SUPPLEMENTARY FIGURES AND LEGENDS FOR:

Exploiting the Therapeutic Interaction of WNT Pathway Activation and Asparaginase for Colorectal Cancer Therapy

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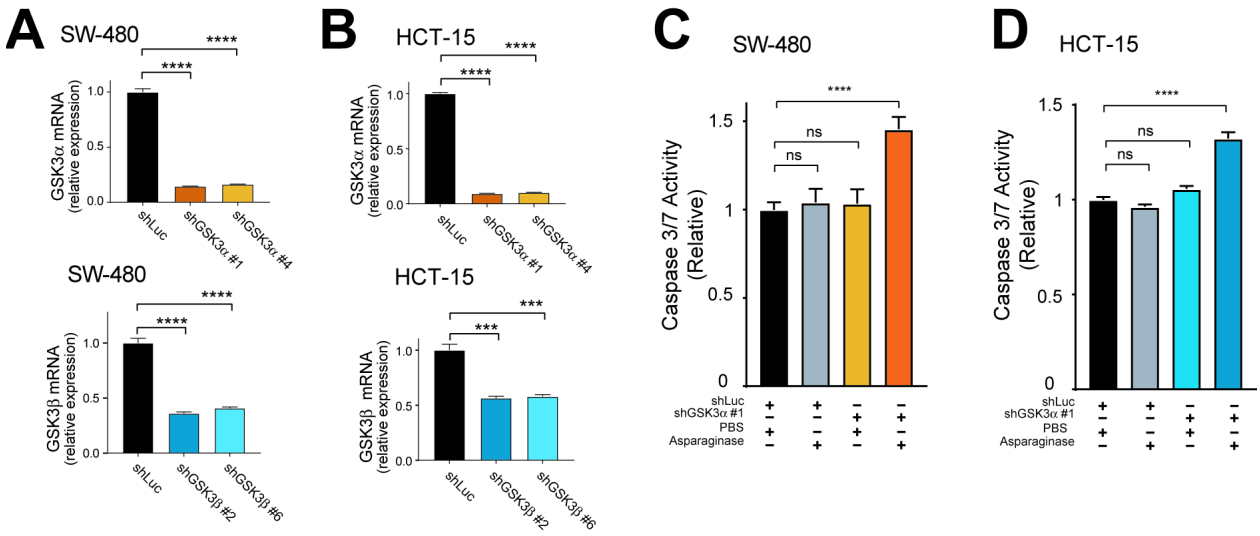


Supplementary Figure S1. The combination of GSK3α knockdown and asparaginase induces apoptosis in colorectal cancer cells.

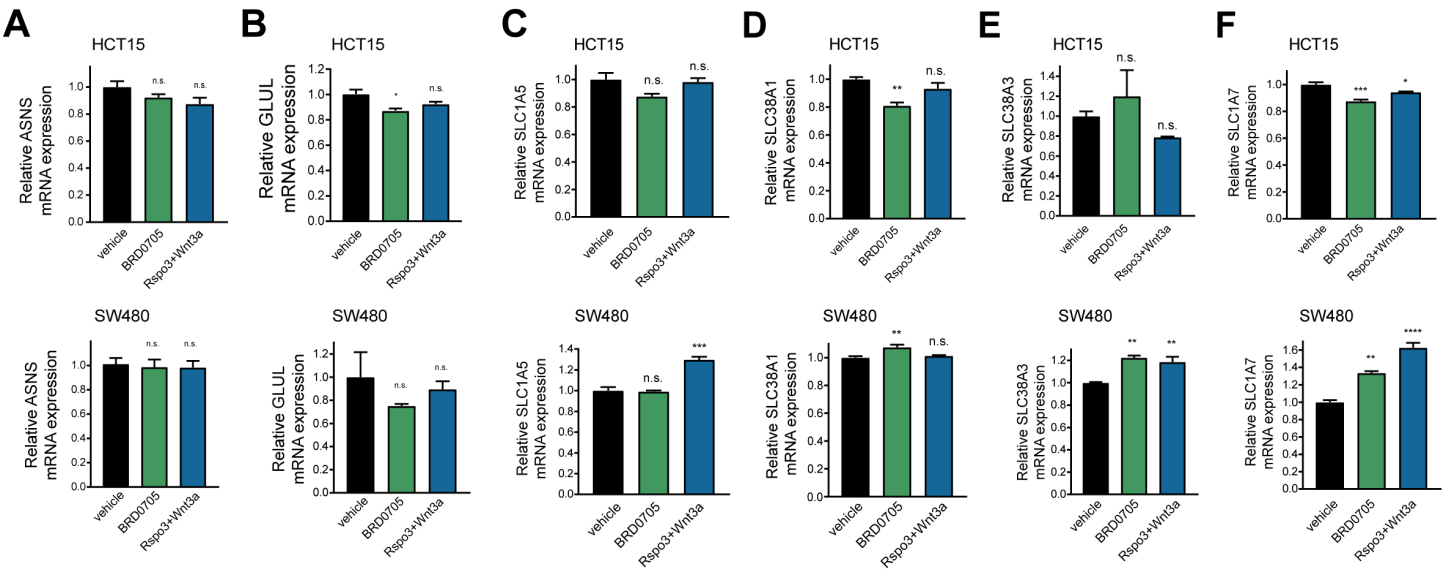
A SW480 cells were transduced with the indicated shRNAs, and knockdown efficiency was assessed by RT-PCR analysis. Statistical analysis was calculated using a one-way ANOVA with Dunnett's adjustment for multiple comparisons, with shLuc used as the reference.

B HCT15 cells were infected with the indicated constructs and knockdown efficiency was assessed as described in (A).

C SW480 cells were infected with shLuciferase or shGSK3α, and subsequently treated with vehicle (PBS) or asparaginase (100 U/L). Caspase 3/7 activity was assessed using the Caspase Glo 3/7 Assay and normalized to shLuciferase, vehicle control. Statistical Significance was calculated by a one-way ANOVA with Dunnett's adjustment for multiple comparisons.

D HCT15 cells were infected with the indicated constructs and treated with vehicle (PBS) or asparaginase (100 U/L). Caspase activity was assessed as described in (D).

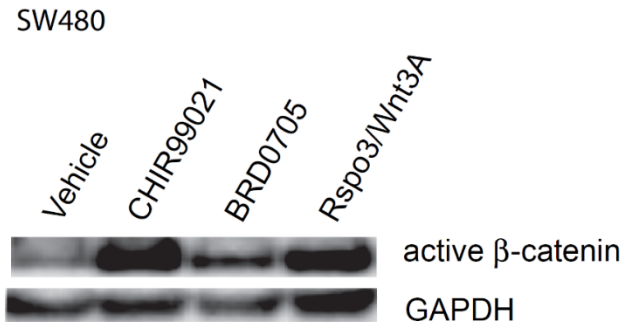
**** $p \leq 0.0001$; *** $p \leq 0.001$; n.s., $p > 0.05$. Error bars denote SEM.



Supplementary Figure S2. WNT-activating ligands or the GSK3α inhibitor BRD0705 have no consistent effect on expression of asparagine synthetase (ASNS), glutamine synthetase (also known as glutamine-ammonia ligase, GLUL), or transporters of asparagine or glutamine.

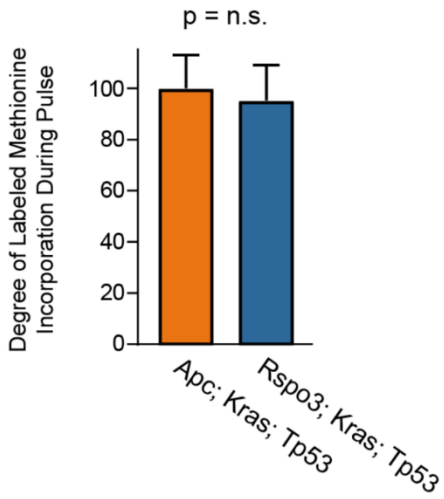
A-F, Expression of the indicated genes was assessed by RT-PCR analysis in the indicated cell types 48 hrs after treatment with vehicle, the GSK3α inhibitor BRD0705 (1 μM) or the combination of Rspo3 (75 ng/ml) and WNT3a (100 ng/ml). B-Actin was the control gene for RT-PCR, and results are normalized to those in vehicle controls. The reported substrates of each amino acid transporter (1) include: SLC1A5, glutamine. SLC38A1, glutamine. SLC38A3, glutamine, asparagine. SLC1A7, glutamine.

**** $p \leq 0.0001$; *** $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$; n.s., $p > 0.05$.



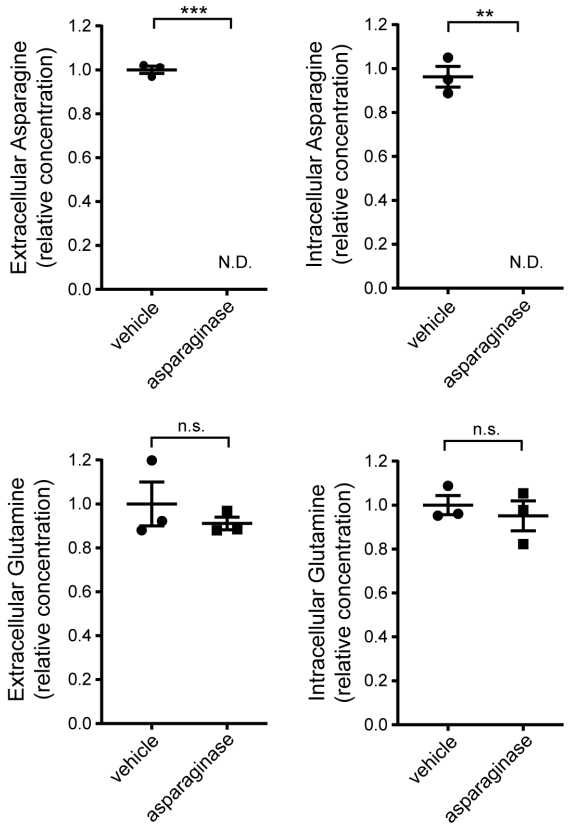
Supplementary Figure S3. Effect of distinct perturbations that activate WNT/STOP and trigger asparaginase sensitivity on β-catenin.

Human SW480 CRC cells were treated with the indicated drugs (1μM CHIR99021, 1μM BRD0705, 100 ng/ml WNT3a, 75 ng/ml Rspo3) for 48 hrs. Protein levels of active (non-Ser33/37/Thr41-phosphorylated) β-catenin were assessed by Western blot analysis. Of note, SW480 cells have a biallelic p.Q1338* APC mutation (2), which retains its N-terminal domain and is predicted to be hypomorphic, but not null, for inhibition of β-catenin (3). Note differential activation of canonical WNT/β-catenin by each of these perturbations, despite similar effects on asparaginase sensitivity (see Fig. 1).



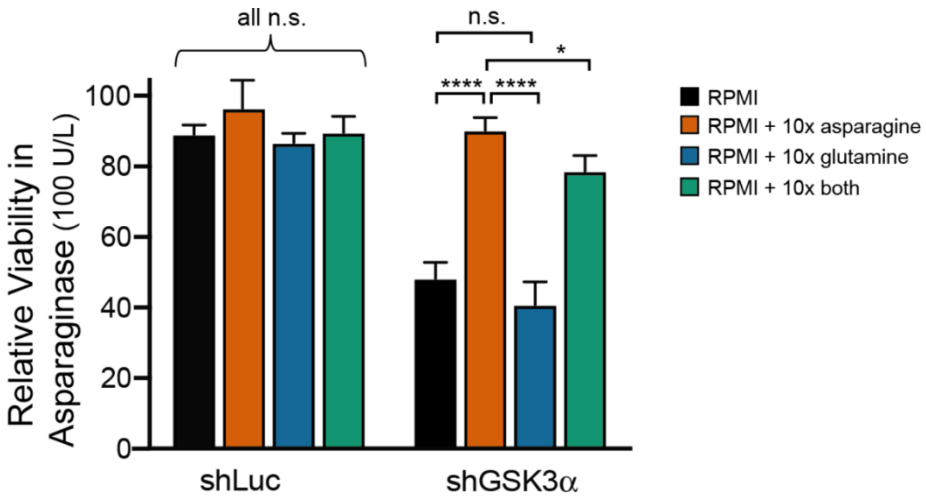
Supplementary Figure S4. Rspo3 expression does not alter the basal rate of incorporation of the methionine analog azidohomoalanine during the pulse period. Mouse intestinal organoids of the indicated genotypes were incubated with a pulse of the methionine analog 5-azidohomoalanine (AHA) for 18 hrs, and the degree of label incorporation was assessed by flow cytometry. Results are shown relative to the degree of label incorporation in Apc -/-; Kras; Tp53 mutant organoids, which was normalized to 100%. Significance was assessed by two-sided Welch t test. Error bars represent SEM.

n.s., $p > 0.05$.



Supplementary Figure S5. Asparaginase treatment of HCT15 cells reduces levels of asparagine, but not glutamine. HCT15 cells were treated with vehicle or asparaginase (100 U/L) for 24hr. Levels of asparagine and glutamine were measured in the media (extracellularly) or in the cell pellet (intracellularly) using liquid chromatrograph-mass spectrometry. Results are normalized to levels in vehicle controls. Significance was assessed by a two-sided Welch t test. Error bars denote SEM. N.D., not detected (limit of detection of the assay < 1.0 mg/L).

*** $p \leq 0.001$; ** p 0.01; n.s., $p > 0.05$.

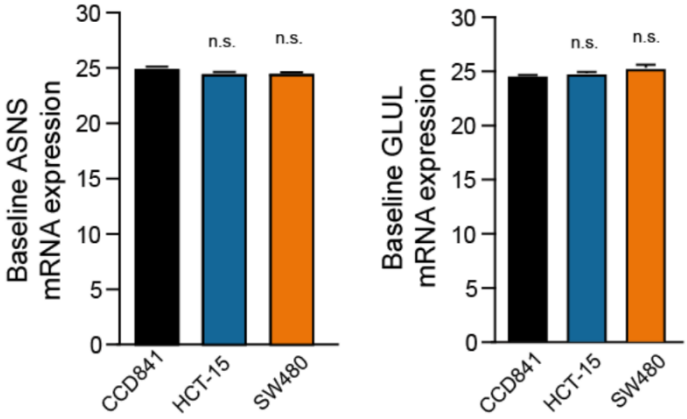


Supplementary Figure S6. The toxicity of asparaginase and GSK3α inhibition can be rescued by replenishing free asparagine, but not glutamine.

HCT15 cells were transduced with the indicated shRNAs, treated with asparaginase (100 U/L), and every 12 hours a 10-fold excess of the indicated amino acid was added to the media (vehicle, asparagine, glutamine, or both asparagine and glutamine). Cell viability was assessed after 72 hours of treatment by trypan blue vital dye exclusion. Viability is normalized to that in shLuc, no-asparaginase, RPMI-only control cells.

Significance was assessed by 2-way ANOVA analysis with Tukey adjustment for multiple comparisons. Error bars represent SEM.

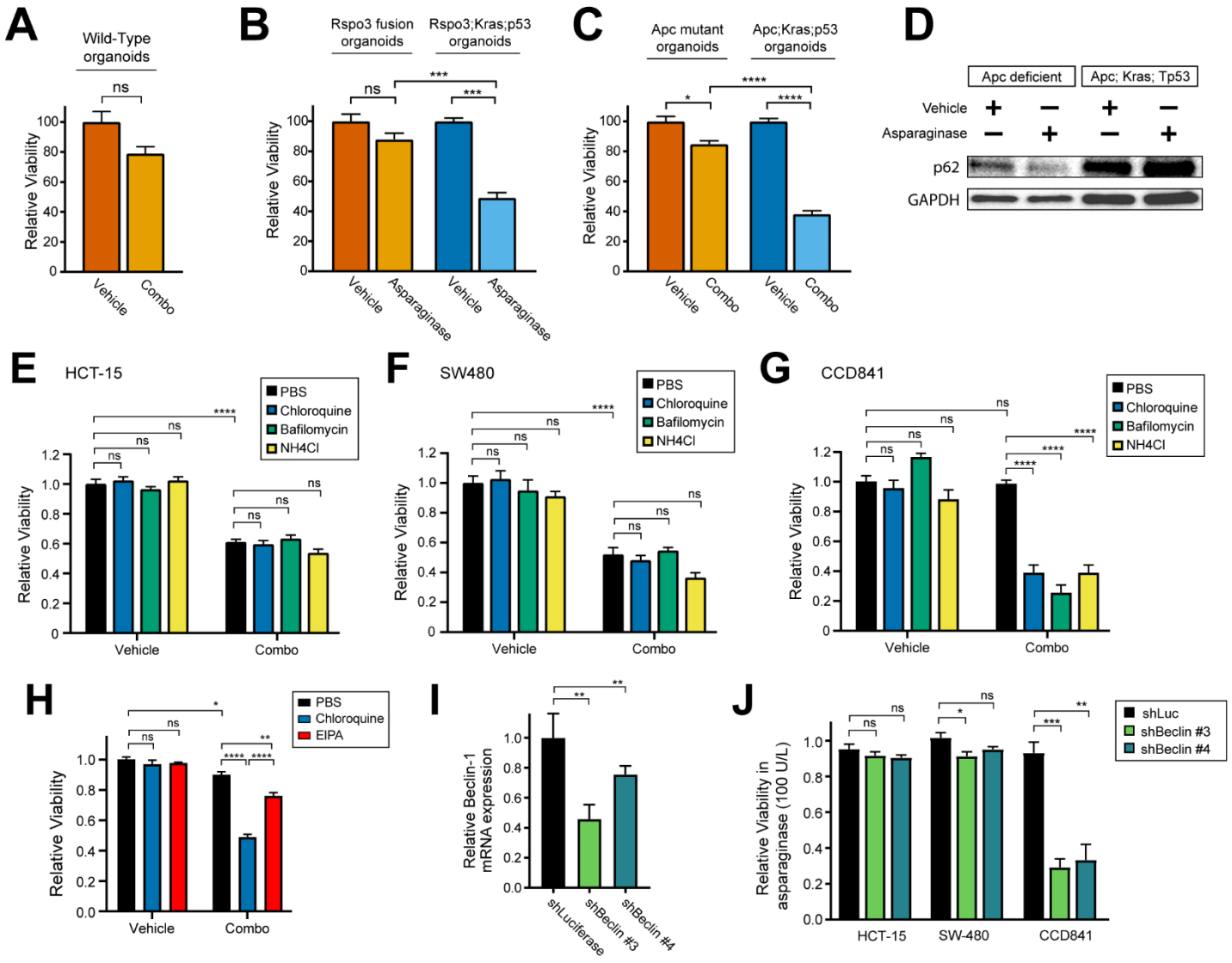
**** $p \leq 0.0001$; * $p \leq 0.05$; n.s., $p > 0.05$.



Supplementary Fig. S7. Expression of asparagine synthetase (ASNS) or glutamine synthetase (GLUL) in cells derived from normal versus malignant intestinal epithelium.

Baseline expression of asparagine synthetase (ASNS) or glutamine synthetase (also known as glutamine-ammonia ligase, GLUL) was assessed in an equal number of the indicated cells by quantitative reverse transcriptase PCR analysis (RT-PCR) analysis. Results shown are Ct values. Error bars represent SEM.

n.s., $p > 0.05$.



Supplementary Figure S8. *Kras* and *p53* mutations sensitize mouse intestinal organoids to the toxicity of asparaginase and GSK3α inhibition.

A Wild-type mouse intestinal organoids lacking *Kras* and *p53* mutations were treated with vehicle (PBS) or combination (combo) therapy using 100 U/L asparaginase and 1 μM BRD0705 for 5 days. Relative Viability was assessed by counting viable organoids, and counts were normalized to the vehicle control. Statistical significance was assessed by a two-sided Welch t-test.

B Mouse intestinal organoids of the indicated genotypes were treated as described in (A). Statistical significance was calculated by a one-way ANOVA with Dunnett's adjustment for multiple comparisons.

C Mouse intestinal organoids of the indicated genotypes were treated as indicated and relative viability was assessed as described in (A). Statistical significance was calculated by a one-way ANOVA with Dunnett's adjustment for multiple comparisons. Note that the 5-day time points used in A-C are earlier than the 10-day timepoints used in Figure 1, based on the idea that sensitization to the toxicity of BRD0705 + asparaginase would be more obvious at earlier timepoints when this combination exhibits less toxicity.

D Mouse intestinal organoids of the indicated genotypes were treated with vehicle or asparaginase 100 U/L for 48 hrs. Western blot analysis for the autophagy substrate P62 were performed. Note the significant increase in P62 levels in Kras/Tp53 mutant organoids, suggesting that these mutations impair autophagy.

E HCT15 cells were treated with vehicle (phosphase-buffered saline, PBS) or combo (100 U/L asparaginase + 1μM BRD0705) therapy in the presence of PBS, 10 μM chloroquine, 100 nM bafilomycin or 20 mM ammonium chloride (NH4Cl). Relative viability was assessed after 5 days of treatment by counting trypan blue viable cells. Statistical significance was calculated by two-way ANOVA with Tukey adjustment for multiple comparisons.

F SW480 cells were treated as indicated, and relative viability was assessed as in (E).

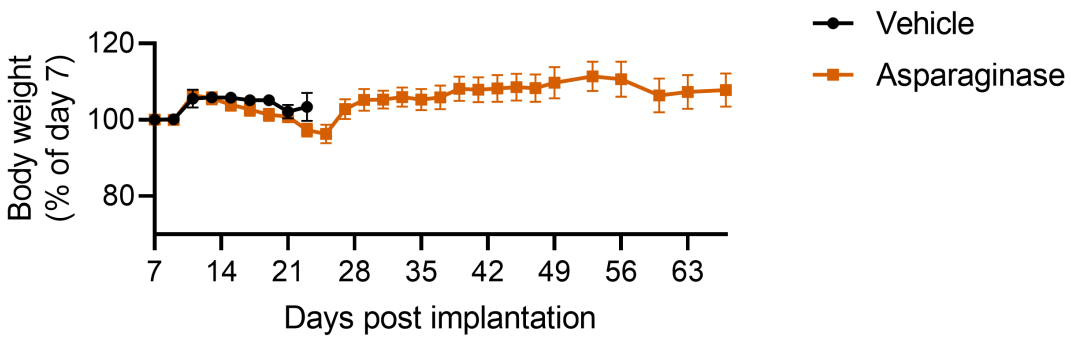
G CCD841 cells were treated as indicated, and relative viability was assessed as in (E).

H CCD841 cells were treated with vehicle or the combination of asparaginase and BRD0705 as in (A), in the presence of PBS, 10 μM chloroquine, or 25 μM of the macropinocytosis inhibitor EIPA. Relative viability was assessed as in (E). Significance was assessed by 2-way ANOVA with Tukey adjustment for multiple comparisons.

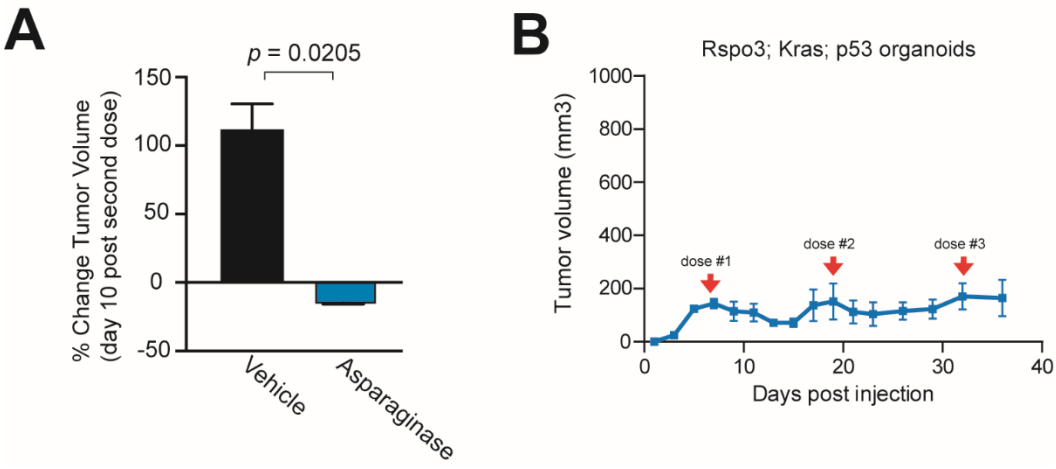
I CCD841 cells were transduced with the indicated shRNAs, and Beclin-1 (BECN1) mRNA expression was assessed by RT-PCR analysis. Significance was assessed by one-way ANOVA with Dunnett adjustment for multiple comparisons.

J The indicated cells were transduced with shRNAs targeting Beclin-1 or control (shLuciferase), treated with vehicle or asparaginase (100 U/L) for 5 days, and relative viability was assessed by counting trypan blue viable cells. For each cell type, results are normalized to those in vehicle-treated, shLuciferase controls. Significance was assessed within each cell line by one-way ANOVA with Dunnett adjustment for multiple comparisons.

**** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s., $p > 0.05$. Error bars denote SEM.



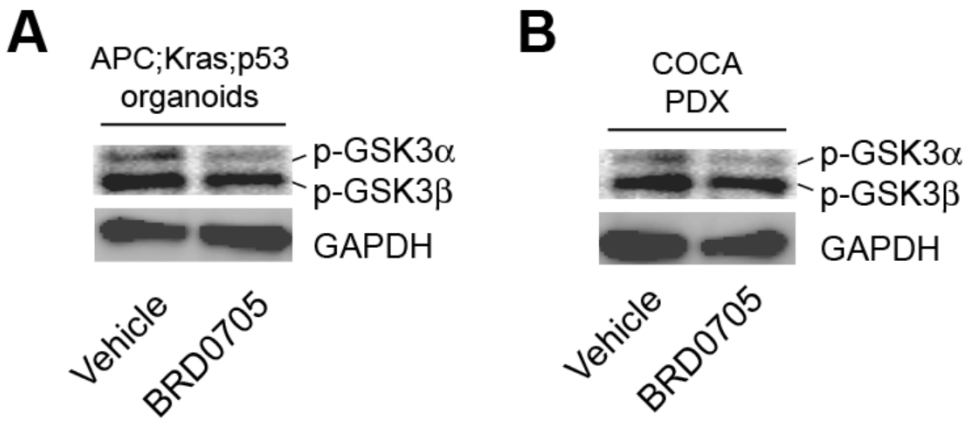
Supplementary Figure 9. Body weights of mice engrafted with mouse intestinal organoids (from Figure 3), with mice grouped based on treatment regimen. Treatment was with a single dose of asparaginase 1,000 U/Kg or vehicle via tail vein injection on day 7. Body weights are shown normalized to those on the day of treatment start.



Supplementary Figure S10. Repeated asparaginase doses can provide sustained disease control in Rspo3 fusion colorectal cancer.

A Mice injected with Rspo3, *Kras, p53* fusion organoids whose tumors progressed following a single dose of asparaginase (from Figure 2) were re-treated with a second dose of PBS or asparaginase at the time of disease progression (n=3 per group). Plots indicate % change in tumor volume post second dose versus measurements before re-treatment. Significance was assessed by a two-sided Welch t-test. Error bars represent SEM.

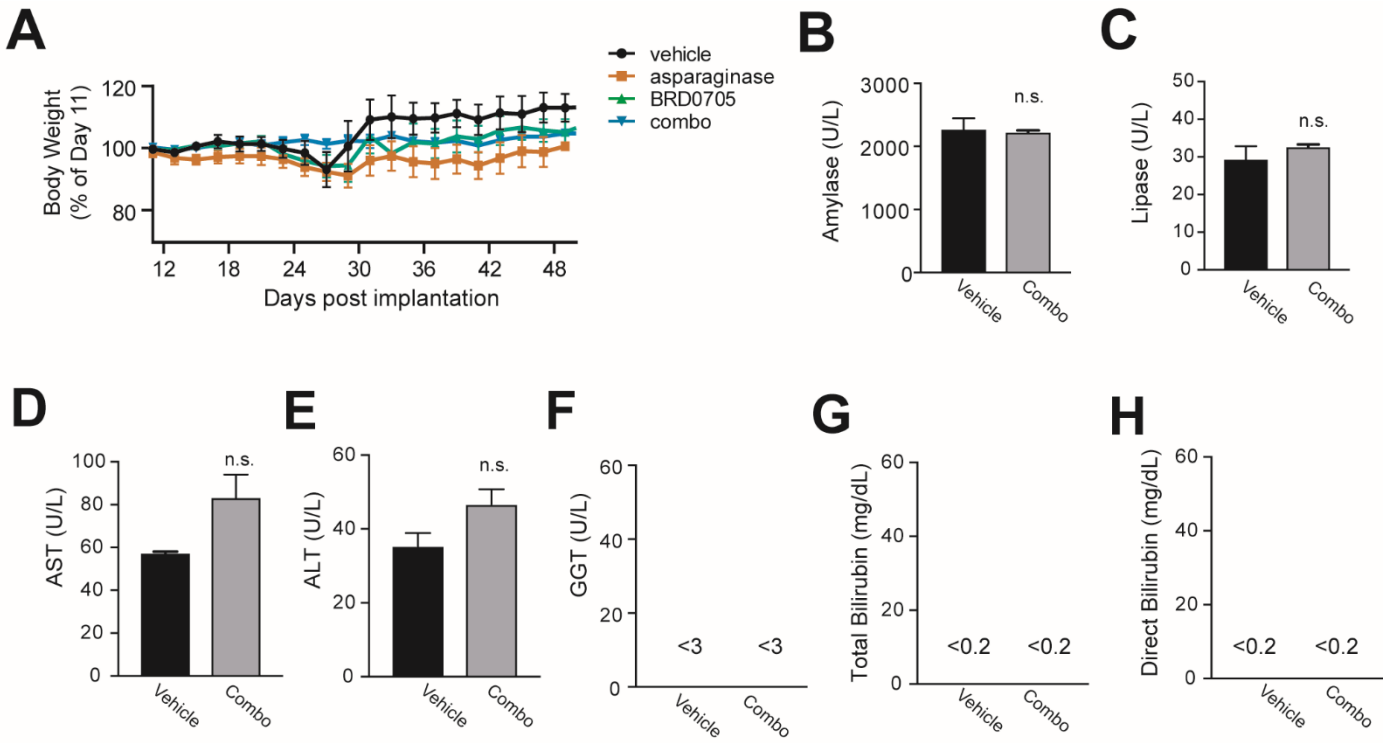
B Mice were injected subcutaneously with Rspo3, *Kras, p53* fusion organoids (n = 5 per group). Beginning at 7 days post injection, mice were dosed with asparaginase (1000 IU/kg) by tail vain injection. Additional asparaginase doses were given every 12 days, and tumor volumes were assessed by caliper measurements. Error bars represent SEM.



Supplementary Figure S11. Effect of BRD0705 on GSK3α and GSK3β activity in mouse and human APC-mutant CRC models, as assessed by GSK3 autophosphorylation.

A, Apc; Kras; Tp53 triple-mutant mouse intestinal organoids were treated in culture with vehicle or 1 μM BRD0705 for 48 hours, and Western blot analysis for GSK3 autophosphorylation was performed.

B, The COCA9 CRC PDX was engrafted into immunodeficient mice. Mice were treated with vehicle or BRD0705 (25 mg/kg by gavage every 12 hours) for 5 days. Tumors were harvested and Western blot analysis was performed.



Supplementary Figure S12. Treatment with asparaginase and the GSK3α inhibitor BRD0705 is well-tolerated and does not induce weight loss or hepatotoxicity in Nude mice.

A Body weights of mice implanted with the human CRC PDX COCA8. Values were normalized to the body weight at the start of treatment (day 12). Error bars represent SEM.

B-H Blood from mice implanted with the human CRC PDX COCA9 and treated with vehicle or the combination of asparaginase and BRD0705 (combo) was collected after 20 days of treatment. Serum levels of indicated parameters were assessed, and differences between groups were calculated using a two-sided Welch t-test. Bars denote the mean, and error bars denote SEM. In panels F-H, all levels were below the lower limit of detection of each assay, which is shown on each plot.
n.s., $p > 0.05$.

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