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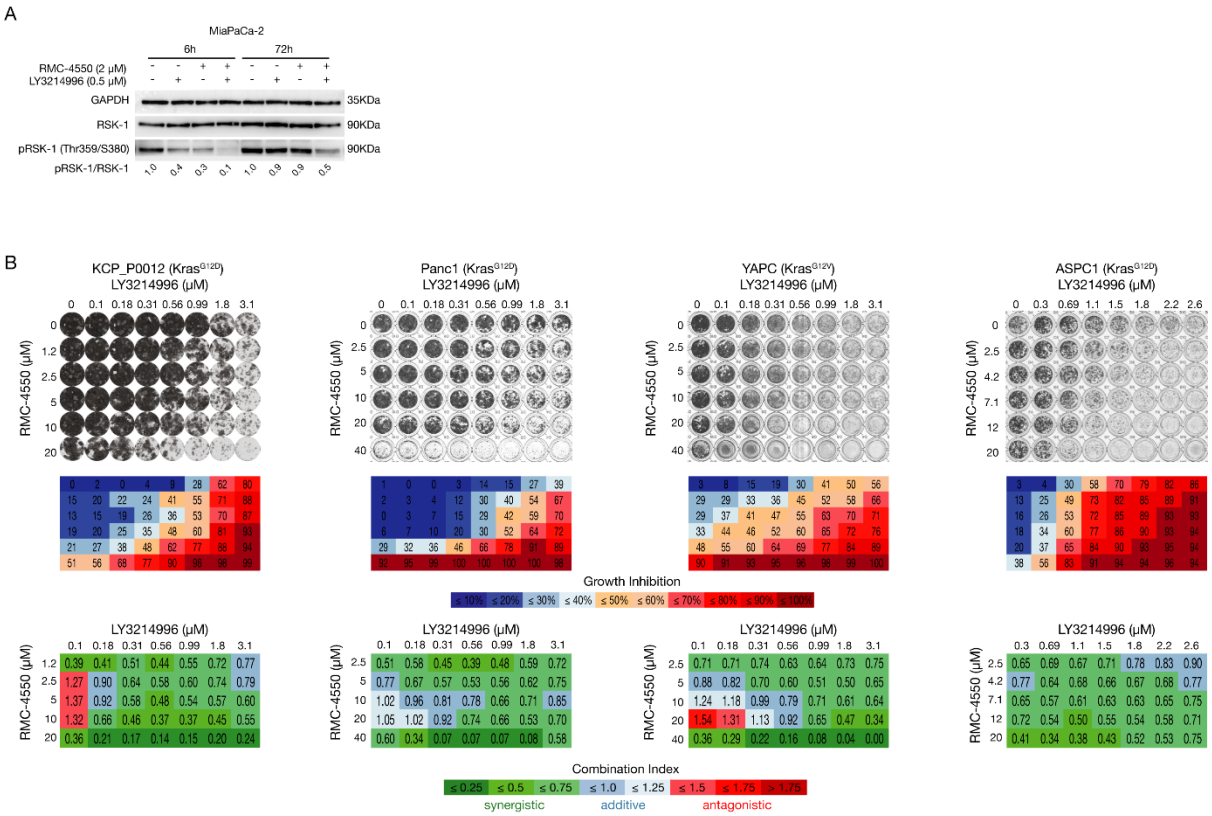
Supplemental information

Extensive preclinical validation of combined RMC-4550 and LY3214996 supports clinical investigation for KRAS mutant pancreatic cancer

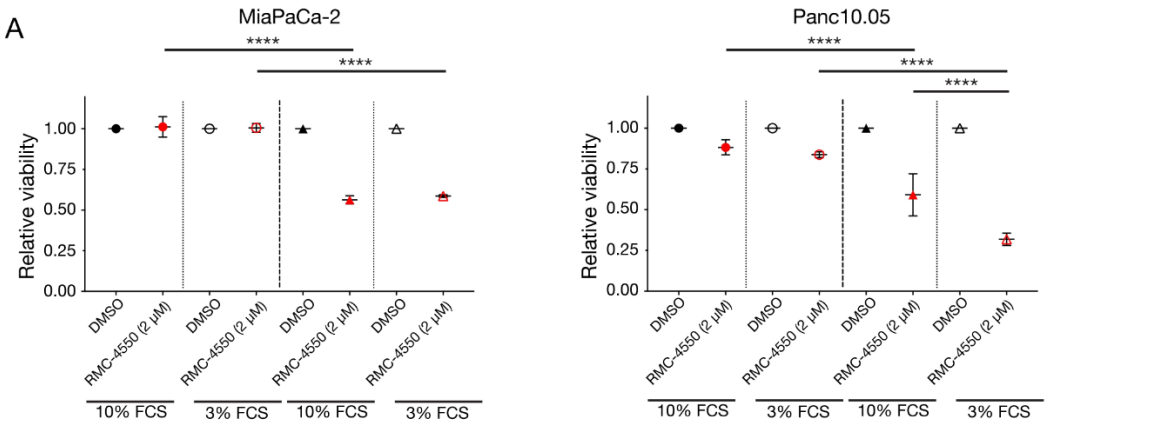
Katrin J. Frank, Antonio Mulero-Sánchez, Alexandra Berninger, Laura Ruiz-Cañas, Astrid Bosma, Kıvanç Görgülü, Nan Wu, Kalliope N. Diakopoulos, Ezgi Kaya-Aksoy, Dietrich A. Ruess, Derya Kabacaoğlu, Fränze Schmidt, Larissa Kohlmann, Olaf van Tellingen, Bram Thijssen, Marieke van de Ven, Natalie Proost, Susanne Kossatz, Wolfgang A. Weber, Bruno Sainz Jr., Rene Bernards, Hana Algül, Marina Lesina, and Sara Mainardi

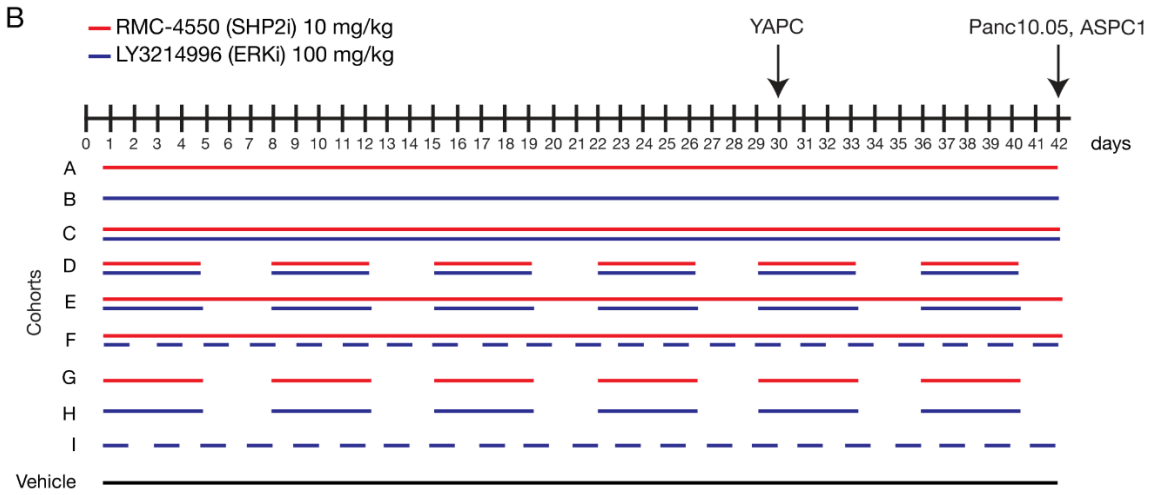
Supplemental material

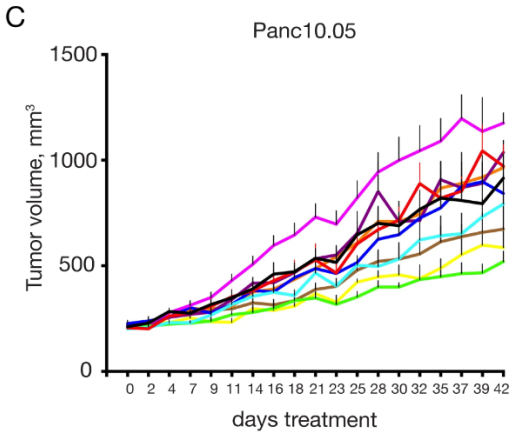


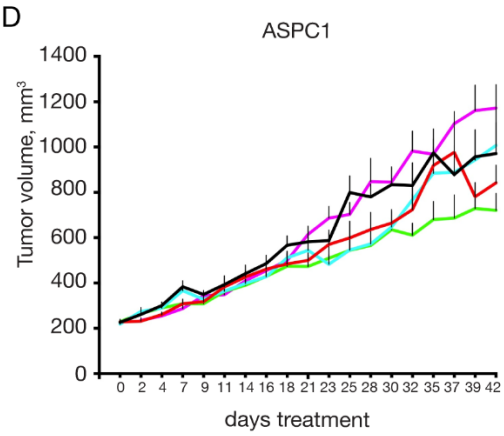
Supplementary Figure S1. Assessing the treatment response in murine and human *KRAS*-mutant pancreatic cancer cell lines, related to Figure 1

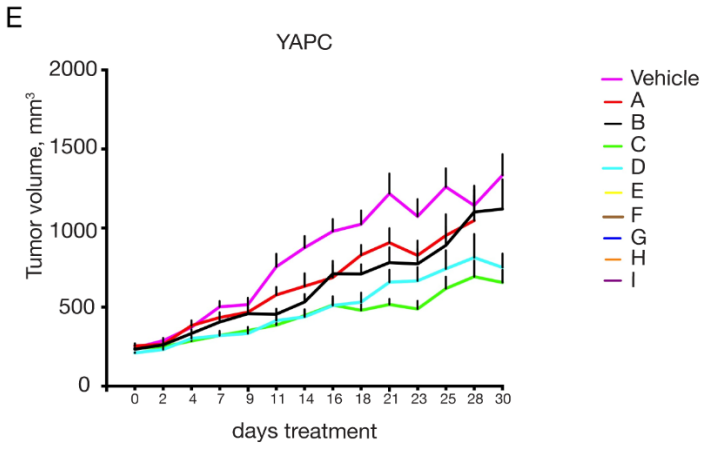
A: Western blot analysis with human cancer cell line MiaPaCa-2. Cells were treated as depicted and collected for lysis at the indicated time points. Protein extracts were probed with specific antibodies against total RSK-1, phosphorylated RSK-1 (pRSK-1), and GAPDH (as loading control). Numerical values indicate the pRSK-1/RSK-1 ratio quantified by densitometry. The blots are representative of at least three independent experiments. RSK-1 = Ribosomal s6 kinase 1. **B**: Synergistic effects of SHP2i and ERKi administration were evaluated by colony formation assay with murine cancer cell line KCP_P0012 derived from KCP mouse model ($Kras^{G12D}$) of spontaneous tumor formation and human cancer cell lines: Panc1 ($KRAS^{G12D}$), YAPC ($KRAS^{G12V}$), and ASPC1 ($KRAS^{G12D}$). SHP2i and ERKi were combined at the concentrations indicated. Representative crystal violet staining of cells is shown (top panel). Box-matrices below the plate-scans depict quantification of growth inhibition in relation to control wells (middle panel). Bottom panel: Calculation of the Combination Index (CI) Scores from the growth inhibition values (shown above) via CompuSyn software demonstrating strong synergism between SHP2i and ERKi across a wide range of combinatorial concentrations. CI < 0.75 (shades of green) indicates synergism, CI = 0.75 – 1.25 (shades of blue) indicates additive effects and CI > 1.25 (shades of red) indicates antagonism. Experiments were repeated independently at least three times each, with similar results.


A
MiaPaCa-2
Panc10.05
****
Relative viability
1.00
0.75
0.50
0.25
0.00
DMSO
RMC-4550 (2 μM)
10% FCS
3% FCS


B
RMC-4550 (SHP2i) 10 mg/kg
LY3214996 (ERKi) 100 mg/kg
YAPC
Panc10.05, ASPC1
days
Cohorts
A
B
C
D
E
F
G
H
I
Vehicle

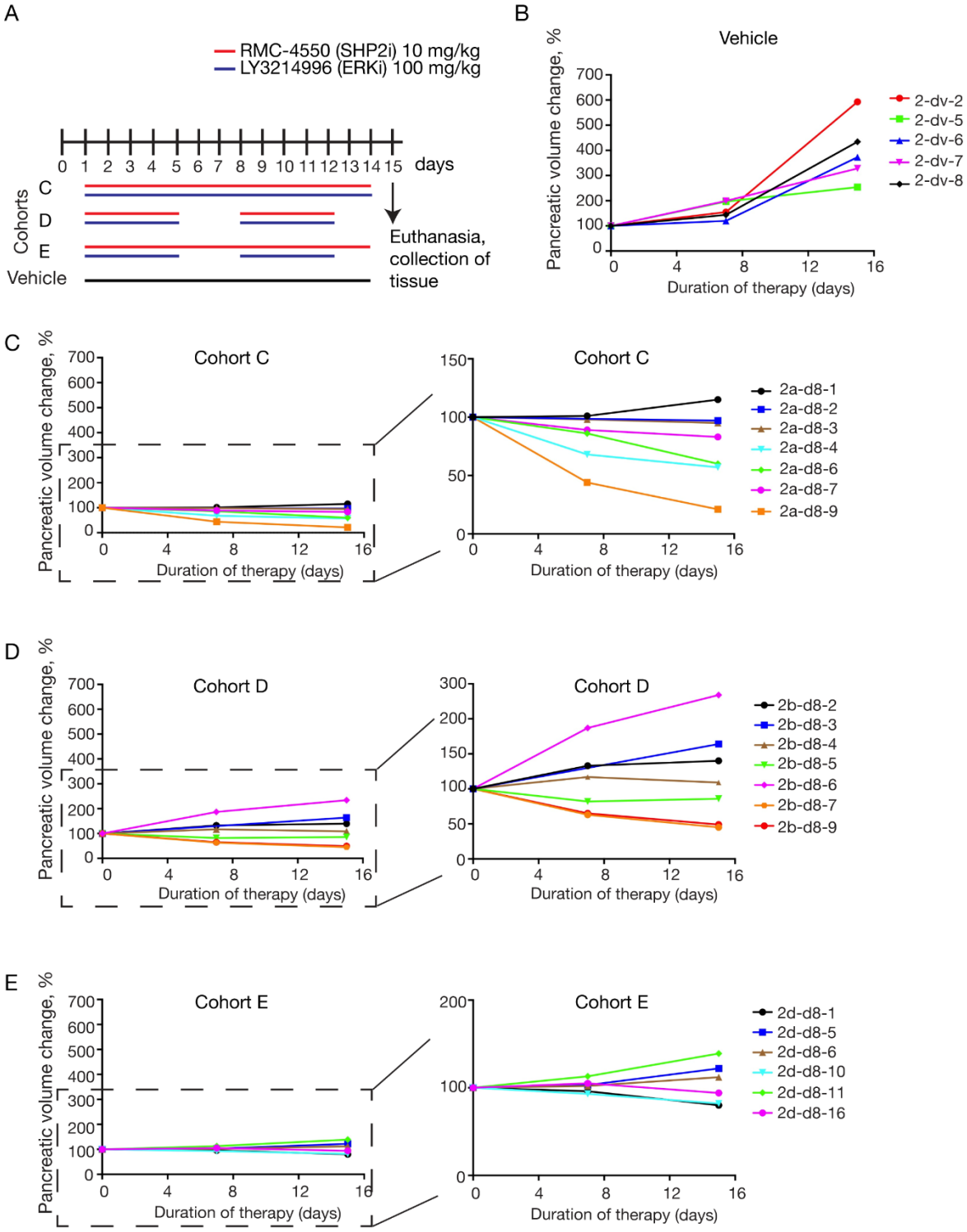

C
Panc10.05
Tumor volume, mm3
days treatment


D
ASPC1
Tumor volume, mm3
days treatment


E
YAPC
Tumor volume, mm3
days treatment
Vehicle
A
B
C
D
E
F
G
H
I

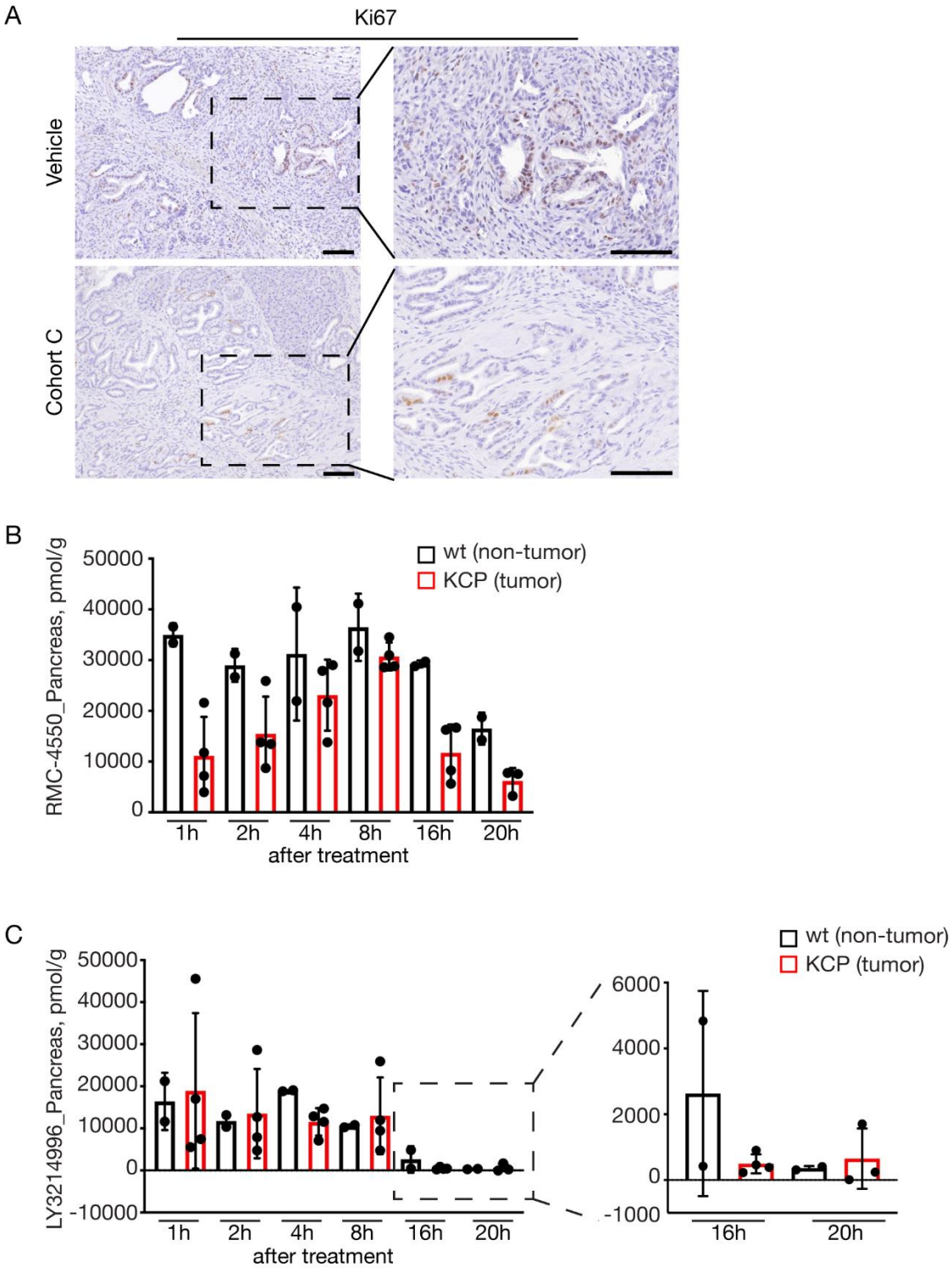
Supplementary Figure S2. *In vivo* evaluation of the combined administration of RMC-4550 (SHP2i) and LY3214996 (ERKi) on tumor growth in different PDAC models, related to Figures 3 and 4

A: Comparison of 2D vs. 3D growth and 10% serum vs. 3% serum conditions in the indicated human cancer cell lines treated with 2 µM RMC-4550. Cell viability was measured after 4 days. Statistical analyses compared the effect of SHP2 inhibition between 2D and 3D growth both in 10% serum and 3% serum using ordinary one-way ANOVA test. Experiments were repeated independently at least three times each, with similar results. **B**: Schematic representation of the treatment schedule applied in MiaPaCa-2, ASPC1, and YAPC xenograft models. Cohort A: Continuous treatment with SHP2i alone daily; Cohort B: Continuous treatment with ERKi alone daily; Cohort C: Continuous treatment with the combination of SHP2i and ERKi daily; Cohort D: Intermittent treatment with the combination of SHP2i and ERKi 5 days on / 2 days off; Cohort E: Semi-continuous treatment schedule with daily dosing of SHP2i and intermittent dosing with ERKi 5 days on / 2 days off. Cohort F: Continuous treatment with SHP2i and on alternate days with ERKi. Cohort G: Intermittent dosing with SHP2i alone 5 days on / 2 days off. Cohort H: Intermittent dosing with ERKi alone 5 days on / 2 days off. Cohort I: Treatment with ERKi alone on alternate days. Control mice were continuously treated with vehicle. For all the xenograft experiments, 5×10^6 cells were subcutaneously injected into the right flank of NOD scid gamma (NSG) mice, respectively. When tumors reached 200 - 250 mm^3, mice were randomly assigned into cohorts and treated by oral gavage with inhibitors or vehicle according to treatment schedule. Mice were sacrificed after 1, 3 or 6 weeks of treatment (n = 8 mice per time point and per cohort) **C-E:** Treatment response was assessed through tumor volume change using caliper measurements 3 times/week in different xenograft models: Panc10.05 (**C**), ASPC1 (**D**), and YAPC (**E**). Results represent mean ± SD.



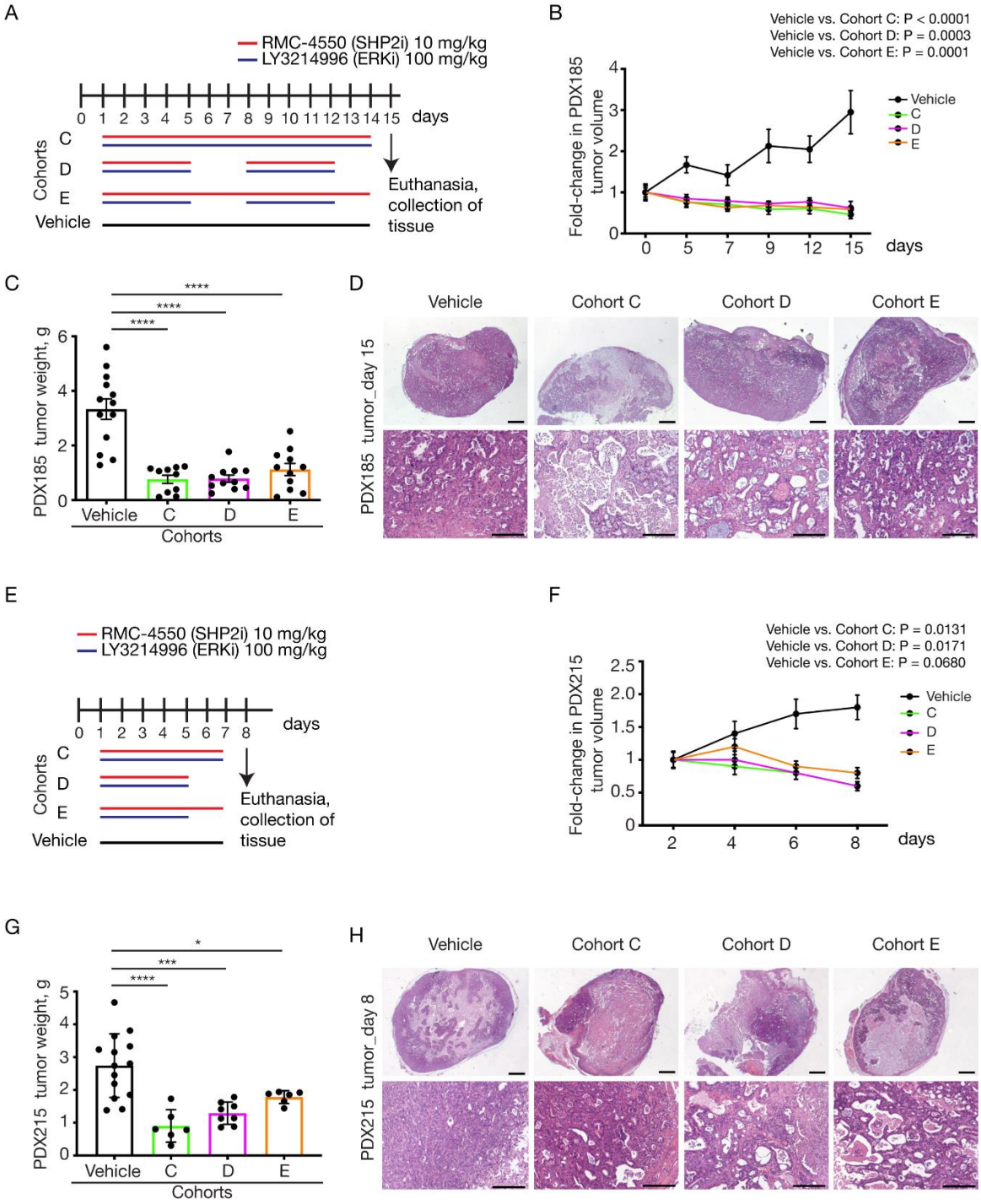
Supplementary Figure S3. *In vivo* assessment of treatment response in an endogenous murine PDAC model, related to Figure 5

A: Schematic representation of the treatment schedule applied in an endogenous (KCP) murine model of spontaneous tumor formation. Cohort C: Continuous treatment with the combination of SHP2i and ERKi daily (n = 9); Cohort D: Intermittent treatment with the combination of SHP2i and ERKi 5 days on / 2 days off (n = 7) ; Cohort E: Semi-continuous treatment schedule with daily dosing of SHP2i and intermittent dosing with ERKi 5 days on / 2 days off (n = 6). Control mice (n = 5) were treated with vehicle for 14 consecutive days. **B - E**: Volume-tracking curves for individual mice over the whole course of therapy. The y axis shows tumor volume change in %. Tumor volume at baseline before commencement of therapy is indicated by 100%.



Supplementary Figure S4. Evaluation of drug distribution of RMC-4550 and LY3214996 and of tumor growth in an endogenous murine PDAC model, related to Figure 5

A: Immunohistochemical analysis of paraffin-embedded pancreatic tissue samples of mice, treated with vehicle (n=5) or with the combination of SHP2i and ERKi daily (Cohort C, n = 9), using Ki67 antibody. Scale bars represent 100 µm. **B, C**: Pharmacokinetic analysis regarding pancreatic tissue derived from KCP mouse model of spontaneous tumor formation and non-tumor bearing littermates over time. Mice were administered a single dose of 10 mg/kg RMC-4550 (SHP2i) (**B**) or 100 mg/kg LY3214996 (ERKi) (**C**) and sacrificed after 1h (n = 4), 2h (n = 4), 4h (n = 4), 8h (n = 4), 16h (n = 4) and 20h (n = 3). Distribution for each drug in the tumor and healthy pancreas was determined by LC-MS/MS. Results represent mean ± SD.



Supplementary Figure S5. Treatment response in Patient-Derived Xenograft (PDX) models, related to Figure 6

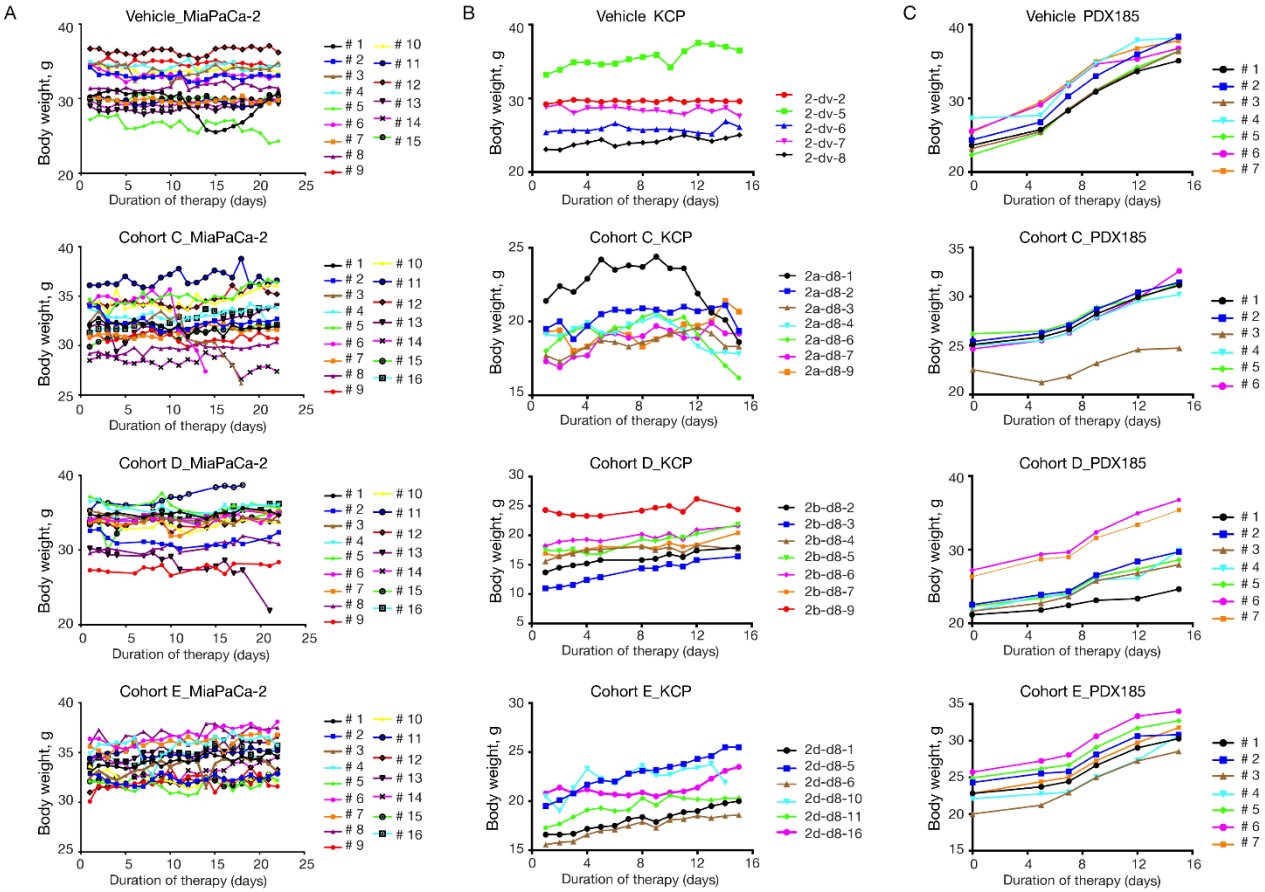
A: Treatment schedule for the PDX185 model. Mice were treated with the combination of RMC-4550 (SHP2i) and LY3214996 (ERKi) once per day via oral gavage for 14 consecutive days (Cohort C) or 5 days on / 2 days off (Cohort D) or with SHP2i continuous and ERKi5 days on / 2 days off (Cohort E). For the PDX models, tumor pieces of 50 mm^3 were subcutaneously implanted into both flanks of NSG mice (n = 7 mice per cohort). When tumors reached 200 - 250 mm^3 (approximately 6 - 8 weeks after subcutaneous transplantation), mice were randomly assigned into cohorts and treated by oral gavage with inhibitors or vehicle according to treatment schedule for the indicated times. **B**, **C**: Treatment response of PDX185 was assessed

through tumor volume changes using daily caliper measurements (**B**) and tumor weight at endpoint (**C**). Results represent mean ± SD. **** P <0.0001. Significance was determined by one-way ANOVA with Bonferroni's multiple comparison test. **D**: Representative H&E-stained sections of vehicle- and combination therapy-treated PDX185 tumors at day 15. Scale bars represent 1000 µm (top) and 200 µm (bottom). **E**: Treatment schedule for the PDX215 model. Mice were treated with the combination of RMC-4550 (SHP2i) and LY3214996 (ERKi) once per day via oral gavage for 7 consecutive days (Cohort C) or 5 days on / 2 days off (Cohort D) or with SHP2i continuous and ERKi5 days on / 2 days off (Cohort E). **F, G**: Treatment response of PDX215 was assessed through tumor volume changes using daily caliper measurements (**F**) and tumor weight at endpoint (**G**). Results represent mean ± SD. * P <0.05, *** P <0.001, **** P <0.0001. Significance was determined by one-way ANOVA with Bonferroni's multiple comparison test. **H**: Representative H&E-stained sections of vehicle- and combination therapy-treated PDX215 tumors at day 8. Scale bars represent 1000 µm (top) and 200 µm (bottom).

Supplementary Table S1: Summary of Pathogenic and Likely Pathogenic Mutations in cells derived from PDX354 and PDX185, related to Figures 6 and S5

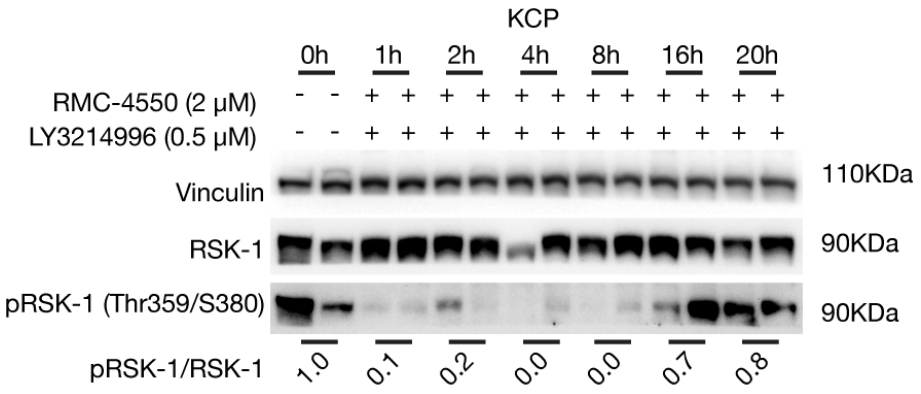
	CDKN2A	***EGFR***	***KRAS***	***SMAD4***	***TP53***
PDX 354	ENST00000498124.1:c.158T>G(p.Met53Arg)	NM_005228.5:c.2291A>G(p.Ty r764Cys)	NM_033360.4:c.35G>A(p.Gly 1 2Asp)	no pathogenic or likely pathogenic variants detected	ENST00000269305.4:c.1100+2 T>C
	ENST00000498124.1:c.221A>C(p.Asp74Ala)				
	ENST00000498124.1:c.206A>G(p.Glu69Gly)				
PDX 185	no pathogenic or likely pathogenic variants detected	NM_005228.5:c.868A>G(p.T hr 290Ala)	NM_033360.4:c.35G>A(p.Gly 1 2Asp)	NM_005359.6:c.1055del(p.Gly3 52AspfsTer32)	ENST00000269305.4:c.520A>G(p.Arg174Gly)
				NM_005359.6:c.1001A>G(p.Gl n334Arg)	
				NM_005359.6:c.1022T>C(p.Val 341Ala)	ENST00000269305.4:c.1100+2 T>C

HGVS-nomenclature is used for each variant. NM = single nucleotide variant; ENST = splice variant



Supplementary Figure S6. Comparison of body weight–time profiles between the different combination therapy cohorts in the endogenous murine PDAC model, the MiaPaCa-2 xenograft as well as the PDX model, related to Figures 3, 5 and S5.

A: Individual body weight–time profile of the vehicle and combination therapy groups in MiaPaCa-2 xenograft bearing mice: Vehicle (n = 15), Cohort-C (n = 16), Cohort-D (n = 16), Cohort-E (n = 16). **B**: Individual body weight–time profile of the vehicle and combination therapy groups in the endogenous (KCP) murine model of spontaneous tumor formation: Vehicle (n = 5), Cohort-C (n = 7), Cohort-D (n = 7), Cohort-E (n = 6). **C**: Individual body weight–time profile of the vehicle and combination therapy groups in PDX185 bearing mice: Vehicle (n = 7), Cohort-C (n = 6), Cohort-D (n = 7), Cohort-E (n = 7).



Supplementary Figure S7. Evaluation of pRSK-1 protein expression over time after administration of RMC-4550 (SHP2i) and LY3214996 (ERKi) in an endogenous murine PDAC model, related to Figures 1 and 7

Western blot analysis with murine pancreatic tissue derived from KCP mouse model ($KRAS^{G12D}$) of spontaneous tumor formation. Mice were treated with a single dose 8 of RMC-4550 (SHP2i) and LY3214996 (ERKi) and sacrificed after 1h (n = 2), 2h (n = 2), 4h (n = 2), 8h (n = 2), 16h (n = 2) and 20h (n = 2). Non-treated mice served as controls (0h, n = 2). Protein extracts of pancreatic tissue were probed with specific antibodies against total RSK-1, phosphorylated RSK-1 (pRSK-1), and alpha-tubulin (as loading control). Numerical values indicate the pRSK-1/RSK-1 ratio quantified by densitometry. The blots are representative of at least three independent experiments. RSK-1 = Ribosomal s6 kinase 1.