/7 assay. To assess apoptosis, 1,000 cells per well were seeded in 96-well plates and treated after 24 h with trametinib (10 nM) or nintedanib (2 μM) alone or in combination as indicated. After 24 h from the start of treatment, caspase-3/7 activity was determined measuring luminescence using caspase-3/7 assay (Promega), according to the kit instruction. All assays were performed independently at least three times.

Kinobead pulldowns. Kinobead pulldown assays were performed as previously described15. In-gel digestion was performed according to standard procedures16. Peptide/protein identification and quantification were performed with MaxQuant (v.1.5.7.4) by utilizing the Swissport database (murine, 16,996 entries, downloaded 23 November 2018) using the search engine Andromeda. Data analysis was performed as previously described15.

Automated combination drug screen. The screened compounds were purchased from SelleckChem either in DMSO or in water. A total of 1,000–2,000 cells were seeded in 96-well plates using a Multidrop Combi (Thermo Fisher Scientific) dispenser. The optimal cell number for each cell culture was determined to ensure that the cell line under the condition of at least two cell doublings by the end of the caspase-3/7 assay, 96 h after seeding (~85% confluence). After overnight incubation, cells were treated with a seven-point dilution series (seven concentrations of each compound, threefold dilutions, highest concentration 10 μM) and DMSO as control, using liquid handling robotics (CyBio Felix), and assayed 72 h later. Each cell culture was treated with a library of 418 compounds either in monotherapy or in combination with trametinib (final concentration 2 nM). This library was measured using a CellTiter-Glo Luminescent Assay (Promega). CellTiter-Glo reagent was added using the Multidrop Combi dispenser, and cells were shaken and incubated for 10 min in the dark. Luminescence was measured using an Infinite Pro 2000 Lumi (Tecan) Lumimeter.

Dose–response curves were generated for both monotherapy and combination using the R package GRmetrics (v.3.14)17 to derive both growth-rate-adjusted and traditional measures of drug sensitivity (half-maximal inhibitory concentration (IC50), area under the curve (AUC), efficacy (E50) and half-maximal effective concentration (EC50)). Only drugs for which a sigmoid curve could be fitted (coefficient of determination, r2 > 0.9) were considered for further analysis. For each drug, we calculated an expected effect of the combination with trametinib using the Bliss independence model. We used the delta of the AUC between the expected and the measured response to the combination as a proxy for synergy. Drug sensitivity parameters for each cell culture are summarized in Supplementary Table 2.

Phosphoprotein array sample preparation and analysis. The mPDAC cells 9091 and 8661 were plated in 10-cm dishes. The next day, they were treated for 6 h with DMSO (vehicle) or T/N (10 nM trametinib and 2 μM nintedanib) and analyzed using the Phospho Explorer antibody microarray, which contains 1,318 antibodies (Full Moon Biosystems), according to the protocol of the manufacturer.

Full enrichment analysis was based on the Reactome gene set and performed through Cytoscape (v.3.8.2) with ClueGO (v.2.5.8)18, a Cytoscape plug-in to decipher functionally grouped pathway annotation networks. The functionally grouped networks used for visualization present terms as nodes and are linked based on their k score level (≥ 0.4). The node size represents the term-enrichment significance and functionally related groups are depicted by similar colors.

Cloning of focused Cas9 sgRNA library. For the customized sgRNA Cas9 library, four sgRNAs (from the Brie library (Addgene, catalog no. 73633)) per target, nontargeting controls, common essential genes and trametinib-sensitizing/resistance genes were included to a total of 350 sgRNA sequences, which were embedded into an oligo sequence with flanking PCR handles and BsmBI restriction sites19. The oligo pool (Twist Bioscience) was then amplified at 5 nM input with NEBNext Ultra II polymerase and primers binding the PCR handles, followed by purification with AmpureXP beads (Beckman Coulter). For Golden Gate cloning, BsmBI-v2 (New England Biolabs)-digested plenti-guide puro (Addgene, catalog no. 52963) was mixed with T4 DNA ligase (New England Biolabs), 10X T4 buffer, BsmBI-v2, linearized backbone and amplified insert (1:3 molar ratio; 30 cycles, Golden Gate). The assembled product was purified with AmpureXP beads and electroporated into Endura Competent cells (Lucigen) using a BioRad MicroPulsar (1.8 kV in 0.1-cm gap cuvets (Sigma-Aldrich)). Bacteria were grown at 33 °C overnight (LB Medium (Sigma) with 100 μg/ml ampicillin). DNA was extracted using the NucleoBond Xtra Midi EF Kit (Macherey-Nagel). Libraries for next-generation sequencing (NGS) were constructed according to the protocol given below to determine sgRNA abundance. Sequences of oligonucleotides for all described methods from the present study are provided in Supplementary Table 7.
Lentivirus production and titration. For virus production of sgRNA libraries, HEK293FT cells were seeded into 15-cm dishes to reach confluence of 60% the following day. Cells were transfected with 14.3 µg of library plasmid, 10.9 µg of PspPA2 (Addgene, catalog no. 12260) and 7.1 µg of PMD2.G (Addgene, catalog no. 12259) per plate using 119 µl of TransIT-LT1 (Mirus Bioscience) in 850 µl of OptiMEM ( Gibco). Then, 48 h and 72 h post-transfection, the supernatant was collected and filtered (0.45 µm). For other constructs, HEK293FT cells were seeded in 10-cm plates and transfected with 2 µg of viral plasmid. For each transfection, cells were harvested and genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue kit (focused libraries) or the Blood & Cell Culture DNA Maxi Kit (both from QIAGEN) according to the manufacturer's instructions.

For transplantation experiments, cancer cells (2,500–10,000) were orthotopically grafted into the pancreas of syngeneic immunocompetent C57Bl/6 wild-type, or T cell deficient C57Bl/6 Cd3ε-knockout mice. When tumors grew to a size ~100 mm³, mice were randomized into the different treatment arms. The following drugs were used: trametinib (3 mg/kg−1, 5 d a week, oral gavage), nintedanib (50 mg kg−1, 5 d a week, oral gavage), anti-PD-L1 antibody (200 µg per mouse, every third day, intraperitoneal injections) and tamofoxin (4 mg per mouse, every third day, intraperitoneal injections). One week after implantation, mice were scanned by magnetic resonance imaging (MRI) for the presence of tumors. Animals were sacrificed when individual mice reached the human endpoint or at the completion of treatment.

Electroporation of CRISPR–Cas9 RNPs and indel depletion assay. For multiplexed validation of targets conferring sensitivity to trametinib, sgRNAs were designed as CRISPR (cr)RNAs (Integrated DNA Technologies (IDT); Supplementary Table 7) and complexed with a trans-activating crRNA (tracrRNA; IDT) according to the manufacturer's instructions. Targeting and depletion of genes were assessed in 24-well plates (1,000 cells per well) in the presence and absence of trametinib as described above.

Histology and IHC. Mouse pancreatic tumors were fixed in 4% paraformaldehyde (PFA; Carl Roth), embedded in paraffin and cut into 1-µm sections. Hematoxylin and eosin (H&E) staining of tissue sections was performed according to standard protocols. The following primary antibodies were used for immunohistochemistry (IHC): rat anti-Ck19 (DHSB, 1:250), rabbit anti-Ki67 (Thermo Fisher Scientific, 1:50), rat anti-CD3 (Dianova, 1:100), rabbit anti-phospho-histone H2A X (Ser5) (Cell Signaling Technology, 1:500), rabbit anti-MEK1 (30C8) (Cell Signaling Technology, 1:50), rabbit anti-MEK2 (13E3) (Cell Signaling Technology, 1:50), rabbit anti-pERK (p-p44/42 MAPK Thr202/Tyr204) (Cell Signaling Technology, 1:30), rabbit anti-p70S6K (Cell Signaling Technology, 1:100), rabbit anti-phospho-PI3Kα (Ser473) (Cell Signaling Technology, 1:100), rabbit anti-phospho-EGFR (Cell Signaling Technology, 1:100), rabbit anti-AMPKα (Thr172) (Cell Signaling Technology, 1:50). Antibody detection was performed by the BioCare Medical antibody detection kit (BioCare Medical, Concord, CA). Sections were counterstained with hematoxylin.
was performed using the Bond Polymer Refine Detection Kit (Leica) or rabbit anti-rat immunoglobulin (IgG) (Vector Laboratories, 1:200) secondary antibody or followed by a secondary antibody conjugated to biotin (Vector Laboratories). Detailed protocols of individual antibodies are available on request. Images were acquired using Leica AT2 Scanner (Leica) and processed by Aperio Image Scope (Leica, v.2.13.3) and FIJI (National Institute of Health (NIH), v.2.1.0). For quantification of Ki67, CD31, and γH2AX staining, five fields of view of individual tumors per treatment condition were analyzed in a blinded fashion. In at least six individual tumors per treatment condition, mitoses were counted per high power field in areas showing increased mitotic activity on scanning magnification. M. Jesinghaus, a board-certified pathologist, performed all quantifications.

**SA-β-Gal staining.** PFA-fixed, OCT-embedded tumor tissues were cut into 5-μm sections and mounted on slides. SA-β-Gal staining was performed using a Senescent-β-Galactosidase Staining Kit (Cell Signaling Technology) at pH 6.0. Images were acquired using Aperio Versa Scanner (Leica) and were processed by FIJI (NIH, v.2.1.0).

**Immunofluorescence staining and imaging.** PFA-fixed, OCT-embedded tumor tissues were cut into 5-μm sections and mounted on slides. The immune tissues were incubated with acetone (Sigma-Aldrich) for 6 min at 4°C. After rehydration with PBS for 10 min, the tissues were blocked for 1 h at RT with 10% goat serum and 10% donkey serum in PBS. The following primary antibodies were used for immunofluorescence staining of T cells, endothelial cells, and epithelial and mesenchymal tumor cells: rat anti-CD3 (BioLegend, 1:50), rat anti-CD31-AF647 (BioLegend, 1:20), rabbit anti-CD4 (BioLegend, 1:1,000), rabbit anti-β-catenin (Cell Signaling Technology), and goat anti-RhoA (Cell Signaling Technology). Secondary antibodies were goat anti-rat AF680 (Invitrogen, 1:200), donkey anti-rat AF488 (Invitrogen, 1:200), goat anti-goat AF568 (Jackson ImmunoResearch, 1:200), and goat anti-chicken AF680 (1:200) were used as secondary antibodies (staining for 1 h at RT diluted in 3% BSA in PBS). Nuclear staining was performed for 10 min at RT with DAPI (Biotium, 1:500) in 3% BSA in PBS. The following primary antibodies were used for immunofluorescence staining of macrophage subpopulations: rat anti-CD68 (BioRad, 1:500), rabbit anti-ARGB (Thermo Fisher Scientific, 1:300) and rabbit anti-CD80 (Abcam, 1:300). Donkey anti-rat AF594 (Invitrogen, 1:200) and goat anti-rabbit AF488 (Invitrogen, 1:200) were used as secondary antibodies (staining for 1 h at RT diluted in 3% BSA+A+6% Triton X-100 in PBS). Nuclear staining was performed with DAPI (Biotium, 1:1,000) in 0.25% BSA in PBS. After three washes in PBS, slides were mounted using Vectashield Mounting Medium (Vector Laboratories). Images were acquired using a TCS SP8 Confocal Laser Scanning Microscope (Leica) and were processed by FIJI (NIH, v.2.1.0). For quantification of T cells, ten fields of view of five individual tumors per treatment condition were analyzed. For quantification of macrophage subpopulations, five fields of view of five individual tumors per treatment condition were analyzed.

**Immunophenotyping by flow cytometry.** Fresh tumor samples were minced and enzymatically digested with the tumor dissociation kit (Miltenyi, catalog no. 130-096-730) for 40 min at 37°C with agitation. The cell suspension was strained through a 100-μm strainer, spun down and resuspended in 3% BSA/PBS. Cells were blocked for 10 min on ice with anti-mouse CD16/CD32 FC block (BioLegend, 1:100). Cells were transferred into RLT buffer (QIAGEN) containing 2-mercaptoethanol. RNA was isolated with the RNeasy kit (QIAGEN) from 80% confluent primary breast and immediately transferred into RLT buffer (QIAGEN) containing 2-mercaptoethanol.

**RNA-seq library preparation and sequencing.** The sorted cells were counted, diluted in 2% FCS/PBS and up to 20,000 cells were loaded per lane on a 10x Chromium chip to generate gel beads in emulsion (GEMs). Single-cell GEM generation, barcoding and library construction were performed by using 10x Chromium Single Cell 3′ v3 chemistry according to the manufacturer’s instructions. Complementarily, DNA expression analysis was performed on a 10x Chromium Cell Controller and analyzed on a Chromium TIME-11 instrument. The data were analyzed using Chromium v.1.26.0. A gene was considered to be differentially expressed with a Benjamini–Hochberg-adjusted P value of 0.05 and an absolute fold-change >1.

**Sample preparation for scRNA-seq.** Tumor specimens were dissociated and enzymatically digested with the tumor dissociation kit as described above. The cell suspension was strained through a 100-μm strainer, spun down and resuspended in 2% FCS/PBS including RNase inhibitor (New England Biosabs, catalog no. M0314L, 1:100). Debris removal solution (Miltenyi, catalog no. 130-109-398) was used to remove cell debris from the dissociated tissue. Then the dead cell removal kit (Miltenyi, catalog no. 130-090-101) was used to enrich live cells. The cell suspension was spun down and then resuspended in PBS and blocked for nonantigen-specific binding for 10 min on ice with anti-mouse CD16/CD32 Af508/6 block (BioLegend, 1:100). Cells were stained with the following antibodies for FACS sorting: TER-119 BV421 (BioLegend, 1:100), CD45-AF647 (BioLegend, 1:120) and EPCAM-AF647 (BioLegend, 1:20) for 30 min on ice. Cell sorting was performed using the BD FACS Aria Fusion. The sorted cells from the TER-119-negative/CD45-1/CD31-1/EPcam-positive fraction (fibroblasts), of immun-Ant-Mesenchymal markers CD31 (Jackson ImmunoResearch, 1:200), goat anti-chicken AF680 (1:200) and goat anti-anti-chicken AF680 (1:200) were used as secondary antibodies (staining for 1 h at RT diluted in 3% BSA in PBS). Nuclear staining was performed for 10 min at RT with DAPI (Biotium, 1:500) in 0.25% BSA in PBS. After three washes in PBS, slides were mounted using Vectashield Mounting Medium (Vector Laboratories). Images were acquired using a TCS SP8 Confocal Laser Scanning Microscope (Leica) and were processed by FIJI (NIH, v.2.1.0). For quantification of T cells, ten fields of view of four individual tumors per treatment condition were analyzed. For quantification of macrophage subpopulations, five fields of view of five individual tumors per treatment condition were analyzed.

**Dimensionality reduction and clustering.** The Leiden algorithm (v0.8.1) was used for cell clustering and Uniform Manifold Approximation and Projection (UMAP v0.4.6) for dimensionality reduction. The clusters were further annotated by assessment of known cell-type-specific markers. Principal component analysis was performed with default parameters. Neighborgraph neighborhoods were computed based on n = 10 principal components and k = 30 neighbors. The calculation of all UMAP projections was based on default parameters. The number of Leiden clusters was adjusted according to the sample of consideration.

**Cell-type-specific analysis.** Cell-type identification in classical and mesenchymal tumors, scRNA-seq uncovered six different T-cell clusters. The identified CD4+ T cells were enriched in specific cytokine and chemokine expression profiles, with a strong upregulation of IFNγ and IL-17A. The CD8+ T cells were enriched in expression of perforin and granzyme B, indicative of an effector T-cell phenotype. The regulatory T cells were enriched in expression of FoxP3 and IL-10, indicative of a suppressive phenotype.
naive-like populations expressed Cd4 and the naive T-cell marker Sell; in addition, they lacked the expression of Cda4 and T-cell activation genes, such as Icos, Lag3, Haver2 (Tim3), Pdcd1, Tnfrsf4 and Clda4. The activated/effector T cells showed the highest levels of activated markers such as Icos, Tim3, Clda4 and Pdcd1, and intermediate levels of Sell and Cda4. Regulatory T cells expressed high levels of Cda4 and Foxp3, and intermediate levels of the T-cell activation genes Icos, Clda4 and Pdcd1. Central memory T cells were positive for Cda4, Cda2, Cda8, Cda4 and Il7r, and displayed a unique central memory marker signature including Sell and Ccr7. CD8 naivé T cells expressed high levels of Cd74 and Sell. Cytotoxic T cells showed the highest levels of T-cell activation genes Lag3, Tim3, Pdcd1, Tnfrsf18 and Yng1, and cytotoxic markers Gzma, Gzmb and Ptf1.

Cytokine measurements. Cytokine secretion was measured by ELISA in the supernatant of T cells stimulated with PMA plus ionomycin in the presence of Golgi stop (BD, Germany) according to the manufacturer’s instructions. The absorbance was measured at 492 nm using a spectrophotometer (Spectronic Genesys 10 US, Thermo Fisher Scientific).

Data analysis. Statistical analysis was performed using GraphPad Prism (v8). Unless otherwise indicated, all data were determined from at least three independent experiments. For comparisons between datasets, a log(rank) test or two-tailed Student’s t-test with Welch’s correction was employed and the resulting P values are indicated in the respective figures. The significance level was set to 0.05. If more than one statistical test was performed simultaneously on a single dataset, and Bonferroni’s adjusted significance level was calculated to account for the increased possibility of false-positive results. Survival analysis was carried out using the log(rank) test.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The RNA-seq dataset has been deposited in the EBIsArrayExpress repository with accession no. E-MTAB-11187. The MS kinobead pulldown and the MS secretomics data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with accession nos. PXD023267 and PXD027877, respectively. The scRNA-seq data have been deposited in the EBiArrayExpress repository with accession no. E-MTAB-9954. The human pancreatic cancer data were derived from previous studies and are available in the supplementary information of the respective publications5–9. All other data have been provided as supplementary tables or source data files. Mice and cell lines are available from the corresponding author on reasonable request. Key resources are listed in Supplementary Table 8. Source data are provided with this paper.

Code availability

Analyses were performed using open-source software, and in-house scripts in R v.3.6.2 and Python v.3.8.3, which are available from the corresponding author on reasonable request. Codes are provided via the GitHub repository at the following link: https://github.com/stefanie-baerthel/combinatorial_treatment_analysis.

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Data availability. The RNA-seq dataset has been deposited in the EBiArrayExpress repository with accession no. E-MTAB-11187. The MS kinobead pulldown and the MS secretomics data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with accession nos. PXD023267 and PXD027877, respectively. The scRNA-seq data have been deposited in the EBiArrayExpress repository with accession no. E-MTAB-9954. The human pancreatic cancer data were derived from previous studies and are available in the supplementary information of the respective publications5–9. All other data have been provided as supplementary tables or source data files. Mice and cell lines are available from the corresponding author on reasonable request. Key resources are listed in Supplementary Table 8. Source data are provided with this paper.

Code availability. Analyses were performed using open-source software, and in-house scripts in R v.3.6.2 and Python v.3.8.3, which are available from the corresponding author on reasonable request. Codes are provided via the GitHub repository at the following link: https://github.com/stefanie-baerthel/combinatorial_treatment_analysis.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Assessment of differential pharmacologic and genetic dependencies and signaling pathway activities in PDAC subtypes.

a, Clonogenic assay of two hPDAC cell lines (top) and two mPDAC cell cultures (bottom) treated with the MEK inhibitor trametinib. The shown cell lines represent the drug-response of the epithelial and mesenchymal subtypes to trametinib treatment. b, c, β-score distribution of CRISPR/Cas9 genome wide negative-selection (viability) screens performed in one classical (6075, panel (b)) and one mesenchymal (9091, panel (c)) mPDAC cell line. Highlighted in yellow, for the classical line, and blue, for the mesenchymal line, are the β-scores of KRAS and the core genes involved in direct KRAS downstream signaling. d, CRISPR/Cas9 dependency scores of KRAS and core genes involved in direct KRAS downstream signaling. The dependency scores of all hPDAC cell lines were obtained from the DepMap database and are shown in grey. Dependency scores corresponding to classical and mesenchymal cell lines included in the T/N drug screen are represented in the yellow and blue violin plots. Data were obtained from the CRISPR dataset and analyzed through the DepMap release DepMap 21Q2 Public (https://depmap.org/portal/download/). e, f, Mesenchymal (9091) and classical (8661) PDAC cell cultures were used to generate site-specific phospho-array datasets (Phospho Explorer antibody microarray, Full Moon Biosystems). Phospho-array data (supplementary table 1) were used to test for the enrichment of differentially phosphorylated sites between classical and mesenchymal mPDAC cell lines. Functionally grouped networks with reactome terms as nodes, showing pathways overrepresented in classical (e) and mesenchymal (f) cells are represented with the ClueGO plugin of Cytoscape. Only the pathways with an adjusted p value (calculated by two-sided hypergeometric test, Bonferroni corrected) ≤ 0.05 are depicted. The node size represents the term enrichment significance.
Extended Data Fig. 2 | Genetic depletion of Mek1/2 in established PDAC. a, Genetic strategy to delete Mek1 and Mek2 by 4-hydroxytamoxifen (4-OHT)-mediated CreERT2 activation. Pdx1-Flp;FSF-KrasG12D;FSF-CreERT2;Mek1lox/lox;Mek2lox/lox mice were crossed with mice harboring loxP-flanked Mek1 and Mek2 alleles. This allowed MEK1/2 deletion in established PDAC by tamoxifen administration in vitro and in vivo after orthotopic transplantation. b, Genotyping PCR of PDAC cells with indicated genotypes to analyze recombination of the floxed Mek allele. Non-recombined mutant, recombined mutant and wild-type PCR products are indicated on the right side. Representative gel of three independent experiments. c, Western blot analysis of MEK1 and MEK2 expression in primary PDAC cell cultures with indicated genotypes after 4 days of tamoxifen (4-OHT) and vehicle (ethanol, EtOH) treatment. HSP90 served as loading control. Representative gel of three independent experiments. d, Clonogenic assays of mPDAC cells with indicated genotypes. Control cells treated with vehicle (ethanol; EtOH) are shown in the upper row, 4-OHT treated cells in the lower row. e, Schematic representation of the experimental set-up to test the effect of Mek1/2 knockout in vivo by tamoxifen administration using syngeneic immunocompetent PDAC models. mPDAC cells with conditional Mek1lox/lox;Mek2lox/lox alleles were used for the orthotopic transplantation experiments. f, Waterfall plot showing tumor response of vehicle and tamoxifen treated animals after one week of treatment (fold-change compared to baseline before treatment based on MRI-volumetric measurements, y-axis). Each bar represents one mouse. p values calculated with two-tailed unpaired t test. g, Kaplan-Meier survival curve of vehicle and tamoxifen treated PDAC models. Number of mice is indicated in the corresponding panels. P value was calculated with log-rank (Mantel-Cox) test. h, Representative images of HE and IHC for MEK1, MEK2 and KI67 of tissue sections of tumors from orthotopically transplanted Mek1lox/lox;Mek2lox/lox models treated with vehicle or tamoxifen. Representative pictures of three independent experiments. Scale bars, 100 µm.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Pharmacologic and genetic modulation of drug sensitivity in classical and mesenchymal PDAC cell cultures. a, b, Clonogenic assays of a representative human (left) and mouse (right) PDAC cell culture showing antagonism to the trametinib/nintedanib (T/N) combination. Cell cultures were treated with indicated concentrations of T/N. c, Western blot of phospho-ERK and ERK in T/N (10 nM trametinib + 2 μM nintedanib) and vehicle treated classical and mesenchymal primary mPDAC cell lines. HSP90 served as loading control. Classical cell lines are marked in yellow, mesenchymal in blue. Representative gels of three independent experiments. d, Clonogenic assays using increased drug concentrations of the T/N combination of three of the most antagonistic cell lines, as depicted in Fig. 2, panel (g). e, Doxycycline-induced overexpression of KRASG12D in mouse PDAC cells. 2259 mPDAC cells representative of the classical subtype was transduced with lentivirus carrying doxycycline-inducible KRASG12D or GFP-control expression constructs. KRASG12D or GFP expression were induced by doxycycline (100 ng/ml) for one or 14 days. f, Western blot of phospho-ERK and total ERK in cells overexpressing KRASG12D or GFP for one day. HSP90 served as loading control. g, Expression of the marker gene Cdh1 for epithelial cell differentiation was evaluated by qRT-PCR (normalized to Cyclophilin B). Data are shown as mean ± SD; n = 3 biological replicates. P value was calculated with two-tailed unpaired t test. h, Representative picture of three independent experiments of the morphological changes of PDAC cells upon KRASG12D induction for one or 14 days of doxycycline treatment. Scale bars, 200 μm. i, Representative clonogenic assays of mPDAC cells treated with the indicated concentrations of trametinib and nintedanib upon KRASG12D (right panel) or GFP (left panel) overexpression. j, Bliss synergy scores for the mPDAC cell line treated with the combination of trametinib and nintedanib upon KRASG12D or GFP overexpression.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Kinobead-based proteomic identification of the trametinib and nintedanib targets and treatment-induced changes in the phosphoproteome of classical and mesenchymal PDAC. a, b, Representative pictures of the target space of trametinib (a) and nintedanib (b) for 2259 PDAC cells. A phylogenetic tree of all kinases for the 2259 primary mouse PDAC cell culture is shown. The indicated circle sizes indicate potency (apparent dissociation constants (Kdapp)), the color code specifies protein-drug interaction with the designated or other targets. Arrows highlight the identified targets. c, Radar plot showing the overlay of the pKd (−log10Kd) for the targets of nintedanib in the 6 PDAC cell cultures tested. PDAC cells of the classical (n = 4) and mesenchymal (n = 2) subtype are indicated with the color code. d, Heatmap showing the differentially expressed genes between epithelial and mesenchymal cell cultures identified as targets of nintedanib. The color code indicates the Z score. e, Volcano plots representing the change in gene expression of the nintedanib targets (in blue) upon trametinib treatment. The x-axis log2 fold change (treated/control), the y-axis shows the per test adjusted p values, which were calculated by differential expression test (using the DESeq2 package). A gene was considered to be differentially expressed with a Benjamini-Hochberg adjusted p-value of 0.05 and an absolute fold change >1. f, g, Mesenchymal (9091) and classical (8661) PDAC cell cultures were used to generate site-specific phospho-array datasets (Phospho Explorer antibody microarray, Full Moon Biosystems). The cell lines were analyzed at basal condition and in presence of T/N (trametinib 10 nM + nintedanib 2 μM). Phospho-array data (Supplementary Table 3) were used to test for the decrease of differentially phosphorylated sites between T/N and vehicle (DMSO) treated classical and mesenchymal mPDAC cells. Functionally grouped maps, obtained with the ClueGO plugin of Cytoscape, representing pathways overrepresented in mesenchymal (f) and classical (g) mPDAC upon T/N treatment are shown. Only the pathways with an adjusted p value (calculated by two-sided hypergeometric test, Bonferroni corrected) ≤ 0.05 are represented. The node size represents the term enrichment significance.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Pharmacologic assessment of nintedanib targets. a, Combinatorial drug screen on mesenchymal hPDAC cell line MiaPaca2 and mPDAC cell line 9091, as shown in Fig. 2, panel (b). The MEK inhibitor trametinib was used in fixed concentration and combined with 418 additional drugs under preclinical and clinical investigation. Highlighted in orange are the drugs in the high-throughput drug screen showing overlapping targets with nintedanib. b, Venn diagrams showing the target overlap between the drugs identified in (a) and nintedanib (see Supplementary Table 4) as reported from the ProteomicsDB database (https://www.proteomicsdb.org). c, Venn diagrams showing the target overlap between nintedanib and additional drugs with an overlapping target profile chosen for further target assessment. The overlapping targets are listed below each figure. The target information was downloaded from the ProteomicsDB database (https://www.proteomicsdb.org). d, Representative clonogenic assays of mesenchymal mPDAC cell cultures treated with trametinib in combination with the drugs shown in (c) as compared to nintedanib. The cell lines were treated with the indicated concentrations of trametinib and the indicated experimental drug. e, Bliss synergy scores of clonogenic assays shown in (d) integrated with cell morphology for the treated mPDAC cell cultures (classical subtype depicted in yellow and mesenchymal in blue).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Genetic screens to identify relevant nintedanib targets sensitizing mesenchymal PDAC towards trametinib. a, Western blot of Cas9 expression in the clonal cell lines used for CRISPR/Cas9 screens. β-Actin served as loading control. b, Editing efficiency at the Hprt locus. c, Relative viability upon 6-Thioguanine treatment to validate Cas9 function in Hprt proficient and deficient Cas9 cells (mean ± SD; n = 3 biological replicates). d, Relative cell growth (y-axis), assessed by cell counting, in the presence of different concentrations of trametinib (mean ± SD; n = 3 biological replicates). The pink line indicates the trametinib concentration used for the CRISPR/Cas9 screens. e, Phospho-ERK, ERK and Cas9 Western blots of clones used for CRISPR/Cas9 screens. Cells were treated with DMSO or trametinib (1.25 nM, 2.5 nM, 5 nM, 10 nM and 20 nM) for 4 days. HSP90 served as loading control. f, Focused CRISPR/Cas9-based genetic screening in mesenchymal mPDAC cells 8248 and 8570. Trametinib sensitivity (x-axis) represents β-scores calculated as sgRNA representation difference between trametinib-treated cells and their initial representation. Differential sensitivity (y-axis) indicates β-score differences between trametinib- and DMSO-treated arms. In red, genes presenting differential sensitivity ≤ −0.25. g, Network visualization of normalized gene expression (assessed by RNA-seq) of nintedanib targets shown in Fig. 3 (d). h, Lentiviral CRISPR/Cas9-mediated deletion of selected top-scoring nintedanib targets in 8248 and 8570 cells. Knock-out cells were treated with trametinib (5 nM) or DMSO and viability was assessed through clonogenic assays. i, Quantification of panel (h). Data are normalized to DMSO-treated non-targeting controls (mean ± SD; n = 3 biological replicates). The dashed line represents the mean of trametinib-treated non-targeting controls. j, Editing efficiency of each sgRNA used in Fig. 2 (f and g) and in panels (h) and (i) of this figure. k, Combinatorial deletion of nintedanib targets via ribonucleoprotein (RNP) electroporation. Mesenchymal 8248 and 8570 knock-out cells were treated with trametinib (5 nM) or DMSO and viability was assessed through clonogenic assays. l, Quantification of panel (k). Data are normalized to DMSO-treated non-targeting controls (mean ± SD; n = 3 biological replicates). Dashed line represents the mean of trametinib-treated non-targeting controls. The shown gels are representative of three independent experiments.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Characterization of context-specific changes of the tumor vasculature and the adaptive immune system in classical and mesenchymal PDAC subtypes upon therapy. a, Orthotopically transplanted tumors of the indicated subtypes were treated with vehicle (control) and the T/N combination. Representative images of immunofluorescence stainings of tissue sections for P-selectin (upper panel) and α-SMA (lower panel) (magenta). CD31 was used to detect endothelial cells (green). DAPI was used for nuclear staining (blue). Scale bars, 25 µm. b, c, Quantification of the P-selectin+ vessels (b) and α-SMA+ vessels (c) of the immunofluorescence stainings depicted in (a). Individual tumors are shown as single dots in the graph (classical: control n = 3, T/N n = 4; mesenchymal: control n = 4, T/N n = 5). d, Orthotopically transplanted tumors of the indicated subtypes were treated with vehicle (control) and the indicated drugs and drug combinations, explanted, single cell suspended and analyzed by flow cytometry. Panel (d) shows the staining for CD45+ cells. Individual tumors are shown as single points in the graph. e, f, Graphs representing the percentage of CD4+ (e) and CD8+ (f) cells in the PDAC control cohort and in the different treatment conditions as analyzed by flow cytometry. Single points represent individual tumors. g, Left, scheme of the in vivo experimental strategy using orthotopic PDAC cell transplantations into T cell deficient CD3ε knockout (KO) mice. Right, representative FACS plot of immunodeficient CD3ε-KO and wild-type C57BL/6 mice, highlighting the lack of T cells in the CD3ε-KO animals. h, Representative MRI picture of vehicle (control) and T/N treated PDAC bearing CD3ε-KO mice before (week 2) and after 1 week treatment (week 3). P values in (b), (c), (d) and (f) were calculated with two-tailed unpaired t test. T: trametinib, N: nintedanib, T/N: trametinib+nintedanib.
Extended Data Fig. 8 | Characterization of context-specific changes of the innate immune system in classical and mesenchymal PDAC subtypes upon therapy. a, Pie charts representative of the fraction of innate immune cell populations in PDAC from vehicle control and mice that received the combination (T/N) for both classical and mesenchymal orthotopically transplanted tumors as analyzed by flow cytometry. The number of tumors per condition analyzed is depicted in the corresponding panel. b, Graphs representing the percentage of Ly6G+ CD11b+ F4/80+ macrophages in PDAC of the control cohort and in the treatment conditions as analyzed by flow cytometry. Single points represent individual tumors. P values in (d) and (e) were calculated with two-tailed unpaired t test. T: trametinib, N: nintedanib, T/N: trametinib+nintedanib.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | scRNA-seq reveals treatment-induced changes in TME cell subpopulations and activation of the DNA damage pathway in cancer cells. a, Dotplot displaying marker gene expression across each identified cluster of cancer cells and corresponding tumor microenvironment for both classical and mesenchymal tumors. The clusters are indicated on the y axis and the main markers for each identified population are indicated on the x axis. b, Left, UMAP plot showing all identified cell populations within the scRNA-seq experiment. Middle, UMAP plot showing classical (yellow) and mesenchymal (blue) tumors from all treatment and vehicle groups. Right, UMAP plot showing the treatment-induced changes in cell type composition among the identified cell populations across subtypes. Lower part, UMAP density plots showing distribution of annotated clusters upon treatment, cell numbers for each condition are integrated below. c, UMAP plot showing the identified tumor cell clusters. The expression of Cdh1 and Krt18, epithelial markers, and of Cdh2 and Vim, mesenchymal markers, across treatment conditions are shown below. d, Heatmap of the most differentially expressed genes from the gene expression signature in Fig. 7 across subtypes and treatment conditions. e, Gene set enrichment analysis (GSEA) of scRNA-seq data of cancer cells reveals enrichment of DNA damage in both classical and mesenchymal tumors upon treatment with the T/N combination. NES and FDR-q values are indicated. f, Representative images of immunohistochemical staining for γH2AX of control and T/N treated tumor sections for both classical and mesenchymal subtypes. Scale bar, 70 μm. g, Quantification of γH2AX positive cells in (f). Individual tumors are shown as single points in the graph (classical: control n = 6, T/N n = 5; mesenchymal: control n = 8, T/N n = 7). P values were calculated with two-tailed unpaired t test. Endo cells: endothelial cells. T/N: trametinib+nintedanib. T/N + aPD-L1: trametinib+nintedanib+anti PD-L1 antibody.
Extended Data Fig. 10 | Context-dependent reprogramming of the cancer cell derived secretome and cancer associated fibroblasts (CAFs) by the T/N combination therapy. **a**, Volcano plots highlighting the changes in secreted factors upon T/N treatment in classical (left) and mesenchymal (right) PDAC cells. The x-axis shows log2 fold change (treated/control), the y-axis the per test adjusted p values, which were calculated by differential expression test (two-sided t test). **b**, Circos plot showing the key communication signals from tumor cells to T cell subtypes, tumor cells and acinar cells in classical mPDAC. The ligand protein expression fold change, identified from secretome experiments, between T/N and control is shown in the middle. Normalized receptor expression levels obtained from scRNA-seq data are shown in the outer concentric circles. **c**, UMAP plot highlighting the whole population of CAF cells identified in classical and mesenchymal tumors. **d**, Left, UMAP plot showing the CAF population across different treatment conditions in classical tumors. Right, UMAP plots displaying the identified CAF clusters and resulting subpopulations for classical tumors. **e**, UMAP plots of the CAF cluster displaying selected marker gene expression. **f**, Heatmap displaying expression of selected genes in CAFs across the identified clusters. The y-axis shows the selected marker genes, the x-axis represents each of the identified clusters in (d). **g**, Violin plot showing Tgfb1 expression by myoCAFs across the different treatment conditions. **h**, Proportion of CAF subtypes in the indicated different treatment conditions. CAF subpopulations were identified in the fibroblast cell clusters and annotated with the markers described in (f). T/N: trametinib+nintedanib, T/N+aPD-L1: trametinib+nintedanib+anti PD-L1 antibody.
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Sofware used for data collection: Horos{Horosproject.org, v3.3.6}, Aperio Image Scope {Leica, v12.3.3}, FlowJo {v10.6.2}, CellRanger {v3.1.0}, MaxQuant {v1.5.7.4}, MaxQuant3 {v1.6.2.10}. Additional information are reported in the methods section of the manuscript.

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Sample size
No statistical methods were used to predetermine sample size estimates. For in vitro and FACS experiments samples size was determined based on preliminary experiments that defined the adequate number of samples to consistently identify differences between groups. In vivo experiments were performed initially as small pilots and repeated to reach n=10 or more in each group.

Data exclusions
No data were excluded from the analysis.

Replication
All experimental procedures were replicated successfully at least in three independent experiments.

Randomization
For the animal studies, the mice were randomized when their tumors grew to size of ~100 mm3 to achieve equal tumor volume between treatment arms. For other experiments described in this manuscript randomization was not appropriate and the tumors or tumor cells were characterized without further randomized assignment.

Blinding
The investigators were not blinded when performing in vivo treatments. The processing of the samples and their analysis, when possible, was performed in a blinded fashion (e.g. quantification of immunohistochemistry, tumor volume assessment, flow cytometry analysis). Moreover, experiments were analyzed by independent investigators. For experiments not involving mice, e.g. cell culture treatment experiments, the authors were not blinded to the treatment groups.

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| n/a |
| ☐ xx Antibodies |
| ☐ x Eukaryotic cell lines |
| ☐ Palaeontology and archaeology |
| ☐ Animals and other organisms |
| ☐ Human research participants |
| ☐ Clinical data |
| ☐ Dual use research of concern |

| n/a |
| ☐ xx Involved in the study |
| ☐ xx ChIP-seq |
| ☐ xx Flow cytometry |
| ☐ xx MRI-based neuroimaging |

Antibodies

Antibodies used

For flow cytometry:
Validation

All antibodies used in this study were validated for the use in mouse samples by the manufacturers and adequate controls were included (positive and negative controls).

Detailed information can be found on manufacturers' websites:

For flow cytometry:
- CD4 BU805: https://wwwbdbiosciencescom/en-us/products/reagents/fluorescence-reagents/single-color-antibodies-ruo/bu805-antifa-mouse-cd4-612900 (replacement for #564922; additional info can be found here: https://www.citeabcom/antibodies/2409851-564922-bu805-antifa-mouse-cd4)
- CD3e BU395: https://wwwbdbiosciencescom/en-us/products/reagents/fluorescence-reagents/single-color-antibodies-ruo/bu395-hamster-antimouse-cd3e-363565
- CD11c BU737: https://wwwbdbiosciencescom/en-us/products/reagents/fluorescence-reagents/single-color-antibodies-ruo/bu737-hamster-antimouse-cd11c-612796 (replacement for #564986; additional info can be found here: https://www.citeabcom/antibodies/3288721-564986-bu737-hamster-antimouse-cd11c-3288721-564986)
- NK1.1 BU395: https://wwwbdbiosciencescom/en-us/products/reagents/fluorescence-reagents/single-color-antibodies-ruo/bu395-mouse-antimouse-nk-1-1564144
- CD8a BV785: https://wwwbiolcgcom/en-us/products/brilliant-violet-785-antimouse-cd8a-antibody-7975
- CD45 PertC Cy5.5: https://wwwbiolcgcom/en-us/products/percp-cyaninem5-5-antimouse-cd45-antibody-9793
- CD19 FITC: https://wwwbiolcgcom/en-us/products/flt-4antimouse-cd19-antibody-1528
- EpCAM APC/AF647: https://wwwbiolcgcom/en-us/products/alexa-fluor-647-antimouse-cd32-selector-epcam-antibody-4973
- Ly6C BV785: https://wwwbiolcgcom/en-us/products/brilliant-violet-785-antimouse-ly-6c-antibody-8275
- Ly6G BV650: https://wwwbiolcgcom/en-us/products/brilliant-violet-650-antimouse-ly6g-antibody-2634
- F4/80 BV421: https://wwwbiolcgcom/en-us/products/brilliant-violet-f480-antimouse-cd206-antibody-2479
- Ly6G PE: https://wwwbiolcgcom/en-us/products/pe-antimouse-ly-6g-antibody-4777
- CD68 APC-C7: https://wwwbiolcgcom/en-us/products/apc-cyaninem7-antimouse-cd68-antibody-13175
- TruStain FCX CD16/32: https://wwwbiolcgcom/en-us/products/trustin-control-fluor-anti-mouse-cd16-cd32-antibody-7863
- TERT-19 BV421: https://wwwbiolcgcom/en-us/products/brilliant-violet-421-antimouse-ter119-antibody-7259
- CD45 APC: https://wwwbiolcgcom/en-us/products/alexa-fluor-488-antimouse-cd45-antibody-3101
- CD31 APC: https://wwwbiolcgcom/en-us/products/alexa-fluor-488-antimouse-cd31-antibody-3092

For immunofluorescence stainings:
- CD68: https://wwwbio-radantibodiescom/monoclonal/mouse-cd68-antibody-fa-11-mca1957html
- ARG1: https://wwwthermofishercom/antibody/product/arginase-1-antibody-polyclonal-pas29645
- CD80: https://wwwabcacom/cd80-antibody-ab254579.html
- CD3: https://wwwbiolcgcom/en-us/products/purified-anti-mouse-cd3-antibody-48groupid-8lg6738
- CD31: https://wwwabcacom/cd31-antibody-2h8-ahb19341html
- CX3C9: https://wwwsigmaalrichcom/DE/en/produkte/sigmaab4501665
- Vimentin: https://wwwthermofishercom/antibody/product/vimentin-antibody-polyclonal-pai16759
- aSMA: https://wwwabcacom/alpha-smooth-muscle-actin-antibody-ab5684.html
- P-selectin: https://wwwlsioscom/antibodies/ihc-plus-selp-antibody-p-selectin-antibody-cd62p-antibody-ihn-wb-western-ls-b3578-103464
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Human established cell lines were purchased from ATCC; the primary human cell lines were isolated from autochthonous PDAC as described previously [Eser et al., 2013]; the primary mouse cell lines were isolated from genetically engineered mouse models as previously described in Mueller et al., 2018.

The used established human lines were the following: PSN1, HupT4, HPAFII, HPAC, IMIM-PC1, MiaPaCa2, PanTu, DanG, HS766T, ASPC1, CFPCAL1, HEK293T.

The primary human cell lines were the following: hUPDCA7, hUPDCA17.

The primary mouse cell lines employed were the following: 5671, 53578, 5748, 8305, 8296, B590, R1035, 9203, 5821, 9591, 2259, 53646, 8661, 16990, 16992, 8442, 8513, 53631, 8028, 8182, 4072, 5302, 6075, 8570, 53704, 8248, 4706, 5914, 9091, 3202, 5320, mPDAC1, mPDAC2, mPDAC3.

Authentication
All human cell lines were authenticated through STR profiling. The murine cell lines were authenticated through genotyping PCR.

Mycoplasma contamination
All cell lines were routinely checked for mycoplasma contamination and tested negative.

Commonly misidentified lines (See ICLAC register)
No misidentified cell lines according to the ICLAC register were used in this study.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
For orthotopic transplantsations experiments of the cell lines 9091 and 8661, primary murine pancreatic cancer cell lines derived from C57BL/6 mice, the employed animals were C57BL/6 or CD3e: KO on a C57BL/6 background. For orthotopic transplantsations experiments of the mPDAC3, primary murine pancreatic cell line derive from a mouse on a mixed C57BL/6J;129SvEv genetic background, we used F1 hybrids C57BL/6J;129SvEv. All animals presented between 8 and 12 weeks of age when the experiments were performed.

Cell lines isolated from female endogenous mice were transplanted for the study in female recipients, vice versa for male mice. All animals were kept in a dedicated facility, with a light dark cycle or 12:12 hours, a housing temperature between 20 and 24°C and a relative air humidity of 55%.

Wild animals
No wild animals were used in the study.
Field-collected samples
No field-collected samples were used in the study.

Ethics oversight
The animal studies comported the use of mice and were performed in compliance with the ARRIVE guidelines, moreover all animal studies were conducted on compliance with European guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees (IACUC) of the local authorities of Technische Universität München and the Regierung von Oberbayern.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Fresh tumor samples were minced using a scalpel and enzymatically digested with the tumor dissociation kit (Miltenyi #130 096 730) in DMEM medium (Sigma, #D5796 500 mL) for 40 min at 37°C with agitation. The cell suspension was strained through a 100 μm strainer, spun down and resuspended in 2% FCS/PBS. Cells were blocked for 10 min on ice with anti-mouse CD16/32/37 FC block (Biolegend, 1:100) and stained with Zombie Aqua Fixable Viability Kit (Biolegend, 1:500) for 10 min on ice and the antibody cocktails for acquisition of adaptive immune cells as well as innate immune cells (for 30 min on ice).

Instrument
BD LSRFortessa (Immunophenotyping); BD FACS Aria Fusion [Sorting for scRNA-seq]

Software
FlowJo software (Version 10.6.2)

Cell population abundance
- TER-119-negative/CD45+/CD31-/EPICAM-positive fraction: 0.5-8% of total cells
- TER-119+/CD45+/CD31+/EPICAM-negative fraction: 0.4-3% of total cells

Gating strategy
The flow cytometry gating strategy can be found in detail in the source data figure 4. FSC and SSC gates were used to identify cells and exclude doublets. Live/dead cells were discriminated by Zombie Aqua staining (staining of dead cells). Live cells were further analyzed for cell types of interest, which can be analyzed by the following marker:

- Neutrophils: CD45+ Ly6G+ CD11b+
- Macrophages: CD45+ Ly6G- CD11b+ F4/80+
- Dendritic cells: CD45+ Ly6G- F4/80- CD11c+
- NK cells: CD45+ Ly6G- F4/80- CD11c- NK1.1+
- T cells: CD45+ CD3+
- B cells: CD45+ CD19+
- CD4+ T cells: CD45+ CD3+ CD4+
- CD8+ T cells: CD45+ CD3+ CD8a+

For sorting of TER-119-negative/CD45+/CD31-/EPICAM-positive cell fraction (for enrichment of immune, endothelial and epithelial tumor cells and exclusion of erythrocytes) as well as TER-119+/CD45+/CD31+/EPICAM-negative cell fraction (for enrichment of fibroblasts/mesenchymal tumor cells and exclusion of erythrocytes) FSC and SSC gates were used to identify cells and exclude doublets. TER-119-negative cells were used for sorting two fractions:
- CD45+ CD31+ EPICAM+
- CD45+ CD31- EPICAM-

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.