Supplementary Information

Materials and Methods

Zebrafish lines

Primary MPNSTs were harvested from the *nf1a+/-;nf1b-/-;p53m/m; sox10:mCherry* zebrafish line [1], which was maintained under standard conditions as previously described [2]. As recipient fish embryos for the implantation assay, the *roy* and *nacre* double homozygous mutant line, termed Casper, was used [3]. This line is transparent due to loss of light absorption and reflection as a result of combined loss of both melanocytes and iridophores [3]. All experiments involving zebrafish were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute.

Embryonic implantation assay

The embryonic implantation assay was modified from a previously described method [1]. About 100-120 primary MPNST cells derived from the tumors of *sox10:mCherry*-expressing fish or non-transformed cells harboring the *ubi:GFP* gene were injected into the pericardial cavity of each embryo (Casper line) at 2dpf. Successfully implanted embryos were collected and imaged by fluorescence stereomicroscopy at 24 hr postinjection. The imaged embryos were then distributed into the wells of a 96-well plate, and incubated with either DMSO or drugs for 4 days at 28°C. After drug treatment for 4 days, each embryo was imaged again, and the tumor-positive areas were quantified with ImageJ software and compared with controls to determine drug activity. After 4 days of treatment, the drug-responsive embryos were transferred to fresh fish water and fed with

rotifer each day. The remaining tumor in each treated embryo was measured at 10 and 14 dpf with ImageJ software.

Drug treatment

MPNST-implanted embryos were treated with selumetinib (Selleck, S1008), trametinib (Selleck, S2673), PD-3025901 (Santa Cruz Biotechnology, sc-205427A), sunitinib (LC Laboratories, S-8803), rapamycin (Selleck, S1039), everolimus (LC Laboratories, E-4040), AZD2014 (Selleck, S2783), INK128 (Selleck, S2811), KPT-330 (Karyopharm Therapeutics Inc.), vincristine (Selleck, S1241), cyclophosphamide (Sigma Aldrich, PHR1404), etoposide (EMD Millipore, 341205), veliparib (LC Laboratories, V-4703), talazoparib (MedKoo Biosciences, 204710), topotecan (Sigma Aldrich, T2705), or DMSO (American Type Culture Collection) added to the fish water.

Maximum tolerated dose determination

The MTD for each drug was determined with uninjected 3-day-old Casper embryos. Each drug was serially diluted at a 1:2 ratio in 5 wells of a 24-well plate, and Casper embryos at 3 dpf were exposed to each drug dilution for 4 days. After drug treatment, the embryos were transferred to fresh fish water in a 6-well plate, fed with rotifer every day, and the number of surviving embryos counted at 14 dpf. We defined MTD as the maximum tolerated concentration of drug given over 4 days from 3 to 7 dpf, such that 7 of 9 of the Casper embryos survive for at least 7 days after treatment is stopped.

Cell culture and viability assay

NF1-associated human MPNST cell lines (sNF96.2, ST8814, S462, and 90-8TL) were generous gifts from Dr. Karen Cichowski (Brigham and Women's Hospital, Boston, MA). The sporadic MPNST cell line STS26T was a generous gift from Dr. Nancy Ratner (Cincinnati Children's Hospital, Cincinnati, OH). sNF96.2, ST8814, S462, and 90-8TL cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, 11995) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). STS26T cells were maintained in RPMI 1640 (Gibco, 11875093) medium supplemented with 5 % heat-inactivated fetal bovine serum (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). Cells were routinely tested for Mycoplasma contamination and genotyped by short tandem repeats (STR) marker analysis to verify authenticity by the Dana-Farber Molecular Diagnostic Core Facility. The Cell Titer Glo assay (Promega) was used to assess cell viability upon treatment with irinotecan, topotecan, AZD2014, INK128, rapamycin, or DMSO. Cells were plated at a density of 5000 cells per well in a 96-well plate and incubated with DMSO or increasing concentrations of each drug. Cell viability was measured after a 72-hr exposure to each drug and reported as a percentage of DMSO controls using the SpectraMax M5 Microplate reader and SoftMax Pro software (VWR). Cell growth after treatment with a drug was reported as the fold change from the day 0 measurement. The concentration of inhibitor required for 50% inhibition of cell viability (IC_{50}) was determined with GraphPad Prism 7 (GraphPad Software).

Isobologram analysis

Isobologram analysis was performed based on the results of 72 hr of combination treatment with serial dilutions of irinotecan and AZD2014 or INK128, both alone and in combination. Calcusyn 2.0 software (Biosoft) was used to analyze combined drug effects and produce the isobolograms.

Apoptosis analysis

To assess apoptotic cell death, we distributed 1×10^5 cells in 12-well plates and incubated the cells in cell culture media for 24 hr. The cells were then treated with each drug or drug combination for 48 to 72 hr, harvested with Accutase (Stemcell Technologies, 07920), and subjected to analysis with the FITC Annexin V Apoptosis Detection kit I (BD Biosciences, 556547) according to the manufacturer's instructions for adherent cells. Stained cells were analyzed with a BD LSRFortessa flow cytometer (BD Biosciences, Singapore), with FlowJO 10.5.3 software used for data analysis. For TUNEL assay, we distributed 2×10^5 cells in 6-well plates containing poly-L-lysine-mounted glass slides, and incubated them in cell culture media for 24 hr. The cells were then treated with each drug or drug combination for 24 hr, fixed with 4% paraformaldehyde in PBS, and subjected to TUNEL assay with the ApopTag Plus In Situ Apoptosis Fluorescein Detection kit (EMD Millipore, S7111), according to the manufacturer's recommendations. Imaging was performed with a Leica SP5X laser scanning confocal microscope, with the Leica Application Suite X used to process images.

Western blotting

Protein lysates were prepared in 1X RIPA (Cell Signaling) containing, PhosSTOP phosphatase inhibitor cocktail tablet (Roche) and complete protease inhibitor tablet (Roche). The inhibitors were prepared according to the manufacturer's recommendation. Protein lysates were separated by gel electrophoresis, transferred to nitrocellulose membranes and probed overnight at 4℃ with the following primary antibodies: anti-phospho-4E-BP1(Ser65) (Cell Signaling 9451; 1:1000), anti-4E-BP1 (Cell Signaling 9644; 1:1000), anti-phospho-p70S6K (Thr389) (Cell Signaling 9206;

1:1000), anti-p70S6K (Cell Signaling 2708; 1:1000), anti-γH2AX (Cell Signaling 9718; 1:1000) and anti-beta actin (Cell Signaling 4967; 1:1000). Primary antibody binding was visualized with ImageQuant LAS4000 (GE Healthcare) using anti-mouse-HRP (Cell Signaling 7076; 1:10,000) or anti-rabbit-HRP (Cell Signaling 7074; 1:10,000) secondary antibodies together with SuperSignal West Dura or Femto (Thermo Fisher Scientific) chemiluminescent substrates. Stripping was performed using Restore PLUS Western blot stripping buffer (Thermo Fisher Scientific) according to the manufacturer's protocol.

Protein synthesis assay

Protein synthesis was detected with the Click-iT HPG Alexa Fluor 488 protein synthesis assay kit (Molecular Probes, C10428) according to the manufacturer's protocol. Briefly, cells were plated at a density of 5000 cells per well in a 96-well plate and incubated with DMSO or each drug. After drug treatment for 24 hr, drug-containing medium was changed to fresh L-methionine-free medium containing a 1:1,000 dilution of Click-iT HPG reagent (50 μM, final concentration). Cells were incubated at 37 ℃ for 30 min and then rinsed three times with PBS. All samples were fixed with 4% paraformaldehyde in PBS for 15 min, washed with PBS with 3% (w/v) BSA, and incubated for 15 min in 0.5% Triton X-100 in PBS. For the Click-iT reaction, cells were incubated in the dark at room temperature in Click-iT reaction cocktail. After 30 min, the samples were washed with Click-iT reaction rinse buffer and then with PBS with 3% BSA. For analysis, the areas of the fluorescence signals in the digital images for each experiment were measured with Image J software at the same intensity threshold settings, and the measured areas then divided into the number of DAPI positive cells in the image. The plots for the click chemistry protein synthesis were generated in triplicate experiments.

4E-BP1 knockout with the CRISPR-Cas9 system

Human 4E-BP1 exon1-specific sgRNA target sequence was cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene #48138), and the plasmid construct transiently transfected into ST8814 cells using 4D-Nucleofector™ System (Lonza, Switzerland). At 72 h post-infection, GFP-positive cells were distributed into 96-well plates as a single cell using BD FACSAria II cell sorter (BD Biosciences, San Jose, CA). 4E-BP1 knockout in the single cell clones was confirmed by Sanger sequencing and Western blotting. The 20-bp target sequences of the indicated sgRNAs were as follows: sg4E-BP1 exon 1, GGTGCTGAAGAGCGTGCCGC; sgLuciferase, TCACCAAGGAGCTGGACCAG.

4E-BP1 overexpression using retrovirus

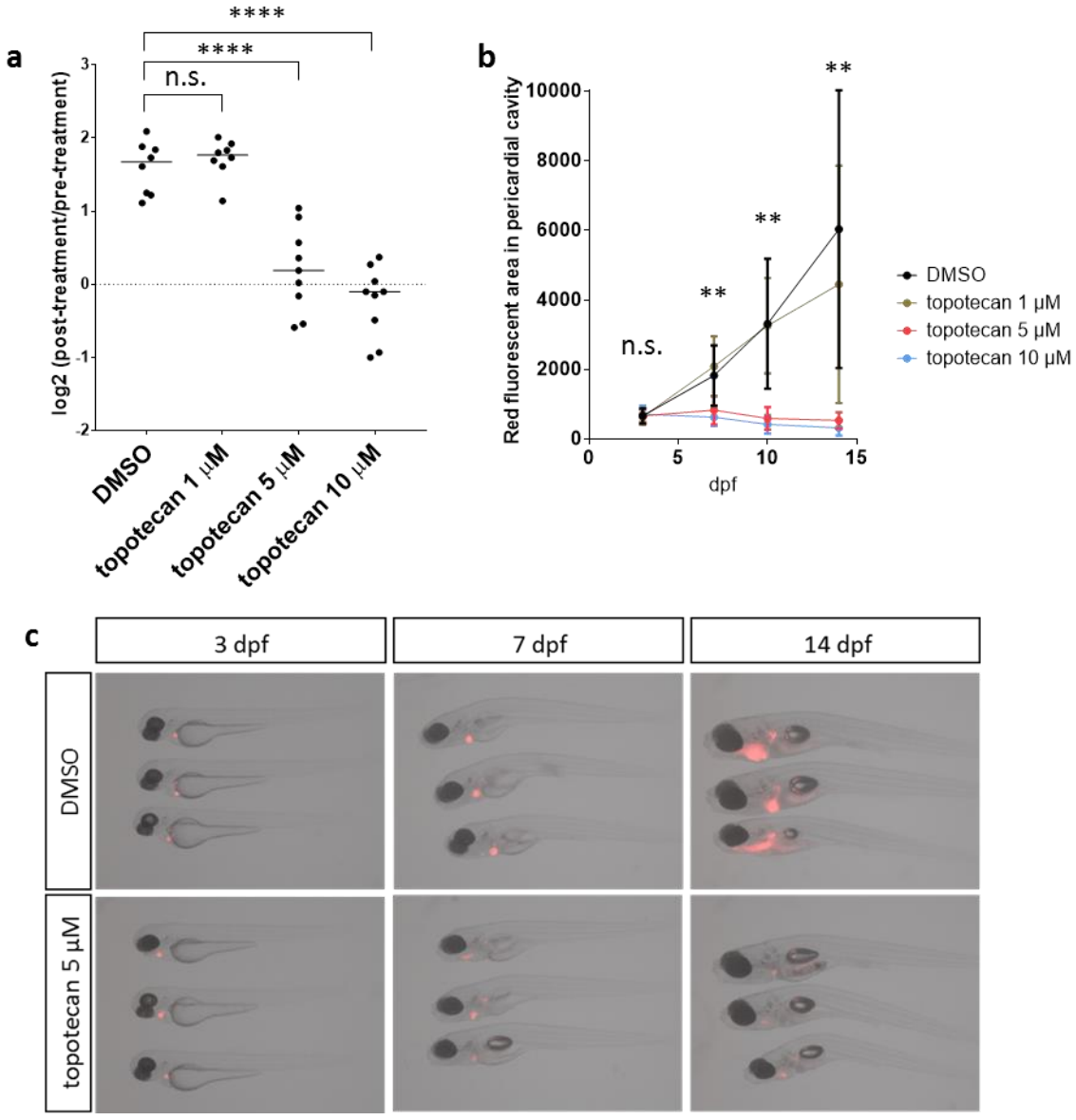
Human 4E-BP1 cDNA was purchased from Integrated DNA Technologies. The 4E-BP1 cDNA was cloned into the MSCV-IRES-GFP retroviral vector to generate MSCV-4E-BP1-IRES-GFP, and was transiently transfected into 293T cells with pkat and VSV-G packaging vectors. ST8814 4E-BP1 knockout cells were infected with the 4E-BP1-IRES-GFP retrovirus for 48 hr. At 72 h postinfection, cells transduced with GFP-carrying retroviruses were sorted using BD FACSAria II cell sorter (BD Biosciences, San Jose, CA) and then used in following experiments.

Statistical analysis

Statistical analysis was performed with Prism 7 software (GraphPad). Exact sample sizes are indicated in each figure legend. A two-tailed unpaired *t*-test was used for the analyses in Figs. 1, 2, 3 and 6. For experiments involving embryonic implantation assay, sample sizes were

determined on the basis of the power to detect a 50% decrease in tumor cells with an agent compared to DMSO, which was 91% when nine embryos are tested per condition, on the assumption that if the mean signal is reduced by 50%, the standard deviation is also reduced by 50%. Thus, at least nine embryos were tested at each condition in experiments reported in this manuscript. After drug treatment, digital images were recorded of the fluorescence signal for every embryo and the cross-sectional areas of the fluorescence signals in the digital images for each experiment were measured quantitatively at the same intensity threshold settings using Image J software.

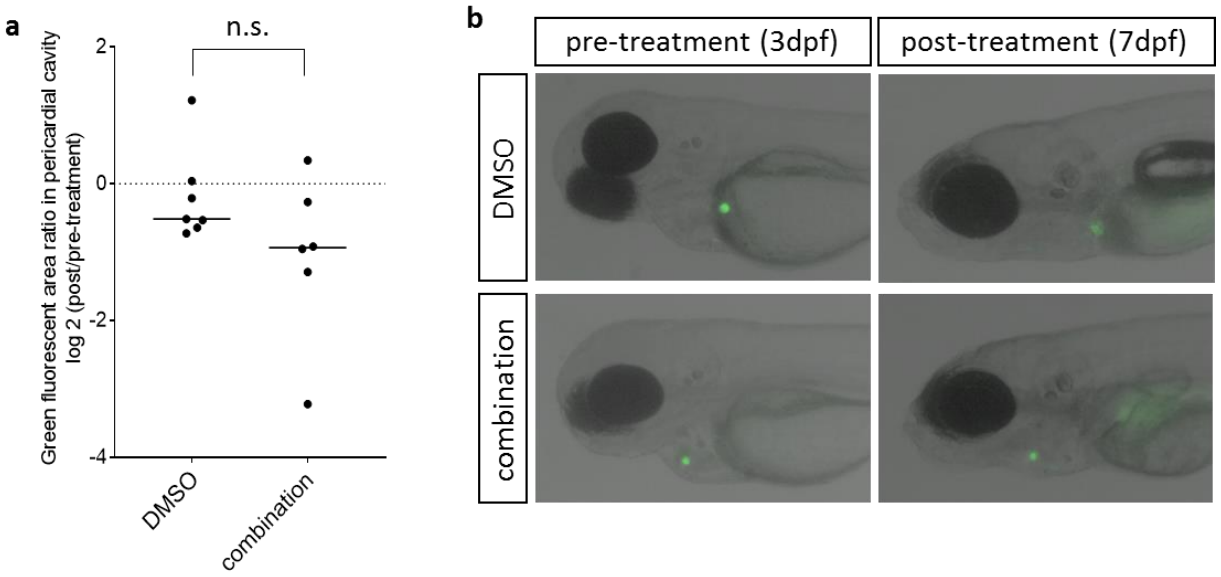
Supplementary Figures



Supplementary Fig. 1. Topotecan shows drug activity against MPNST in an embryonic implantation assay.

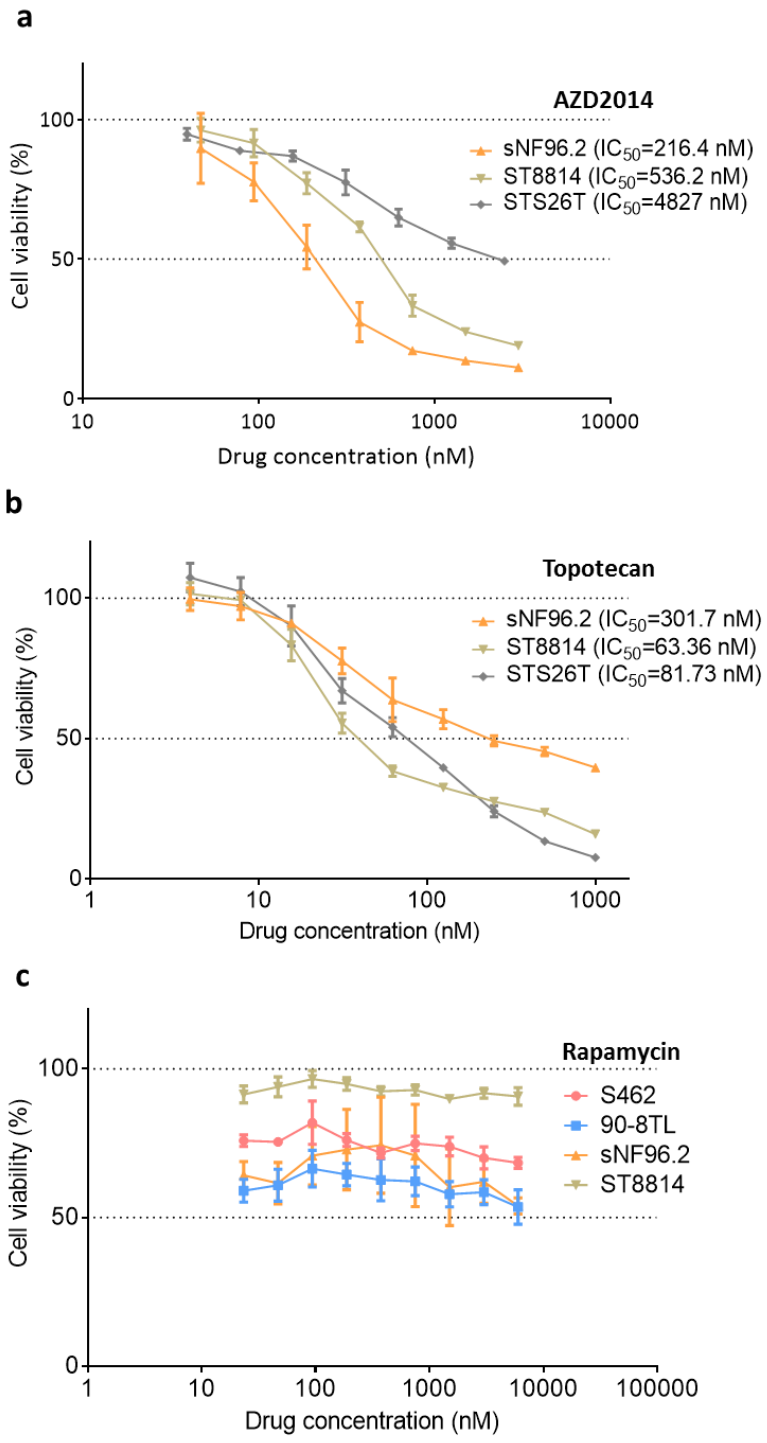
(**a**) MPNST cell growth in the pericardial cavity after 4 days of treatment with 1 µM, 5 µM, or 10 µM of irinotecan or DMSO control. Tumor growth was calculated as a ratio based on the area of mCherry expression in individual embryos at pre- (3 dpf) and post- (7 dpf) treatment times (n=9

embryos per group), and reported as the median and range of individual values. (**b**) Duration of MPNST cell growth inhibition after 4 days of drug treatment. Growth measurements at 3, 7, 10 and 14 dpf were based on red fluorescence areas of the pericardial cavity, and are reported as mean ± SD values. Y-axis represents a cross-sectional red fluorescent area (pixel) of each embryo; **$p<0.01$ for all comparisons in panel B (Student's *t* test); n.s., not significant. (**c**) Representative fish images at 3, 7 and 14 dpf after DMSO control and 5 μM of irinotecan treatment.



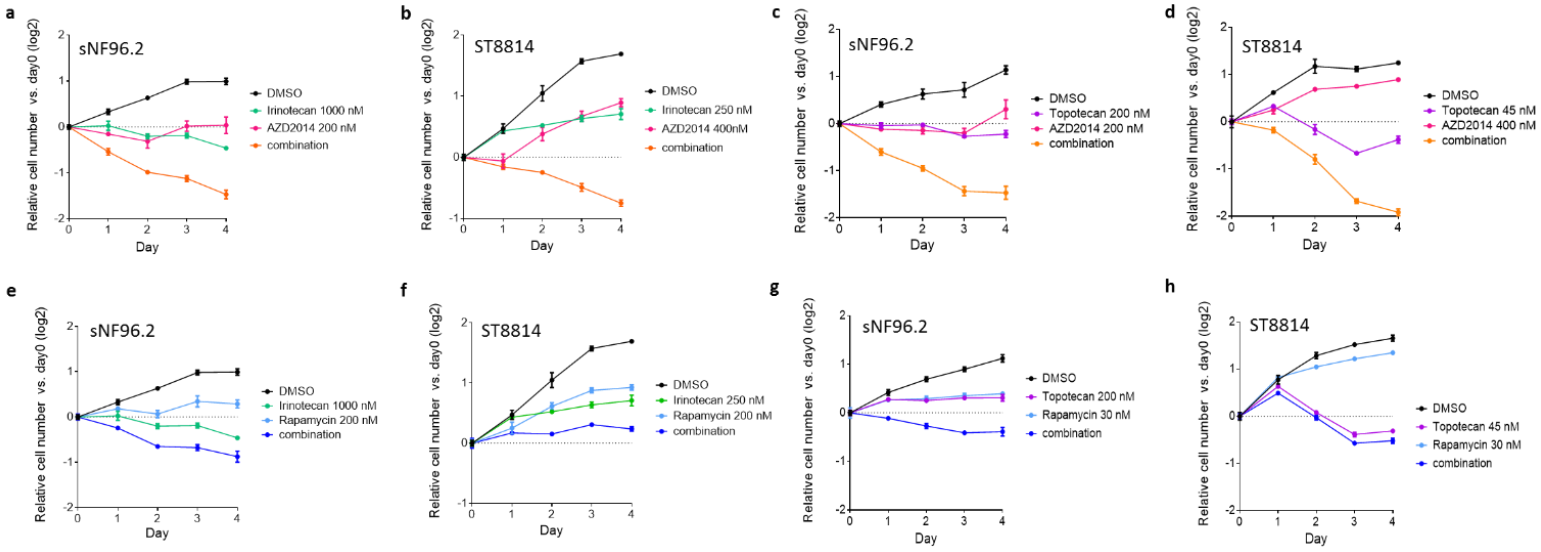
Supplementary Fig. 2. Activity of the irinotecan and AZD2014 combination against nontransformed cells in an embryonic implantation assay.

(**a**) Growth of nontransformed (*ubi*:GFP) cell growth in the pericardial cavity after 4 days of treatment with irinotecan (1.5 μM) combined with AZD2014 (20 μM). Embryos were also treated with a vehicle control. Individual values with medians (black bars) are shown. n.s.= not significant. (**b**) Representative images of embryos after cell implantation and treatment. The embryos in each DMSO or combination panel are identical.



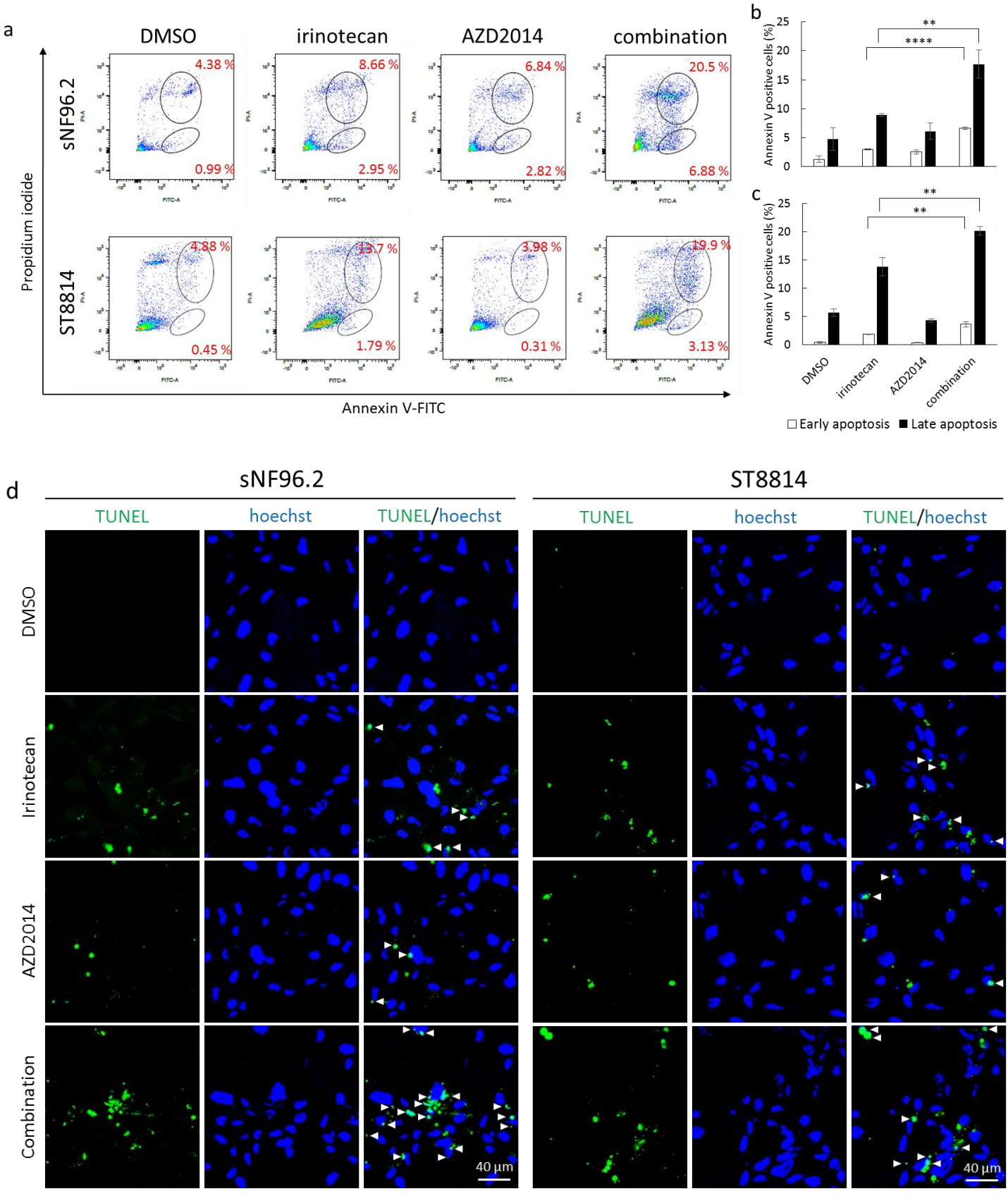
Supplementary Fig. 3. AZD2014 and topotecan are active but rapamycin does not show potent drug effects in human MPNST cell lines.

Single-drug treatment of human MPNST cell lines with AZD2014 (**a**), topotecan (**b**), and rapamycin (**c**) in human MPNST cell lines. sNF96.2, ST8814 and STS26T human MPNST cell lines were cultured with increasing concentrations of either AZD2014 or topotecan for 72 hr. s462, 90-8TL, sNF96.2, and ST8814 cell lines were cultured with increasing concentrations of rapamycin for 72 hr. Cell viability values are mean ± SD percentages of the untreated control value in triplicate experiments. The IC_{50} values of each cell line in this assay are indicated in parentheses. The IC_{50} value of rapamycin in each cell line could not be calculated in the assay.



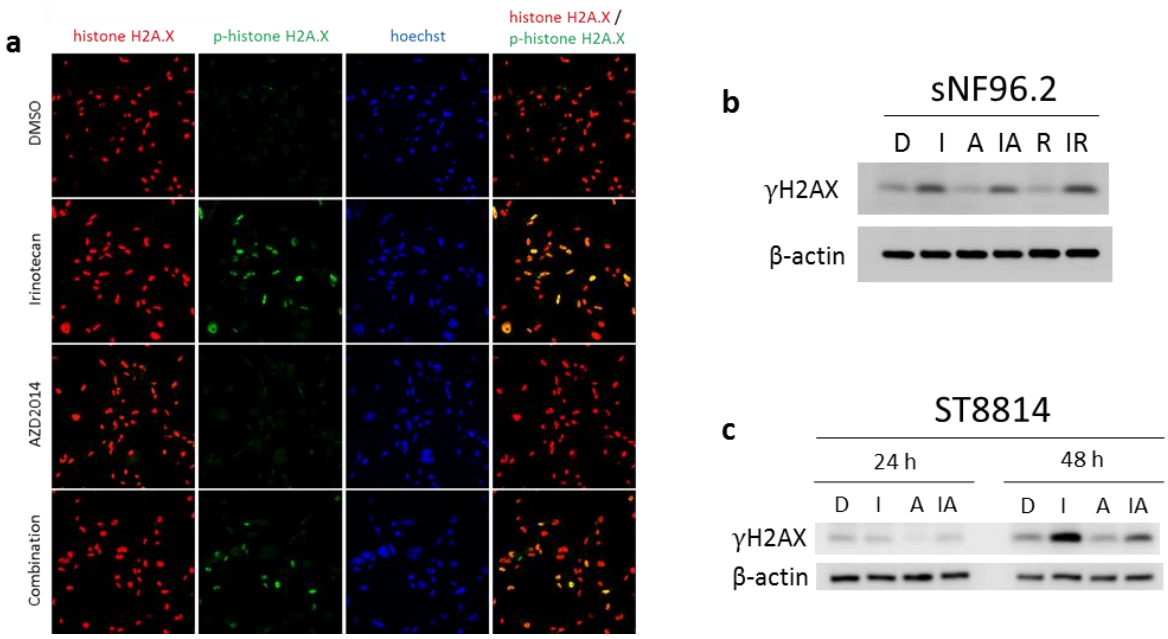
Supplementary Fig. 4. Drug activities of DNA topoisomerase I-targeted drugs are enhanced less with rapamycin than with AZD2014 in human MPNST cell lines.

(**a-h**) sNF96.2 and ST8814 cells were cultured with irinotecan or topotecan, and AZD2014 or rapamycin alone and in combination, and their viability was measured. Used drug doses were labeled on each graph. Values are mean ± SD $\log_2$ fold changes relative to day 0 measurement in triplicate independent biological experiments.

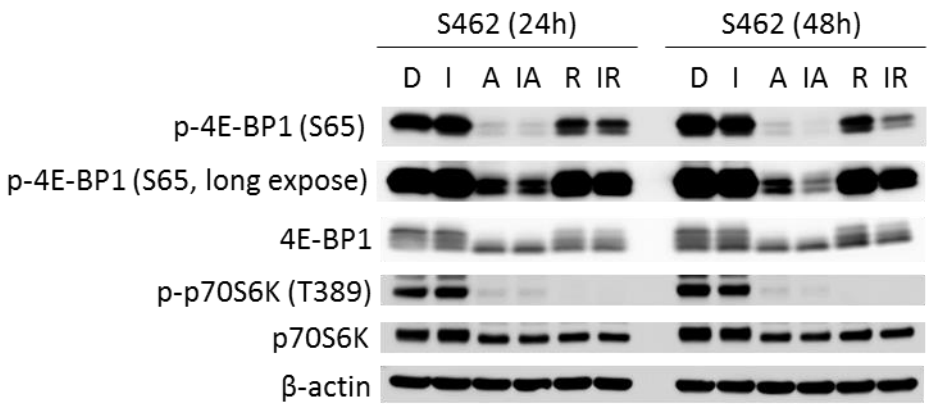

a
DMSO
irinotecan
AZD2014
combination
sNF96.2
ST8814
4.38 %
0.99 %
8.66 %
2.95 %
6.84 %
2.82 %
20.5 %
6.88 %
4.88 %
0.45 %
13.7 %
1.79 %
3.98 %
0.31 %
19.9 %
3.13 %
Propidium iodide
Annexin V-FITC
b
Annexin V positive cells (%)
**
****
c
**
**
DMSO
irinotecan
AZD2014
combination
Early apoptosis
Late apoptosis
d
sNF96.2
ST8814
TUNEL
hoechst
TUNEL/hoechst
DMSO
Irinotecan
AZD2014
Combination
40 µm
40 µm

Supplementary Fig. 5. The combination of irinotecan and AZD2014 induces apoptotic cell death in human MPNST cell lines.

(**a**) Apoptotic sNF96.2 or ST8814 cells detected with Annexin V-FITC/propidium iodide (PI) staining, and analyzed by flow cytometry. sNF96.2 cells were cultured with 1000 nM of irinotecan, 200 nM of AZD2014, or their combination for 48 hr, while ST8814 cells were cultured with 250 nM of irinotecan, 400 nM of AZD2014, or their combinations for 72 hr. Representative flow plots are shown (n=3). (**b, c**) Annexin V-positive cells were counted in triplicate experiments with mean ± SD percentages of positive cells reported in the graphs. The Annexin V-positive and PI-negative cells were defined as early apoptotic cells, and the Annexin V- and PI double-positive cells were defined as late apoptotic cells. **p<0.01, ****p<0.0001 by Student's *t* test. (**d**) Apoptotic sNF96.2 or ST8814 cells detected with TUNEL assay after treatment with irinotecan, AZD2014 or a combination of irinotecan and AZD2014. Cell lines were cultured on a glass coverslip for 24 hr per condition. Blue and green fluorescence reflect Hoechst 33342, and TUNEL staining, respectively. A 40X objective was used for imaging.

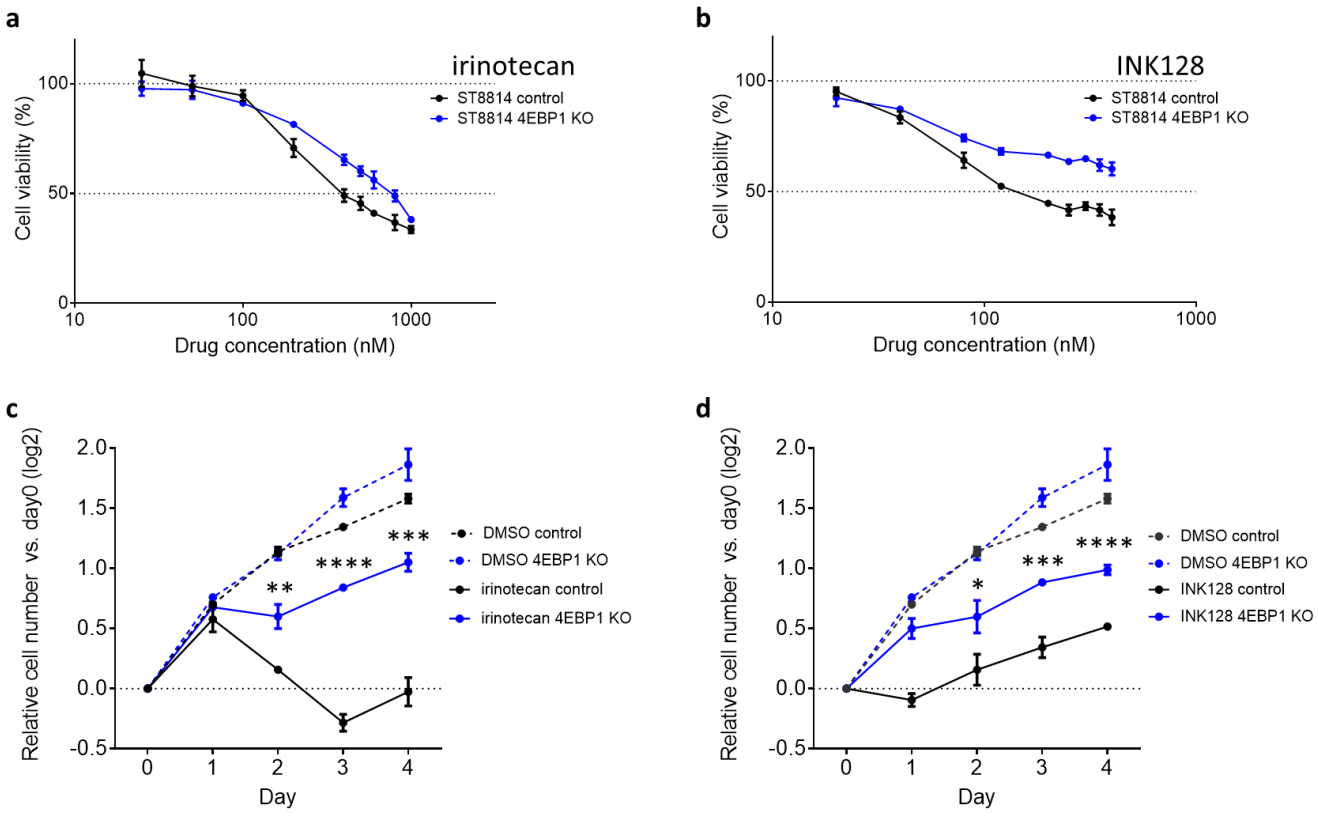


Supplementary Fig. 6. Phosphorylation of the DNA damage-related protein H2AX by irinotecan is not increased with AZD2014 in human MPNST cell lines. Expression of phosphorylated H2AX (γH2AX) was determined by fluorescent immunostaining (**a**) and Western blot analysis (**b, c**). Fluorescence photomicrographs show a typical cell stained with anti-H2AX (red) and γH2AX (green). Nuclei were counterstained with Hoechst 33342 (blue). A representative microscopic field for each treatment is shown. (**a**) sNF96.2 cell was cultured with 1000 nM of irinotecan, 200 nM of AZD2014, or combination. Cell lines were cultured on a grass coverslip in each condition for 24 hr, and immunostaining was performed. The 40X objective was used for imaging. (**b**) sNF96.2 cell was cultured with 1,000 nM of irinotecan (I), 200 nM of AZD2014 (A), a combination of irinotecan and AZD2014 (IA), 200 nM of rapamycin (R), a combination of irinotecan and rapamycin (IR), or DMSO (D). ST8814 cell was cultured with 250 nM of irinotecan, 400 nM of AZD2014, a combination of irinotecan and AZD2014, 200 nM of rapamycin, a combination of irinotecan and rapamycin, or DMSO for 24 hr. (**b**) ST8814 cell was cultured with 250 nM of irinotecan, 400 nM of AZD2014 or combination for 24 and 48 hr.



Supplementary Fig 7. The combination of irinotecan and AZD2014 prevents the phosphorylation of 4E-BP1 in S462 cell line.

S462 cells were incubated with DMSO (D), irinotecan (I), AZD2014 (A), a combination of irinotecan and AZD2014 (IA), rapamycin (R), or a combination of irinotecan and rapamycin (IR) for 24 or 48 hr. Cells were cultured with 200 nM of irinotecan, 500 nM of AZD2014, 200 nM of rapamycin or those combinations.



Supplementary Fig 8. 4E-BP1 knockout decreases sensitivity to irinotecan and INK128.

(**a, b**) Single-drug treatment of 4E-BP1 KO ST8814 cells with irinotecan or INK128. The cells were cultured with increasing concentrations of irinotecan (**a**) or INK128 (**b**) for 72 hr. Cell viability values are mean ± SD percentages of the untreated control value in triplicate experiments. (**c, d**) Control (luciferase targeting CRISPR-transfected ST8814) and 4E-BP1 KO cells were cultured with 300 nM of irinotecan (**c**), 100 nM of INK128 (**d**) or DMSO, and their viability was measured. Values are mean ± SD fold changes relative to the day 0 measurement in three independent experiments. *p<0.05, ** p<0.005, *** p<0.0005 by Student's *t* test (CRISPR control vs. 4E-BP1 KO).

References

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