Supplemental Material For

High-throughput Chemical Screening Identifies Focal Adhesion Kinase and Aurora Kinase B Inhibition as a Synergistic Treatment Combination in Ewing Sarcoma

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SUPPLEMENTAL METHODS

Cell culture

A673, TC32, TC71, EW8, SKNEP, and TTC466 were obtained, verified, and cultured as previously described (1). SKPNDW was purchased from ATCC and was cultured in Dulbeccos' Modified Eagle's Media (Mediatech) with 10% FBS (Sigma-Aldrich). Short-tandem repeat (STR) profiling of SKPNDW matched the ATCC database STR profile for this line. CG-ABPN and CHLA-10 were obtained from the Children's Oncology Group Cell Culture and Xenograft Repository, were cultured in Iscove's Modified Dulbecco's Medium (Invitrogen) with 20% heat-inactivated FBS (Gemini Bio-Products) and supplemented with 10 mg/L insulin, 5.5 mg/L transferrin, and 6.7 µg/L sodium selenite (ITS; Thermo Fisher Scientific) and 4 mmol/L L-glutamine (Thermo Fisher Scientific). We confirmed that CG-ABPN and CHLA-10 express an EWS/FLI rearrangement by RT-PCR (2) and that each have a unique fingerprint when compared to other available Ewing sarcoma cell lines and all cancer cell lines in the Broad Institute's Cancer Cell Line Encyclopedia (CCLE).

Drug synergy analysis

The effects of combination treatments of Ewing sarcoma cells were assessed for synergistic activity using multiple metrics (3). First, models were used to estimate the predicted effect of a combination treatment on viability compared to vehicle control, assuming no synergism. In all models, F_A and F_B represent the fraction of cell viability detected after treatment with drug A or B respectively compared to the viability of

untreated cells and I_A and I_B represent the fractional inhibition of viability compared to untreated cells.

1) The Highest Single Agent (HSA) or Gaddum's Non-interaction model estimates that the combination will be equal to the treatment response of the most potent single agent at the same concentrations such that the expected response (HSA) = max(F_A,F_B) (4).
2) The Bliss Independence model assumes that each agent's activity is independent from the action of the second agent and the expected response (BI) = (I_A + I_B) – (I_A x I_B) (5,6).
3) Loewe Additivity model estimates the effect of combining two drugs based on the concentration of each individual drug that produces the same quantitative effect (7,8).

To assess whether individual treatment combinations were synergistic, additive, or antagonistic, we calculated Excess over BI where the measured inhibition of a specific combination is subtracted from the inhibitor effect predicted by the respective model. In this calculation, positive values are synergistic, negative antagonistic and values close to 0 are additive. We also calculated the combination index (CI) of Lowe Additivity for individual treatment combinations based on the Chou-Talalay Median Effect model such that CI < 0.7 are considered synergistic, CI = 0.7-1.3 are additive and CI > 1.3 are antagonistic (9,10).

To assess the synergistic effects of two compound combinations across a matrix of doses, we used several analytical metrics that were performed using R 3.3.1.

1) Sum of Excess HSA is the sum across the matrix of the differences between the expected responses predicted by the HSA model and the observed responses. The more negative the value the more synergism is observed in the combination (3). In **Figure 1B**, the negative Sum of Excess HSA is plotted so that synergistic interactions have positive numbers for easier visual interpretation of the data.
2) Median Excess is the median of the sum of differences between the observed response and the expected responses predicted by the HSA model. The larger the positive value the more synergism observed by the combination of compounds (3).
3) Beta is obtained by minimizing the Bliss deviation defined by $\sum[F_C - ßF_AF_B]^2$ where F_C is the measured response and F_A and F_B are the single agent responses. The sum runs over all combinations for the pair of compounds. Beta < 1 indicates synergistic activity (4).
4) Gamma is obtained by minimizing the HSA Gaddum deviation defined as $\sum[F_C - γmax(F_A,F_B)]^2$ where F_C is the measured response and F_A and F_B are the single agent responses. The sum runs over all combinations for the pair of compounds. Gamma < 1 indicates synergistic activity (4).

Protein extraction and immunoblotting

Protein was extracted from cell pellets with 1x Cell Lysis Buffer (Cell Signaling) supplemented with EDTA-free protease inhibitors and PhosSTOP phosphatase inhibitors (Roche). Standard western immunoblotting techniques were utilized. Blots were incubated with the following primary antibodies; total FAK (Cell Signaling, #3285),

phospho-FAK (BD Transduction Laboratories, #611722), Aurora kinase B (Cell Signaling, #3094), total Aurora kinase A (Cell Signaling, #3092), phospho-Aurora kinase A (Cell Signaling, #3079), total H3 (Cell Signaling, #3638), phospho-H3 (Cell Signaling, #9701), total PYK2 (Millipore, 06-559), vinculin (Abcam, ab18058), and GAPDH (Santa Cruz, sc-137179). Secondary antibodies conjugated to horseradish peroxidase were added and blots were developed using a chemiluminescent substrate (ThermoFisher) and imaged with a ImageQuant LAS4000 camera (GE Healthcare Lifesciences).

In vivo zebrafish studies

To determine the maximum tolerated dose of the combination of FAK and Aurora kinase B inhibitors in zebrafish, increasing concentrations of PF-562271 and AZD-1152 were added to the water of naturally dechorionated embryos 72 hours post fertilization (hpf). Fish were monitored for an additional 4 days for viability.

Studies to determine the pharmacokinetics and maximum tolerated doses of the combination of FAK and Aurora kinase B inhibitors in zebrafish and mice are described in the **Supplemental Material**. All zebrafish studies were approved by the Dalhousie University Committee on Laboratory Animals and fish were maintained as previously described (11,12). For the zebrafish studies, five million A673 cells were stained with 5 µg/mL Cell Tracker Orange CMTMR Dye (ThermoFisher). Then, naturally dechorionated zebrafish embryos at 48 hours post fertilization were anesthetized with 0.09 mg/mL Tricaine (Sigma-Aldrich) and injected with 50-150 fluorescently labeled cells using a PLI-100A Pico-injector (Harvard Apparatus) (11,13). Embryos were kept at 28ºC for 30

minutes and then at 35ºC for the duration of the experiments. Embryos with a fluorescent cell mass 24 hours post-injection were selected for experiments. For each experimental replicate, 20 embryos were sacrificed immediately to determine the average cell count at the start of therapy as described below. Groups of 30-40 embryos were then treated for 72 hours with 5 µM PF-562271 (Sigma-Aldrich) alone, 6 µM AZD-1152 (Sigma-Aldrich) alone, the combination of 5 µM PF-562271 and 6 µM AZD-1152, or vehicle (DMSO) by addition to the water. Two biological replicate experiments were completed for zebrafish treated with either PF-562271 alone or AZD-1152 alone and four biologic replicates were completed for zebrafish treated with the combination. For each treatment replicate, an equal number of zebrafish were treated simultaneously with vehicle. To determine cell counts, embryos were euthanized with Tricaine (1 mg/ml) and dissociated in 100 mg/mL collagenase (Sigma-Aldrich) for 30 min. The suspension was centrifuged for 5 min at 300g and the supernatant was removed. Cells were resuspended in 10 µL of PBS with 5% FBS for imaging and imaged using an inverted Axio Observer Z1 microscope (Carl Zeiss). Fluorescent cells were counted using ImageJ software (NIH).

In vivo mouse pharmacokinetic, pharmacodynamic and tolerability studies

To determine the effects on pharmacokinetics of combining PF-562271 or VS-4718 and AZD-1152, studies were performed with female NCr nude mice (Charles River Laboratories) at the NIH (Bethesda, MD). All experimental procedures were approved by the Animal Care and Use Committee of the NIH Division of Veterinary Resources. Each animal received either 1) a single oral dose of 100 mg/kg PF-562271 via gavage, 2) a single oral dose of 50 mg/kg VS-4718 via gavage, 3) a single intraperitoneal dose of 25

mg/kg ADZ1152 or 4) a single dose of the combination of PF-562271 and AZD-1152 or the combination of VS-4718 and AZD-1152. Five animals were treated per treatment arm. Blood samples were collected in K2EDTA tubes at 0.167, 0.5, 1, 2, 4, 7, and 24 hr after drug administration, and plasma was harvested after centrifugation at 3000 rpm for 10 min. All plasma samples were stored at -80°C until analysis.

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods were developed to determine PF-562271, VS-4718, and ADZ-1152 concentrations in mouse plasma samples. Mass spectrometric analysis was performed on a Waters Xevo TQ-S triple quadrupole instrument using electrospray ionization in positive mode with the selected reaction monitoring. The separation was performed using an Acquity BEH C18 column (50 x 2.1 mm, 1.7 µ) and a Waters Acquity UPLC system with 0.6 mL/min flow rate. The column temperature was maintained at 60^{o}C. The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. The calibration standards and quality control samples were prepared in the control blank mouse plasma. 10 µL plasma sample was mixed with 200 µL internal standard in acetonitrile to precipitate proteins in a 96-well plate. 1.0 µL supernatant was injected for UPLC-MS/MS analysis. The pharmacokinetic parameters were calculated using the non-compartmental approach (Model 200) of the pharmacokinetic software Phoenix WinNonlin, version 6.2 (Certara, St. Louis, MO).

Studies to determine the tolerability of the combination of PF-562271 and AZD-1152 in mice were performed in CD1 nude mice by Pharmaron and approved by the Animal Care

and Use Committee. Three mice were dosed with vehicle and three with both AZD-1152 and PF-562271 for five days. Mice were monitored with daily weights for five additional days and necropsy was performed after animals were sacrificed. Studies to determine the tolerability of the combination of VS-4718 and AZD-1152 in mice were performed in Ncr nude mice at Dana-Farber Cancer Institute and approved by the Animal Care and Use Committee. Three mice were dosed with the combination of AZD-1152 and VS-4718 for five days. Mice were monitored with daily weights for nine additional days and necropsy was performed after animals were sacrificed.

Summary of reagents and chemicals

Reagent or Chemical	**Manufacturer**
MIPE 4.0 compound library	Supplemental Table S1
PF-562271	SynKinses, Selleck, MedChem Express
MLN-8237	Selleck
AZD-1152	Sigma Aldrich, MedChem Express
VS-4718	Selleck, MedChem Express
GSK-1070916	Selleck
NVP-AEW541	Selleck
Apoptosis Detection Kit-APC	eBioscience
propidium iodide	Invitrogen
BD Cytofix/Cytoperm Kit	BD Biosciences
Cell-TiterGlo	Promega
shRNA constructs	Supplemental Table S2, S3, and S5
CRISPR guides	Supplemental Table S4
X-tremeGene HP	Roche
DNeasy Blood and Tissue Kit	Qiagen
NSG and NCr Nude Mice	Charles River Laboratories
Phycoerythrin (PE) anti-phospho-S6	BD Biosciences, S240
Total FAK	Cell Signaling, #3285

Phospho-FAK	BD Transduction Laboratories, #611722
Aurora kinase B	Cell Signaling, #3094
Total Aurora kinase A	Cell Signaling, #3092
Phospho-Aurora kinase A	Cell Signaling, #3079
Total H3	Cell Signaling, #3638
Phospho-H3	Cell Signaling, #9701
Total PYK2	Millipore, 06-559
Vinculin	Abcam, ab18058
GAPDH	Santa Cruz, sc-137179
Secondary antibodies	ThermoFisher

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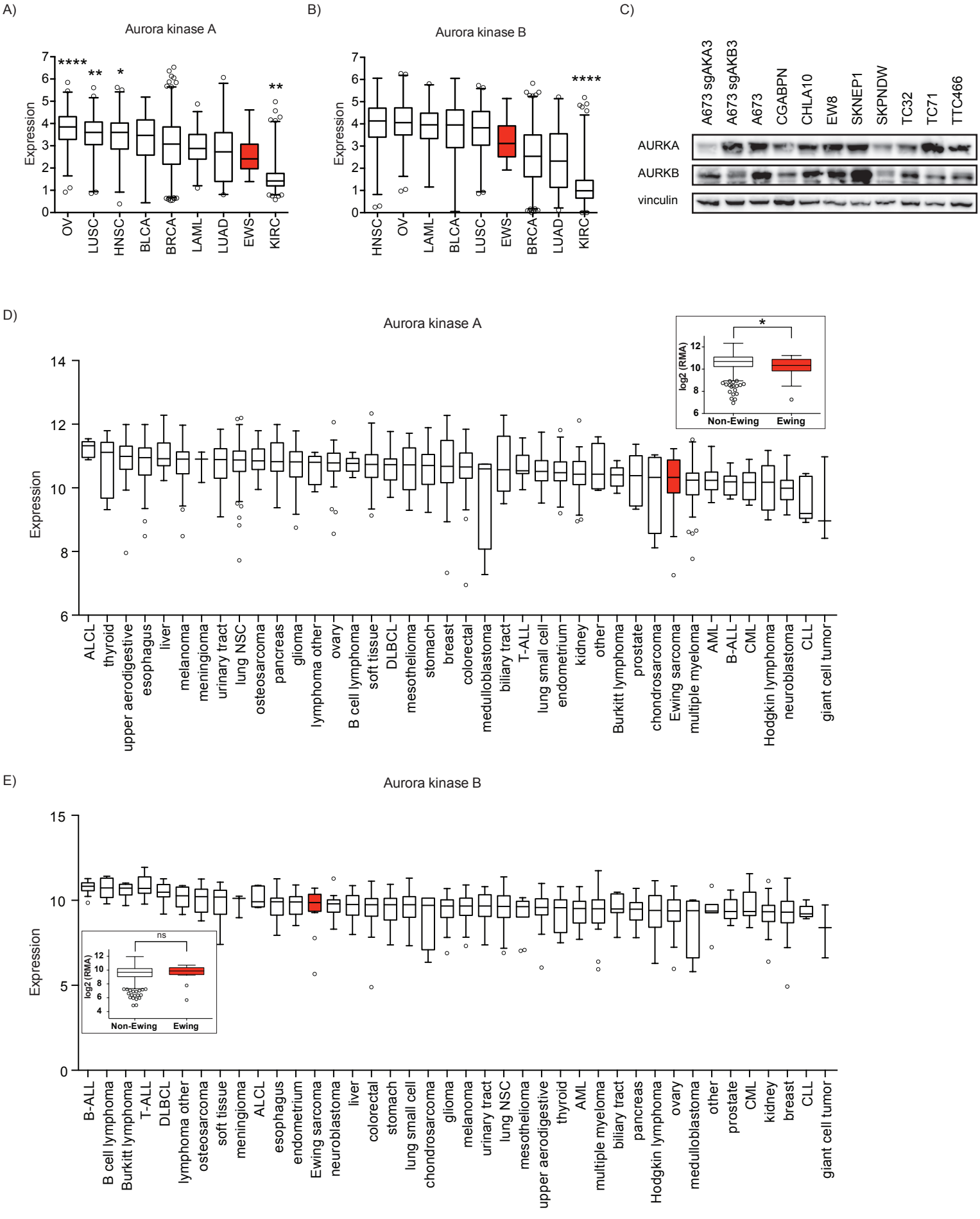
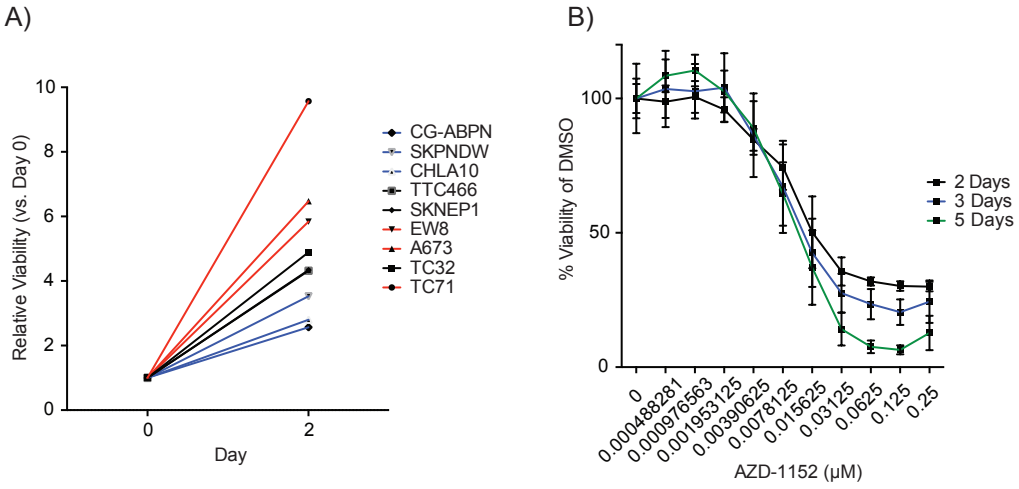
Supplemental Figure S1

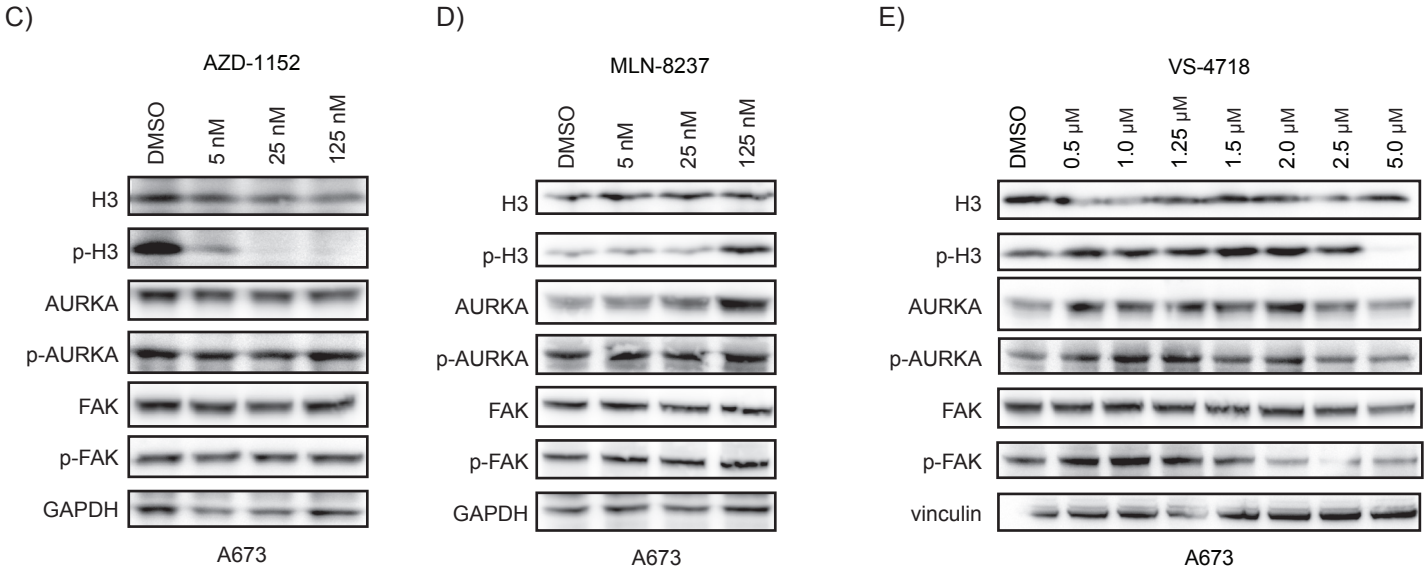


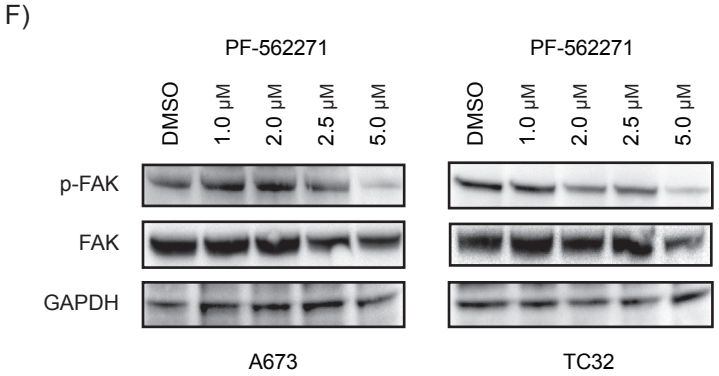
Supplemental Figure S1. Aurora kinase expression in Ewing sarcoma

(A-B) Box plot of tumor expression of **(A)** Aurora kinase A and **(B)** Aurora kinase B was compared to publically available gene expression data of 8 cancer sub-types as defined and abbreviated in TCGA (14,15). All subtypes were compared by one-way ANOVA with Dunn's multiple comparisons test to identify cancer types significantly different to Ewing sarcoma (EWS) (* $P < 0.1$, ** $P < 0.01$, **** $P < 0.0001$). **(C)** Western immunoblot depicting AURKA and AURKB expression levels in Ewing sarcoma cell lines including A673 cells treated with AURKA- or AURKB-targeting CRISPR (sgAKA3 and sgAKB3 respectively) **(D-E)** Box plot of publically available microarray expression data (robust multi-array average; RMA) of cell lines profiled in the CCLE for **(D)** Aurora kinase A and **(E)** Aurora kinase B expression (16). Cell lines were grouped into the indicated cancer subtype. Due to the large number of subtypes, Ewing sarcoma cell line expression was compared to expression in all other lines (boxplot inset) by two-tailed t-test (* $P < 0.1$). For all box plots (A-D), the central box indicates values in the range 25^{th}-75^{th} percentile of all values for that subset of data with the central line indicating the median. Whisker bars extend to the 1^{st} to 99^{th} percentile with points outside that range indicated as empty circles.

Supplemental Figure S2







Supplemental Figure S2. Effects of cell growth on response to AZD-1152 as a function of duration of treatment.

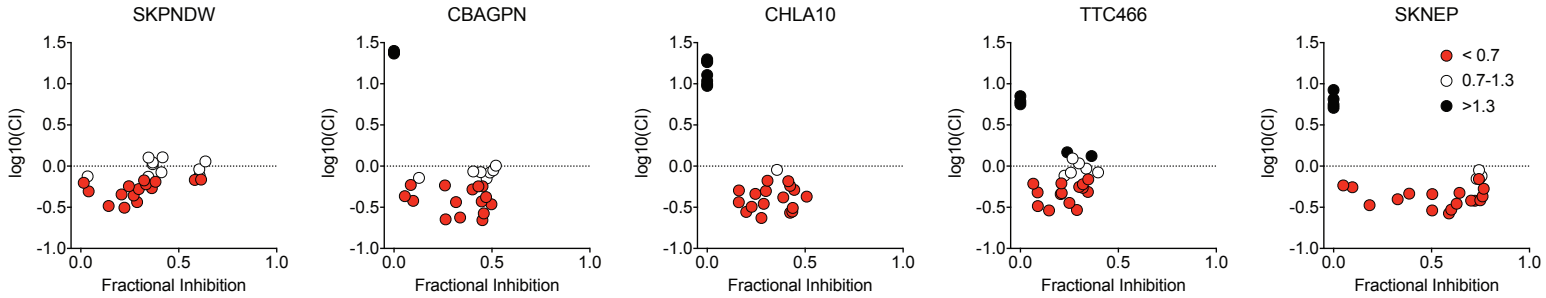
(A) Growth curves for 9 Ewing sarcoma call lines treated with vehicle (DMSO) for two days. Relative viability is normalized to the average day 0 viability. Plotted is the mean normalized viability of four replicates. Cell lines are color coded by their rate of increase of relative viability when treated with vehicle (red are rapidly dividing, black are intermediate, and blue are slowly dividing). **(B)** Effect of additional days of treatment on viability for the TC32 Ewing sarcoma cell line. TC32 cells were treated with AZD-1152 across a range of doses. Viability was normalized to the mean vehicle (DMSO) treated cells. Plotted is the mean normalized viability of four replicates +/- the SD. Cells were treated for the indicated number of days. **(C-F)** Western immunoblots demonstrating total protein and phosphorylation levels in A673 and TC32 cells treated with specified doses of **(C)** an AURKB inhibitor, AZD-1152, **(D)** an AURKA inhibitor, MLN-8237, **(E)** a FAK inhibitor, VS-4718, and **(F)** a FAK inhibitor, PF-562271. Phosphorylated H3 is a well validated marker of AURKB activity and phosphorylation of FAK is an indicator of FAK activity. Studies have shown that changes in AURKA phosphorylation are not correlated with levels of activity or inhibition. Treatment of Ewing cells with AZD-1152 downregulates phosphorylation of H3 across the range of concentrations used in this study but had no effect on FAK or AURKA levels. MLN-8237 did not appear to have off-target activity against FAK or Aurora kinase B as measured by phosphorylated FAK and phosphorylated H3. Treatment with VS-4718 downregulated phosphorylated FAK starting at 1.5 μM but had no effect on AURKA levels and no effect on phosphorylated H3 until cells were dosed with 5 μM, which is above concentrations of VS-4718 used throughout this study. Finally,

treatment with PF-562271 downregulated phosphorylated FAK starting at 2.5 μM and 2 μM for A673 and TC32 cell lines respectively, consistent with previously published results.

Supplemental Figure S3

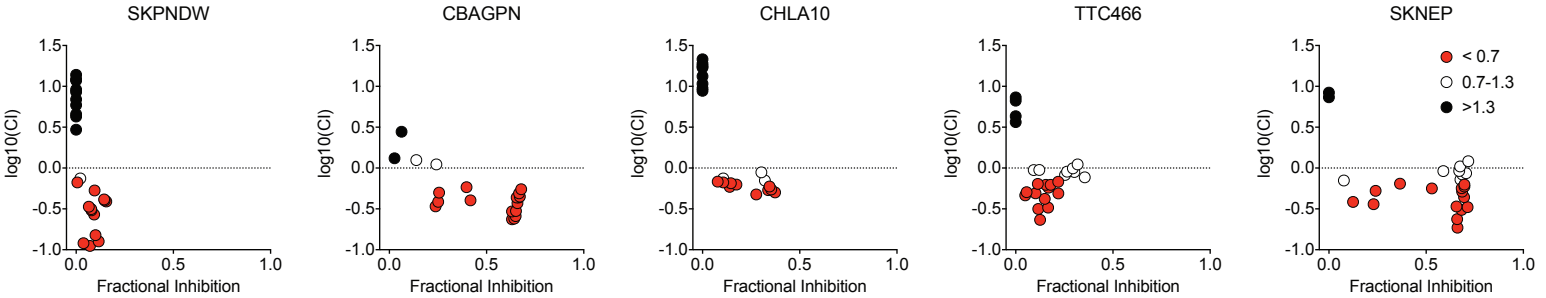
A)

AZD-1152 plus PF-562271



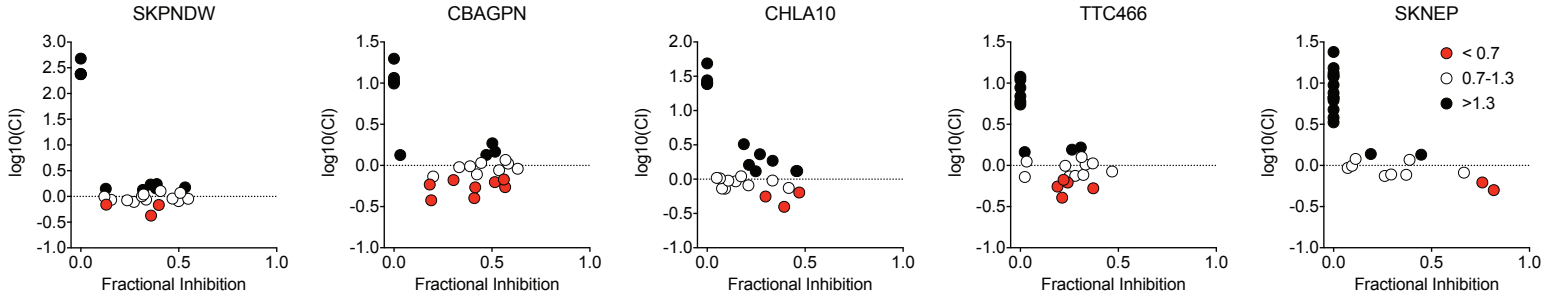
B)

AZD-1152 plus VS-4718



C)

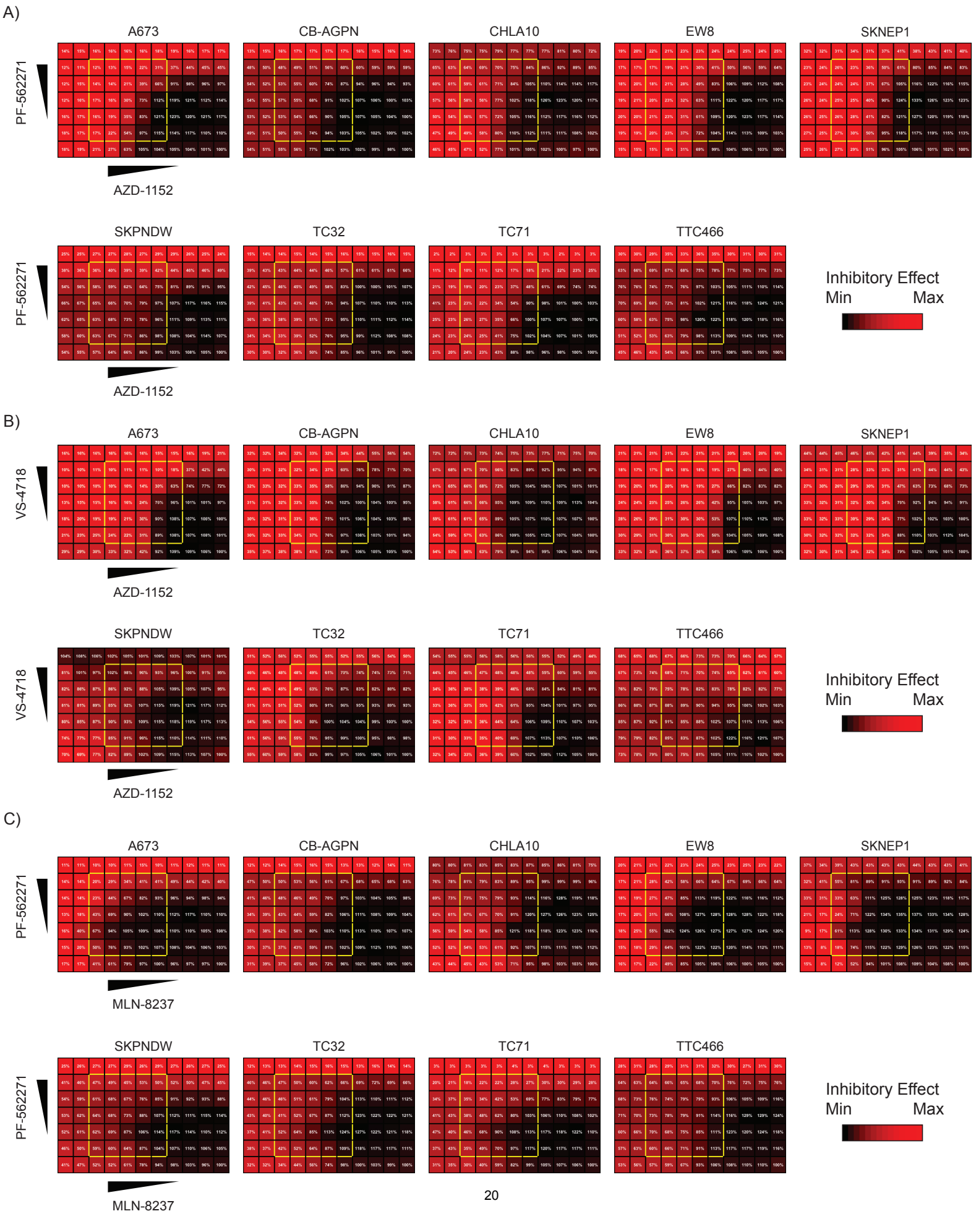
MLN-8237 plus PF-562271



Supplemental Figure S3. Aurora kinase and FAK inhibitor combinations are synergistic in Ewing sarcoma cell lines

Scatter plot of the log10 normalized Combination Index value vs. fractional inhibition of viability for each treatment combinations of **(A)** PF-562271 (FAK inhibitor) and AZD-1152 (Aurora kinase B inhibitor), **(B)** VS-4718 (FAK inhibitor) and AZD-1152, and **(C)** PF-562271 and MLN-8237 (Aurora kinase A inhibitor) for five Ewing sarcoma cell lines with an additional four lines plotted in **Figure 2**. Treatment combinations that are plotted are highlighted in **Supplemental Figure S4** and include the range of concentrations that are active in Ewing sarcoma cell lines. Combinations with a CI <0.7 (indicating synergy) were plotted in red, CI = 0.7-1.3 (indicating additivity) are plotted in white, and CI >1.3 (indicating antagonism) are plotted in black.

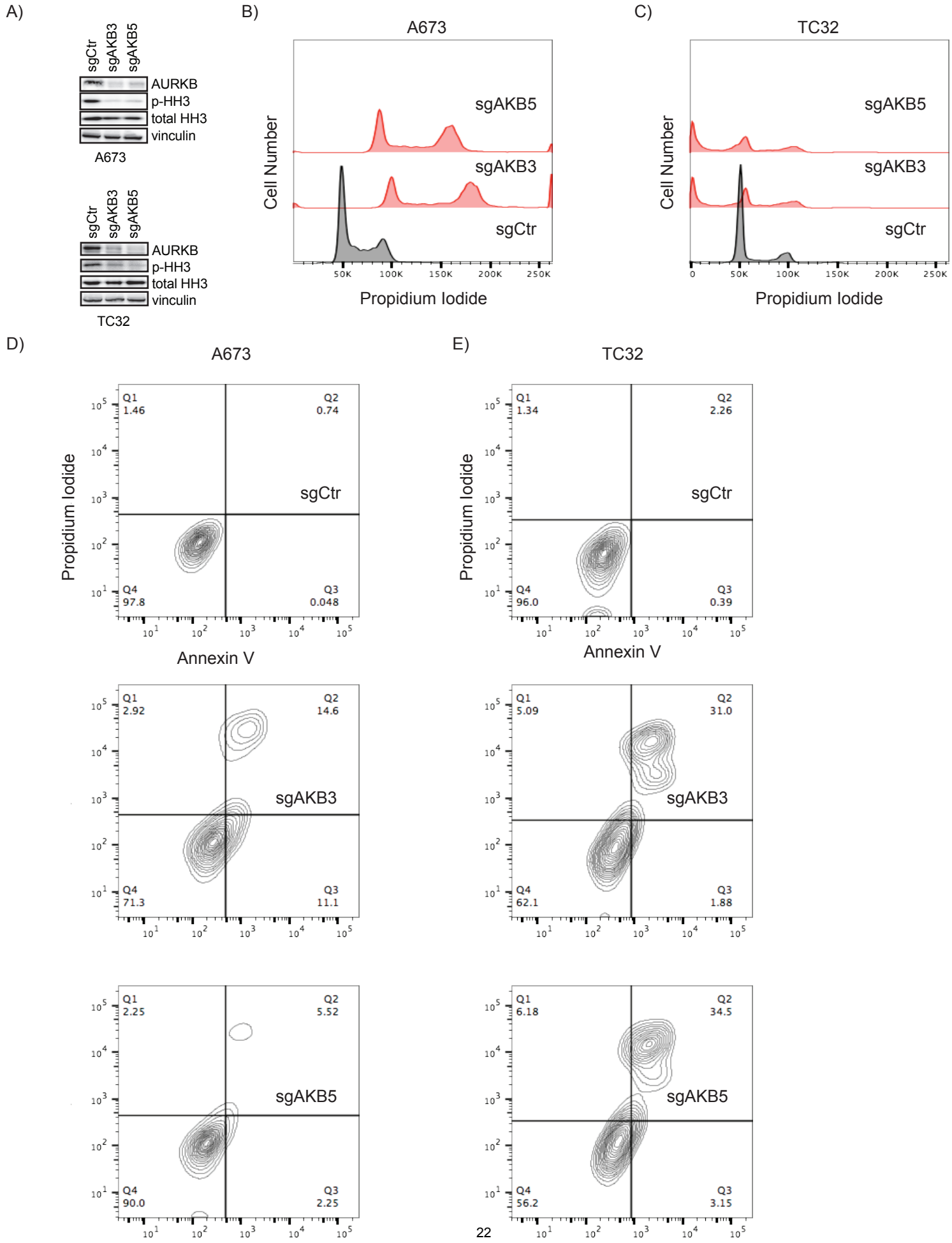
Supplemental Figure S4



Supplemental Figure S4. Response of Ewing cell lines treated with combinations of Aurora kinase and FAK inhibitors

Heatmap depicting percent viability data of the indicated Ewing sarcoma cell lines after treatment with PF-562271 and **(A)** AZD-1152 or **(B)** MLN-8237. Color scheme is the same as in Figure 1C with percent viability relative to vehicle treated cells (normalized within the individual experiment) in white text. Combinations plotted in Figure 2 and Supplemental Figure S3 are outlined in yellow.

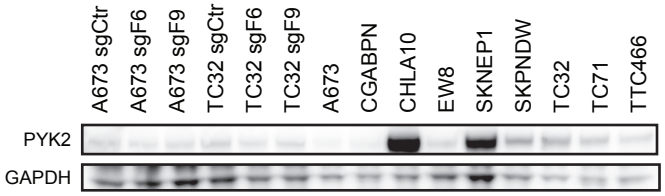
Supplemental Figure S5



Supplemental Figure S5. Cell cycle and apoptotic effects of Aurora kinase B knock out in Ewing sarcoma cell lines

(A) Western immunoblot depicting AURKB, total and phosphorylation of histone H3, and vinculin expression levels in A673 and TC32 Ewing sarcoma cell lines treated with AURKB-targeting CRISPR or non-targeting controls. **(B-C)** Effects of Aurora kinase downregulation on cell cycle in **(B)** A673 and **(C)** TC32 were measured by quantifying cellular DNA content. Plotted are histograms of propidium iodide staining of cells treated with the indicated CRISPR-Cas9 sgRNA. Histograms correspond to data show in Figure 5C-D. **(D-E)** Density plot of Annexin V staining vs. propidium iodide staining in **(D)** A673 and **(E)** TC32 cells treated with the indicated CRISPR-Cas9 sgRNA. Plots are divided into four quadrants gated such that cells treated with sgCtr are considered low Annexin V and propidium iodide staining. The number in the corner of each quadrant indicate the percent of total cells counted in that quadrant. Density plots correspond to data show in **Figure 4D-E**.

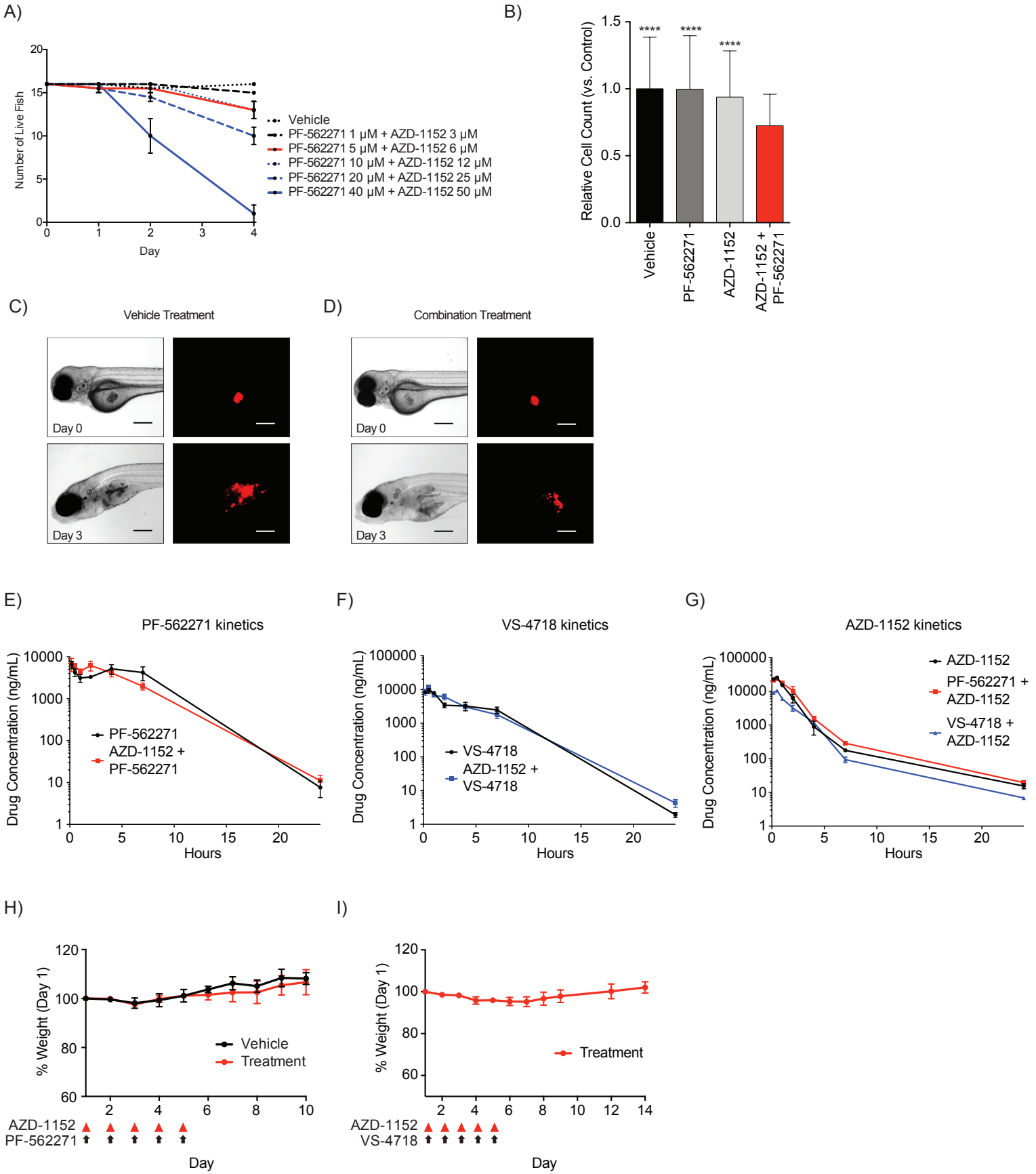
Supplemental Figure S6



Supplemental Figure S6. PYK2 is poorly expressed in Ewing sarcoma cells

Western immunoblot demonstrating poor expression of PYK2 in the majority of Ewing sarcoma cell lines. There is no effect on PYK2 expression levels in A673 and TC32 cells treated with FAK-targeting CRISPR guides (sgF6 and sgF9) compared to control (sgCtr).

Supplemental Figure S7



Supplemental Figure S7. Zebrafish and Murine studies

(A) Zebrafish embryo survival after treatment of fish by addition of the indicated treatment combination to the water. Plotted is the average of two replicate studies +/- SEM. **(B)** Mean +/- SD of relative number of A673 cells that were harvested from zebrafish xenografts after three days of the indicated treatment compared to vehicle-treated animals. Vehicle and single-agent treatment conditions are compared to combination treatment by one-way ANOVA with Bonferroni's multiple comparisons test (**** $P < 0.0001$). **(C-D)** Representative images of zebrafish xenografted with A673 cells and treated with either **(C)** vehicle or **(D)** the combination of 5 μM of PF-562271 and 6 μM of AZD-1152. On the left of each panel are brightfield images and right of each panel are fluorescent images of zebrafish embryos injected prior to treatment (Day 0) and at the study endpoint (Day 3). Scale bar = 200 microns. **(E)** PF-562271, **(F)** VS-4718, and **(G)** AZD-1152 plasma levels in NCr mice treated with single doses of PF-562271, VS-4718, or AZD-1152 and the combination of either PF-562271 pus AZD-1152 or VS-4718 plus AZD-1152. Shown are the mean compound levels +/- SEM for five animals treated in each arm. **(H-I)** Weight of mice over time treated with five days of **(H)** PF-562271 in combination with AZD-1152 or **(I)** VS-4718 plus AZD-1152. Shown is the average of weight of 3 animals per arm +/- SEM.