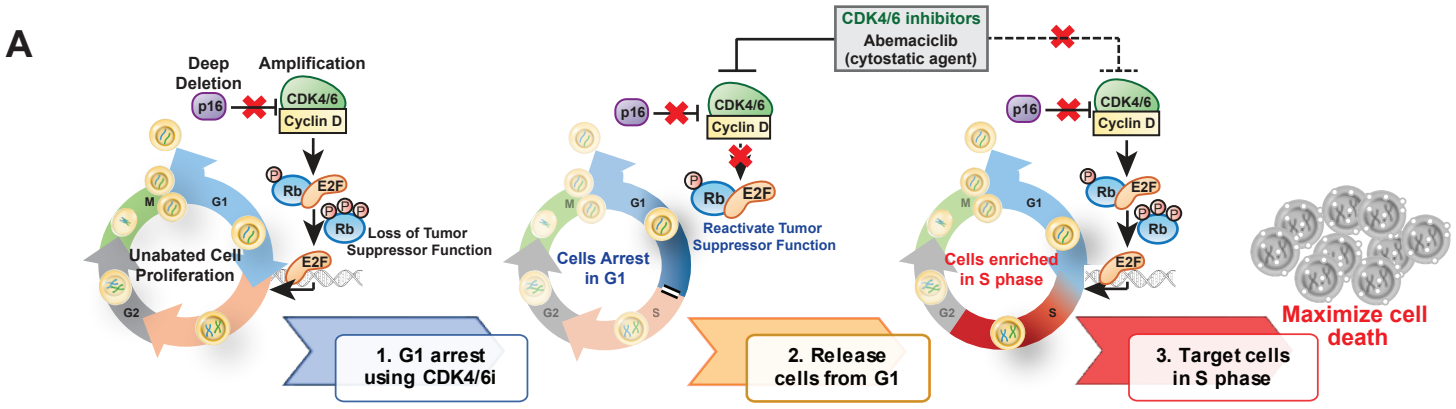
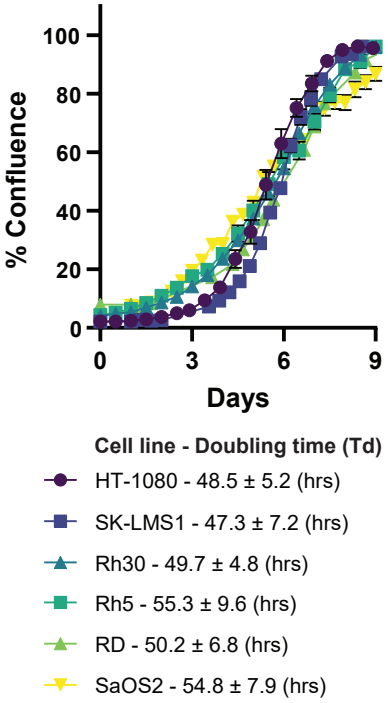
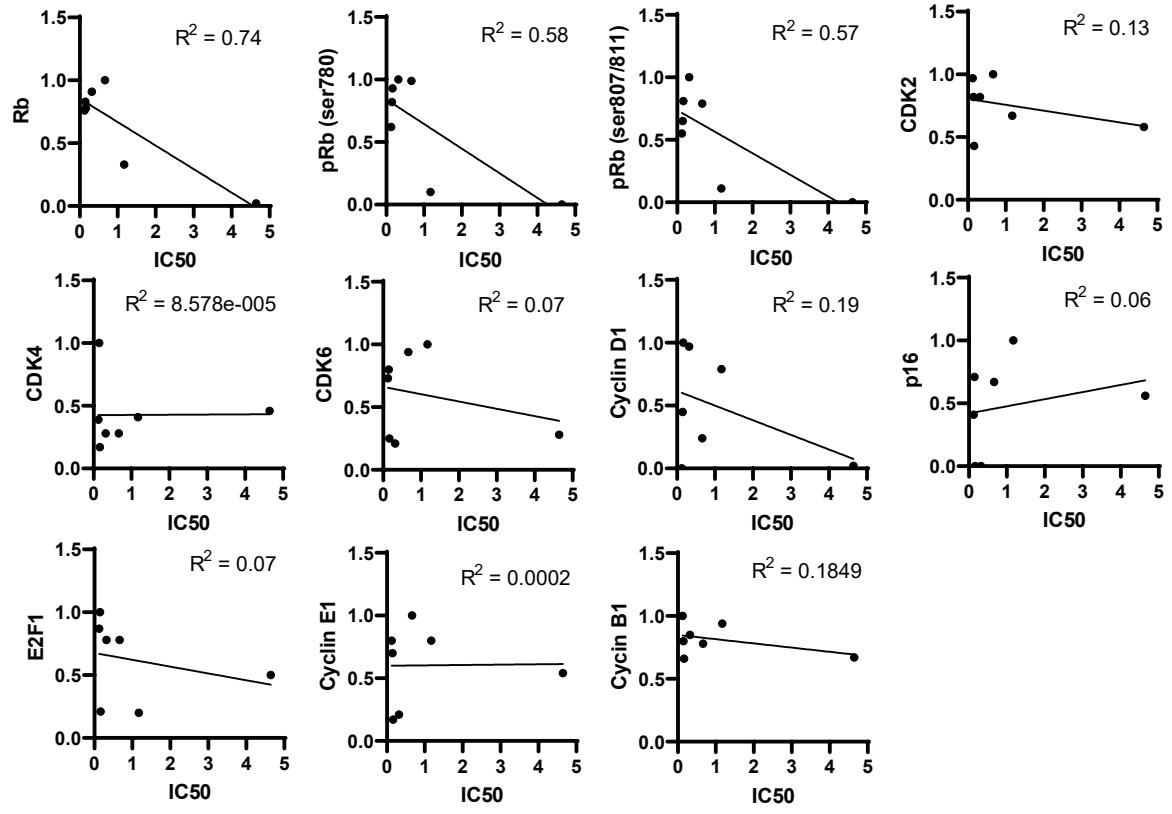
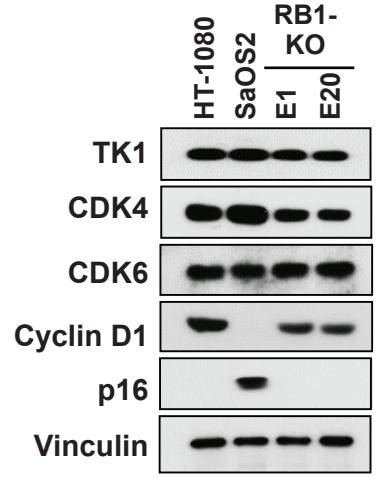
Figure S1 (related to figure 2): Aberration in the p53 and Rb pathways in sarcoma. (A) Schematic diagram illustrating the two-step sequential combination strategy in which CDK4/6i, abemaciclib, is used in the first-step to arrest Rb-positive tumor cells in the G1 phase of the cell cycle. Upon G1 arrest, abemaciclib is removed and cells are allowed to synchronously enter S phase, at which point they are treated with S phase targeting agents in the second-step. **(B)** Proliferation rates of sarcoma cell lines under optimized conditions to achieve similar doubling times, as detected by the IncuCyte Zoom Live Cell Analysis System (n=8/cell line). **(C)** Relative protein expression levels of Rb, CDK2, CDK4, CDK6, E2F1, Cyclin D1, p16, Cyclin E1, and Cyclin B1 were plotted against abemaciclib IC50 concentrations for all cell lines used. Linear regression and Pearson correlation analyses between protein expression and abemaciclib sensitivity were performed using GraphPad Prism 9. All P values were two-tailed with a 95% confidence interval. **(D)** Western blot analysis of the indicated proteins in HT-1080 where Rb is knocked out using CRISPR/Cas9 and sgRNA target exons 1 and 20 in the RB1-encoding gene. **(E)** Flow cytometry histograms showing no change in cell cycle distribution following RB1 knockout in 2 independent clones (E1 and E20 sgRNA) as compared to parental cells. **(F)** Western blot analysis of the indicated proteins in HT-1080 cells in which TP53 is knocked out using CRISPR/Cas9 and sgRNA target exon 9 in TP53 encoding gene. **(G)** Flow cytometry histograms showing similar changes in cell cycle distribution following knockout of TP53. Experiments are representative of three independent biological replicates.

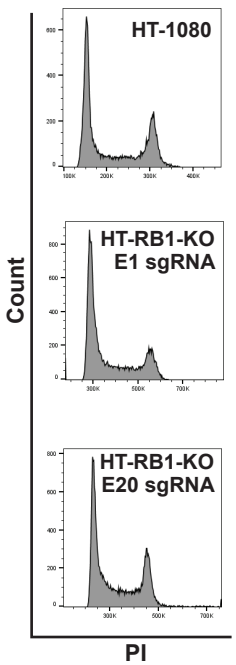
Figure S1-related to Figure 2

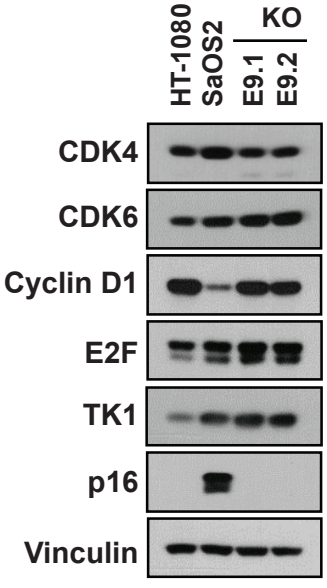












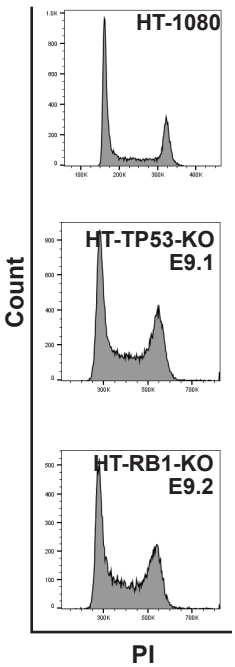


Figure S2 (related to figure 3). Abemaciclib induces a reversible cell-cycle arrest in sarcoma cell lines with intact Rb. (A) Cell cycle distribution of Rb(+ve) and Rb(-ve) cell lines treated with the indicated concentrations of abemaciclib for 6 days (n = 3) showing the dose dependent G1-arrest in Rb (+ve**)** but not Rb (-ve) cell lines**. (B)** Kinetics of cell-cycle progression post-removal of abemaciclib treatments (n = 3). Cells were seeded and treated with abemaciclib for 6 days at IC25 doses; fresh drug-free media was added for the indicated wash out time intervals. The percentage change in the proportion of cells in each phase of the cell cycle was normalized to cells without drug removal. **(C)** Representative cell cycle histograms at 0hr and the maximum recovery time point indicated by * in panels A and B. **(D)** Western blot analysis showing TK1 protein expression at basal level across sarcoma cell lines. **(E)** and **(F)** Western blot analysis of the indicated proteins using cell lysates from the same cells as those in panels **(B)** and **(C)** collected during abemaciclib recovery/washout time intervals. Experiments are representative of three independent biological replicates.

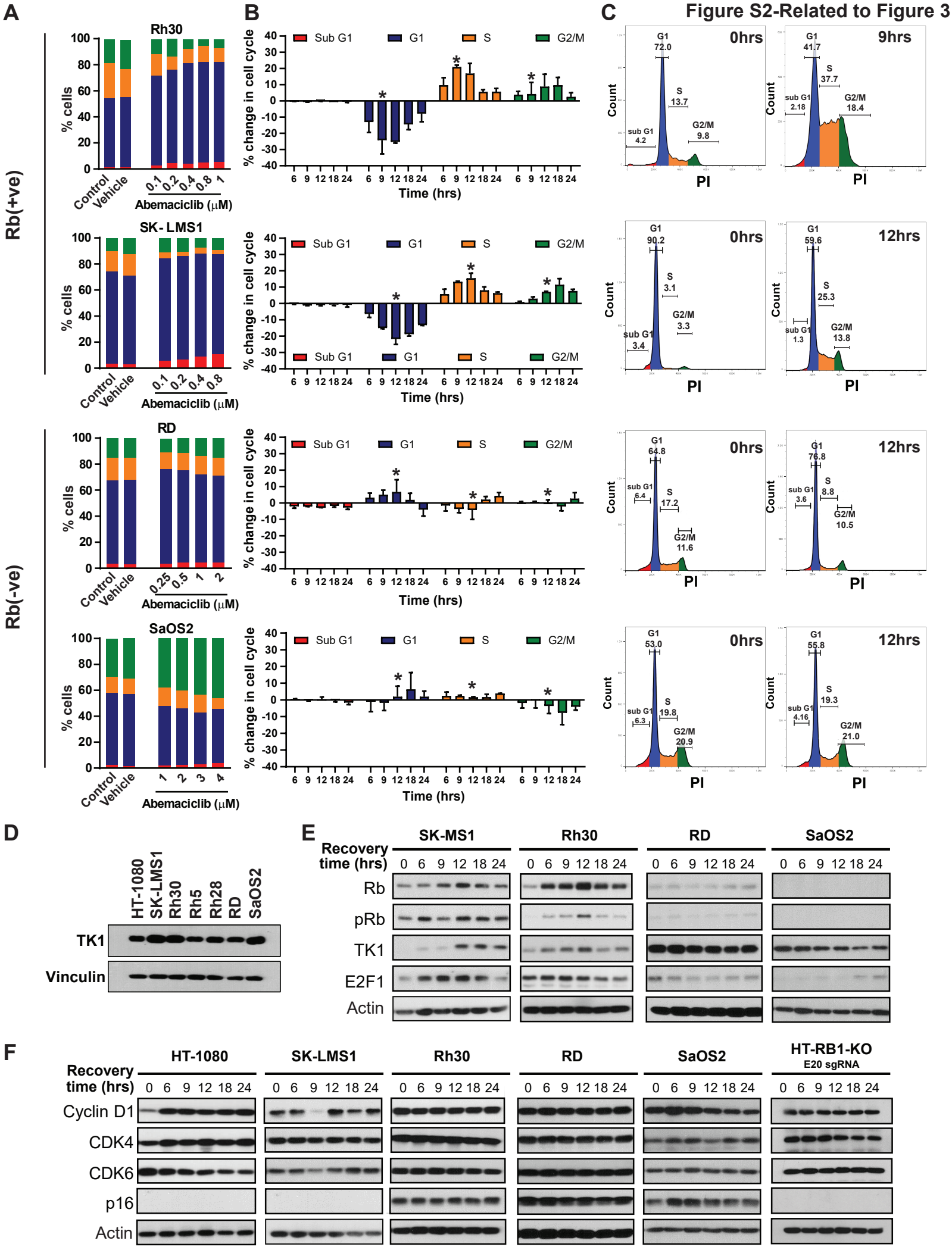


Figure S2-Related to Figure 3
A
B
C
Rb(+ve)
Rb(-ve)
Rh30
SK- LMS1
RD
SaOS2
% cells
Control
Vehicle
Abemaciclib (μM)
Sub G1
G1
S
G2/M
% change in cell cycle
Time (hrs)
Count
PI
0hrs
9hrs
12hrs
G1 72.0
S 13.7
G2/M 9.8
sub G1 4.2
G1 41.7
S 37.7
G2/M 18.4
sub G1 2.18
G1 90.2
S 3.1
G2/M 3.3
sub G1 3.4
G1 59.6
S 25.3
G2/M 13.8
sub G1 1.3
G1 64.8
S 17.2
G2/M 11.6
sub G1 6.4
G1 76.8
S 8.8
G2/M 10.5
sub G1 3.6
G1 53.0
S 19.8
G2/M 20.9
sub G1 6.3
G1 55.8
S 19.3
G2/M 21.0
sub G1 4.16
D
HT-1080
SK-LMS1
Rh30
Rh5
Rh28
RD
SaOS2
TK1
Vinculin
E
SK-MS1
Rh30
RD
SaOS2
Recovery time (hrs)
0 6 9 12 18 24
Rb
pRb
TK1
E2F1
Actin
F
HT-1080
SK-LMS1
Rh30
RD
SaOS2
HT-RB1-KO
E20 sgRNA
Cyclin D1
CDK4
CDK6
p16
Actin

Figure S3 (related to Figure 3). Sequential treatment with abemaciclib, followed by gemcitabine or AZD-1775 significantly decrease cell viability only in Rb-positive sarcoma cells (A) Schematic model depicting the downstream mediators of CDK4/6, following abemaciclib removal: CDK4/6 phosphorylates Rb, releasing the transcription factors E2F1, which can synchronously induce S phase transition, leading to a synergistic cell killing effect when combined with gemcitabine. **(B)** Densitometry depicting the relative protein expression of pRb/Rb, TK1 and E2F normalized to corresponding loading control (actin) at the indicated time point following abemaciclib removal from western blot analysis, as shown in Figure 3G. **(C)** Viability of cells treated with single agents (6 days treatment with abemaciclib or 3 days treatment with gemcitabine alone) versus sequential combination treatment (6 days treatment with abemaciclib followed by 12-18h of recovery followed by 3 days treatment with gemcitabine). At the completion of drug treatment, cultures were continued in drug-free medium (also replaced every other day) until day 12, after which cell confluence was detected by Incucyte. Values were normalized to their no treatment controls. **(D)** Heat map showing p-value comparing cell viability obtained from gemcitabine single treatment with sequential combination of gemcitabine and 0.2 μM abemaciclib. The p-values were obtained from two-way ANOVA statistical analysis using Dunnett's multiple comparison test through Graphpad Prism. **(E)** Viability of cells treated with single agents (6 days treatment with abemaciclib or 3 days treatment with AZD-1775 alone) versus sequential combination treatment (6 days treatment with abemaciclib followed by 12-18h of recovery followed by 3 days treatment with AZD-1775). At the completion of drug treatment, cultures were continued in drug-free medium (also replaced every other day) until day 12, after which cell confluence was detected by Incucyte. Values were normalized to their no treatment controls. **(F)** Heat map showing p-value comparing cell viability

obtained from AZD-1775 single treatment with sequential combination of AZD-1775 and 0.2 μM abemaciclib. The p-values were obtained from two-way ANOVA statistical analysis using Dunnett's multiple comparison test through Graphpad Prism.

Figure S3-related to Figure 3

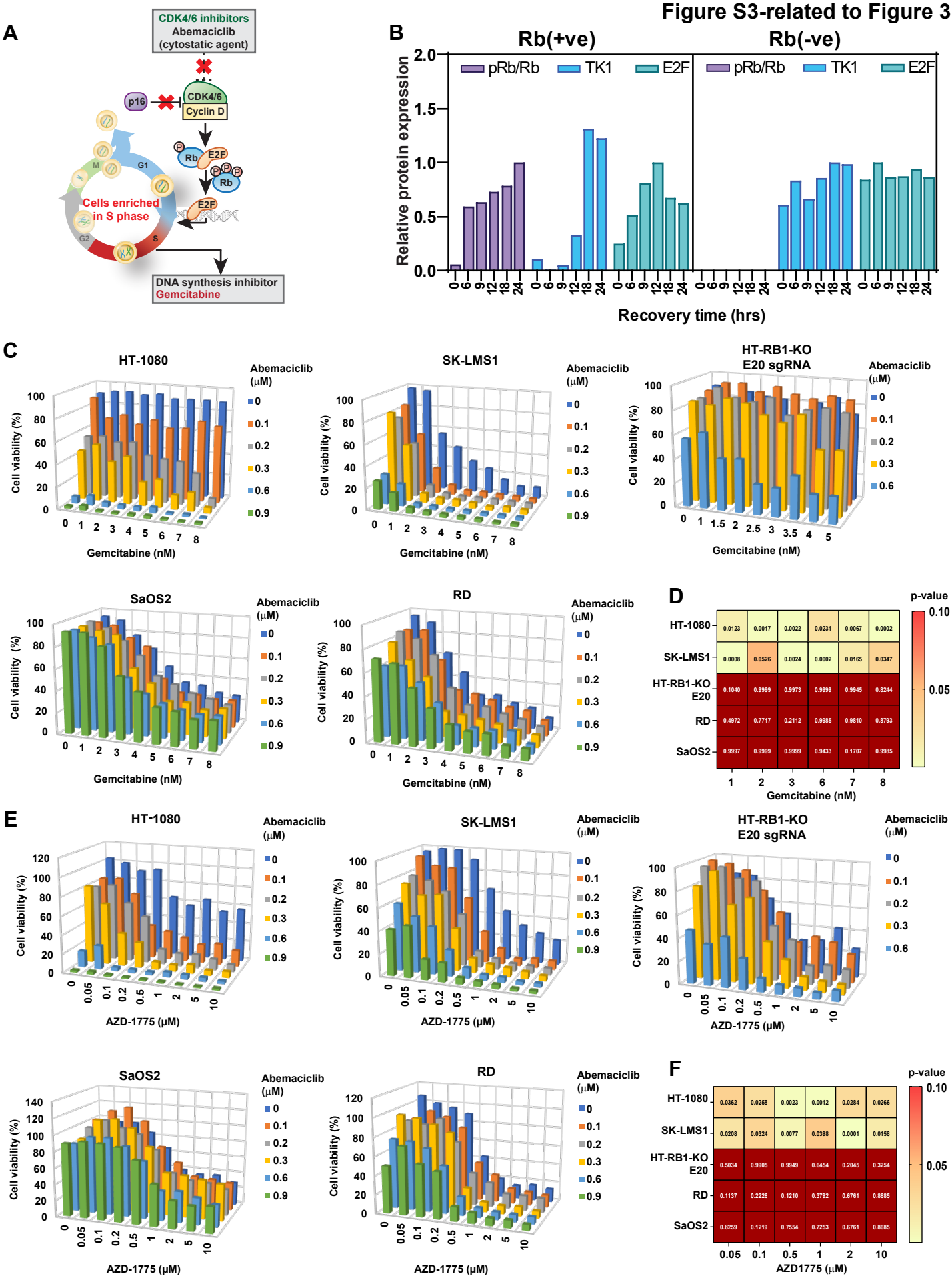
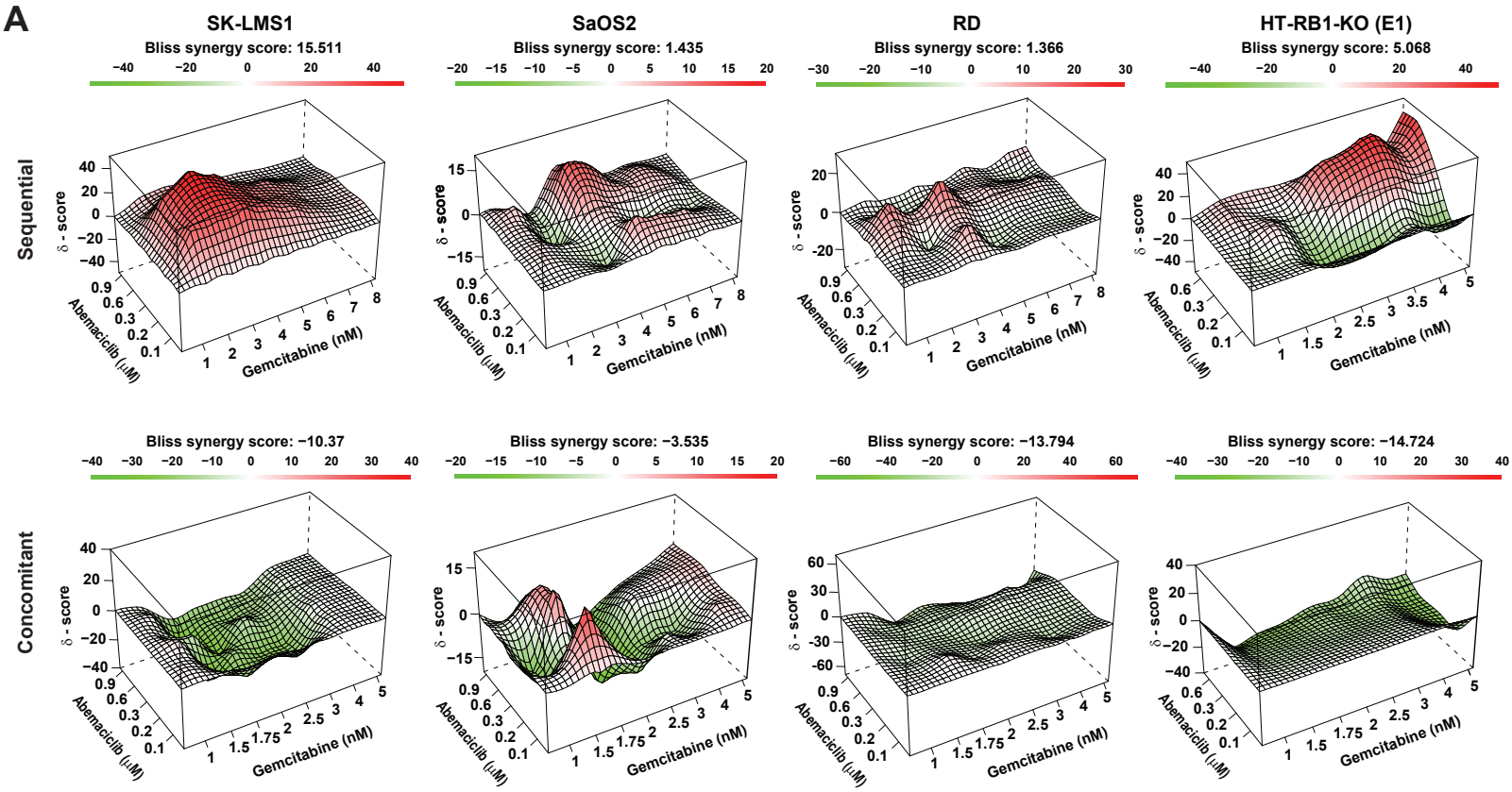
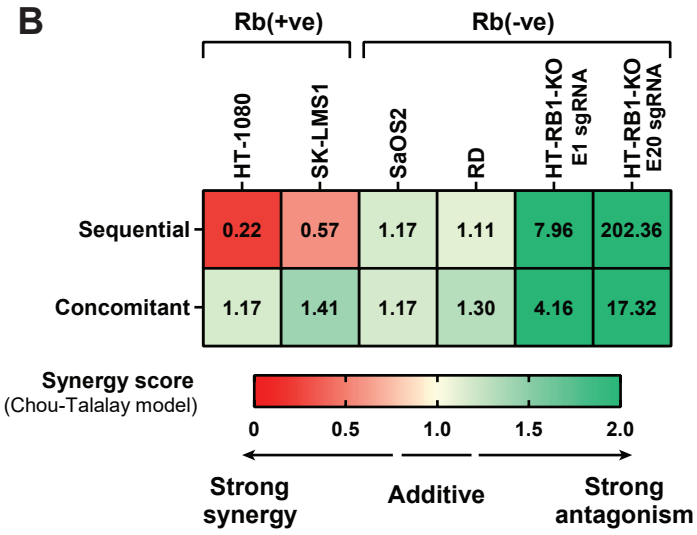


Figure S4 (related to Figure 3). Sequential treatment of Rb-positive sarcoma cells with abemaciclib, followed by gemcitabine, is synergistic (**A)** 3D synergy maps highlight synergistic (red) and antagonistic (green) dose regions of sequential and concomitant treatment on Rb(+ve) and Rb(-ve) cell lines using Bliss independence models. Synergism (synergy score >10), additivity (synergy score from -10 to 10) or antagonism (synergy score < -10) are indicated by red, yellow and green, respectively. **(B)** Heatmap depicts the average synergy scores calculated using Chou-Talaly model. Synergism (synergy score < 1), additivity (synergy score = 1) or antagonism (synergy score > 1) are indicated by red, yellow, and green, respectively. (**C**) Heatmap depicts the average synergy scores calculated using Bayesian dose response framework. Synergism (synergy score > 0), additivity (synergy score = 0) or antagonism (synergy score < 1) are indicated by red, yellow, and green, respectively

Figure S4 related to Figure 3





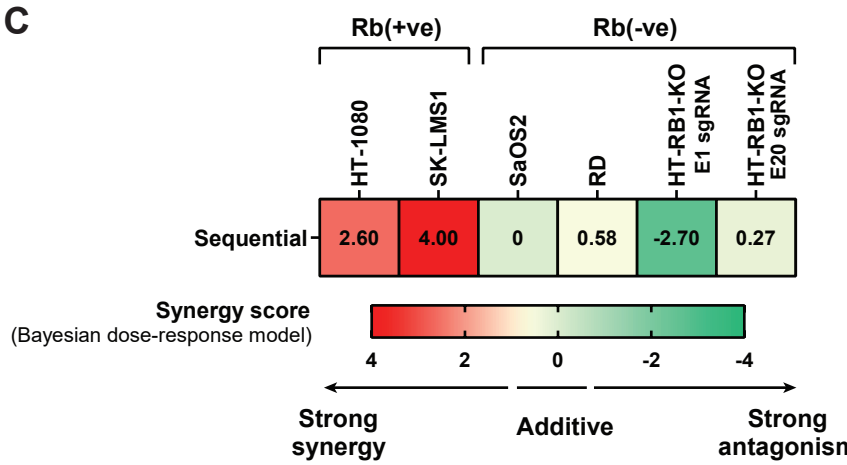


Figure S5 (related to Figure 3). Sequential treatment of Rb-positive sarcoma cells with abemaciclib, followed by Wee-1 kinase inhibitor, is synergistic. (A) Cell cycle model depicting synchronous activation of CDK1 and cyclin B via expression of WEE1/PKMYT1 kinases to sustain replication stress repair and stimulate proliferation upon the removal of abemaciclib. The synergistic cancer cell killing effect can be achieved by inhibiting WEE1/PKMYT1 kinases following abemaciclib removal, which results in the accumulation of DNA damage due to replication stress mediated by abemaciclib. **(B)** Western blot analysis with the indicated proteins using the same cell lysates as those described in Figure 3C and D following abemaciclib removal. **(C)** Densitometry depicting the relative protein expression kinetic of pCDK1/CDK1, cyclin B1, and Wee-1 kinase, normalized to the corresponding loading control (vinculin) at the indicated time point following abemaciclib removal from western blot analysis shown in B. **(D)** Dose response curves of Rb(+ve) and Rb(- ve) cell lines treated with increasing concentrations of AZD-1775 (Wee-1 kinase inhibitor) for 72hrs followed by 9 days of recovery in drug free media. **(E)** 3D synergy maps highlight synergistic (red) and antagonistic (green) dose regions of sequential and concomitant treatment on Rb(+ve) and Rb(-ve) cell lines using Bliss independence models. Synergism (synergy score >10), additivity (synergy score from -10 to 10) or antagonism (synergy score < -10) are indicated by red, yellow, and green, respectively. **(F)** Heatmap depicts the average synergy scores calculated using Bliss independence model for each pair of sequential or concomitant treatment using multiple drug concentrations for each agent. Synergism (synergy score >10), additivity (synergy score from -10 to 10) or antagonism (synergy score < -10) are indicated by red, yellow and green, respectively. **(G)** Heatmap depicts the average synergy scores calculated using Chou-Talaly model. Synergism (synergy score < 1), additivity (synergy score = 1) or antagonism (synergy score > 1) are indicated by red, yellow, and green, respectively. (**H**)

Heatmap depicts the average synergy scores calculated using Bayesian dose response framework. Synergism (synergy score > 0), additivity (synergy score = 0) or antagonism (synergy score < 1) are indicated by red, yellow, and green, respectively. The combination treatment is synergistic only in Rb(+ve) cell lines when they are treated with abemaciclib and AZD1775 sequentially. Experiments are representative of three independent biological replicates.

Figure S5 related to Figure 3

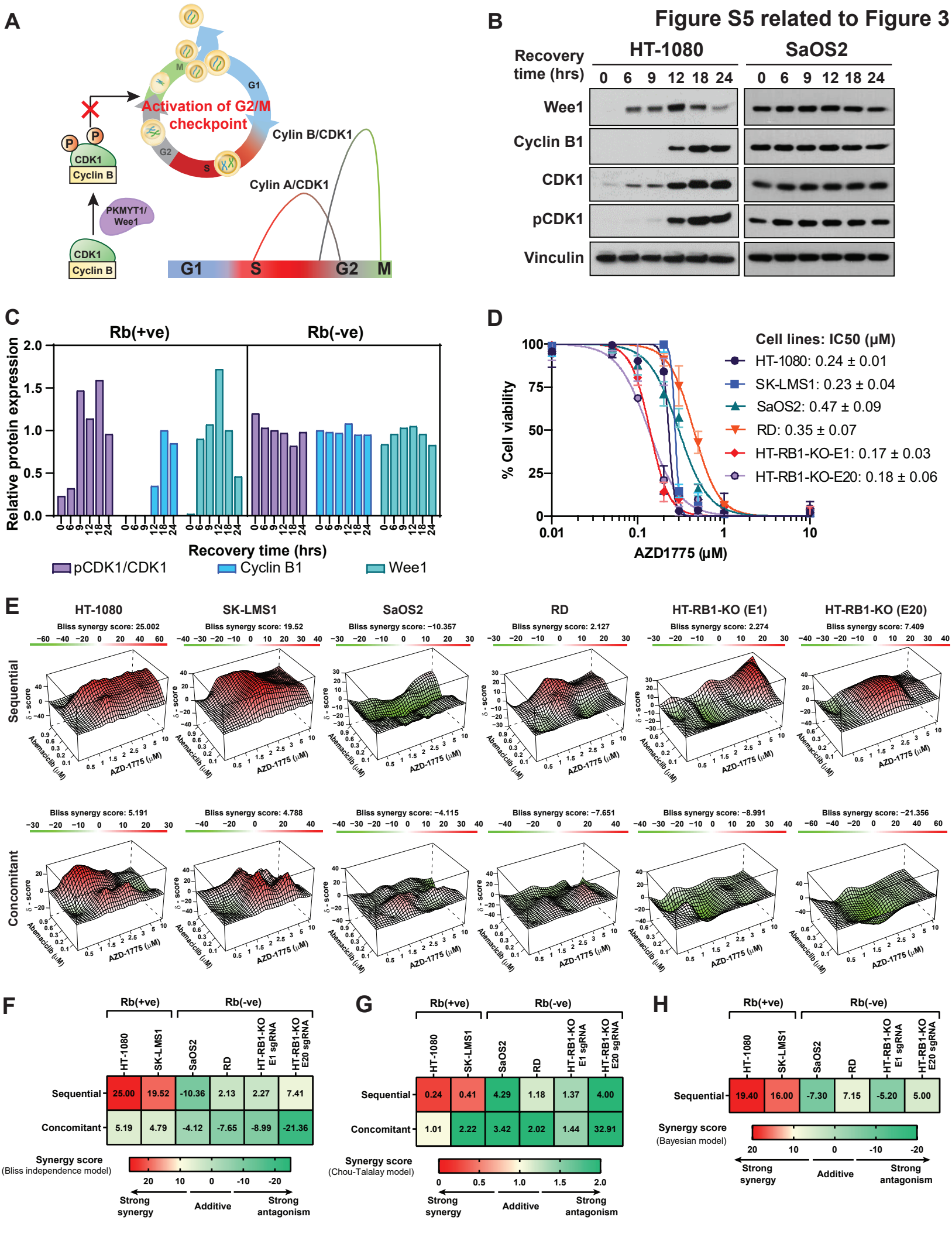


Figure S6 (related to Figure 3 and 4). Sequential combination treatment of abemaciclib followed by gemcitabine is highly effective at killing Rb(+ve) sarcoma cells through apoptosis. (**A**) Scatter plots showing the progression of cell death after the indicated treatment (Q1: double negative for Annexin-V and PI, indicating a healthy cell population. Q2: Annexin-V positive, PI negative, indicating early apoptotic cells. Q3: Annexin-V and PI double positive, indicating late apoptotic cells. Q4: Annexin-V negative and PI positive, indicating necrotic cells. (**B**) Percentage of Annexin-V positive cells from 2 biological replicates was quantified and presented as mean ± SEM. (**C**) Histograms showing cell cycle distribution and its quantification in **(D)** after treatment of abemaciclib or gemcitabine as single agents or in combinations, as illustrated in the study schema shown in Figure 4A. Experiments are representative of three independent biological replicates. The statistical analyses were performed using one- or two-way ANOVA. *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

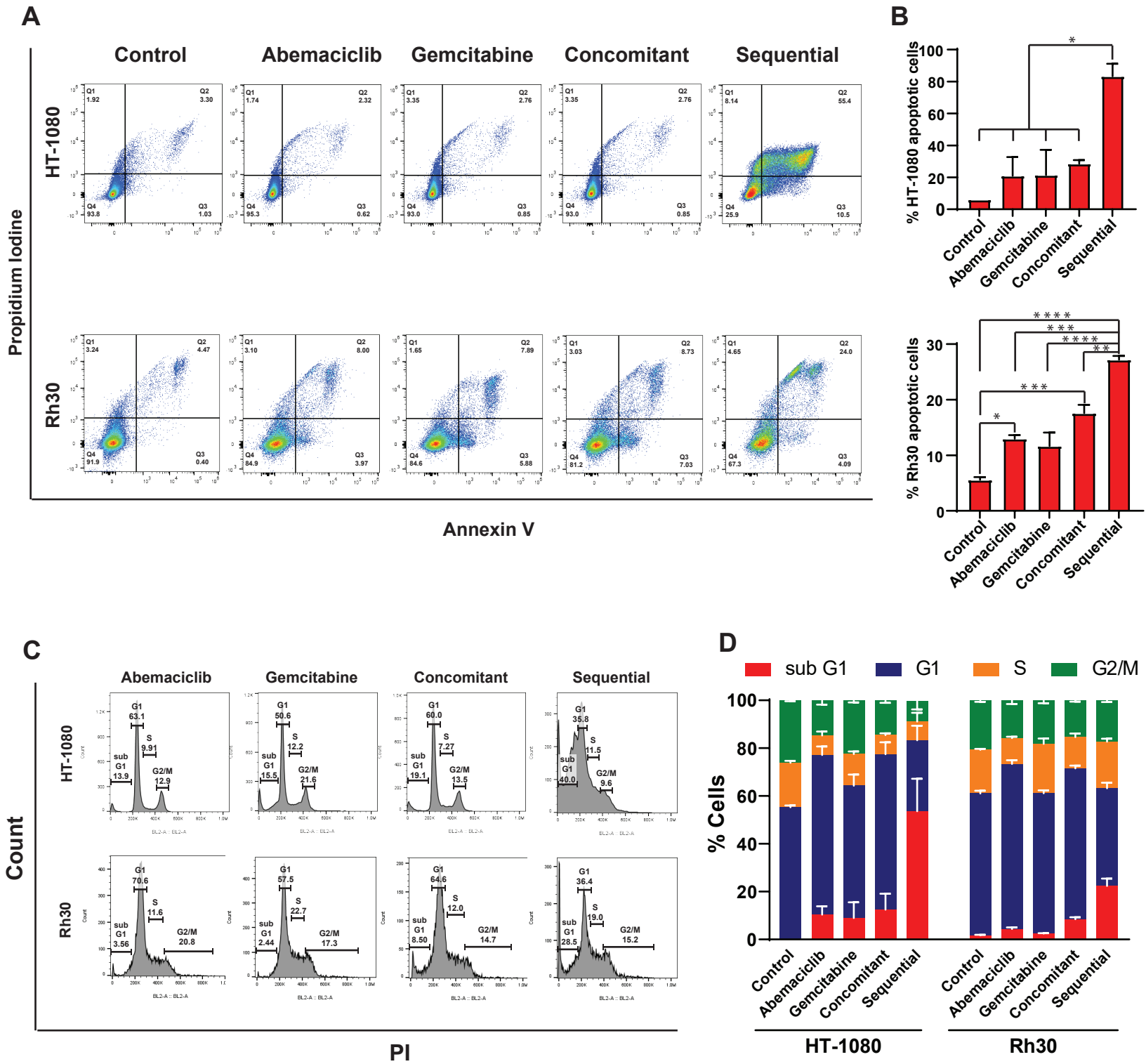
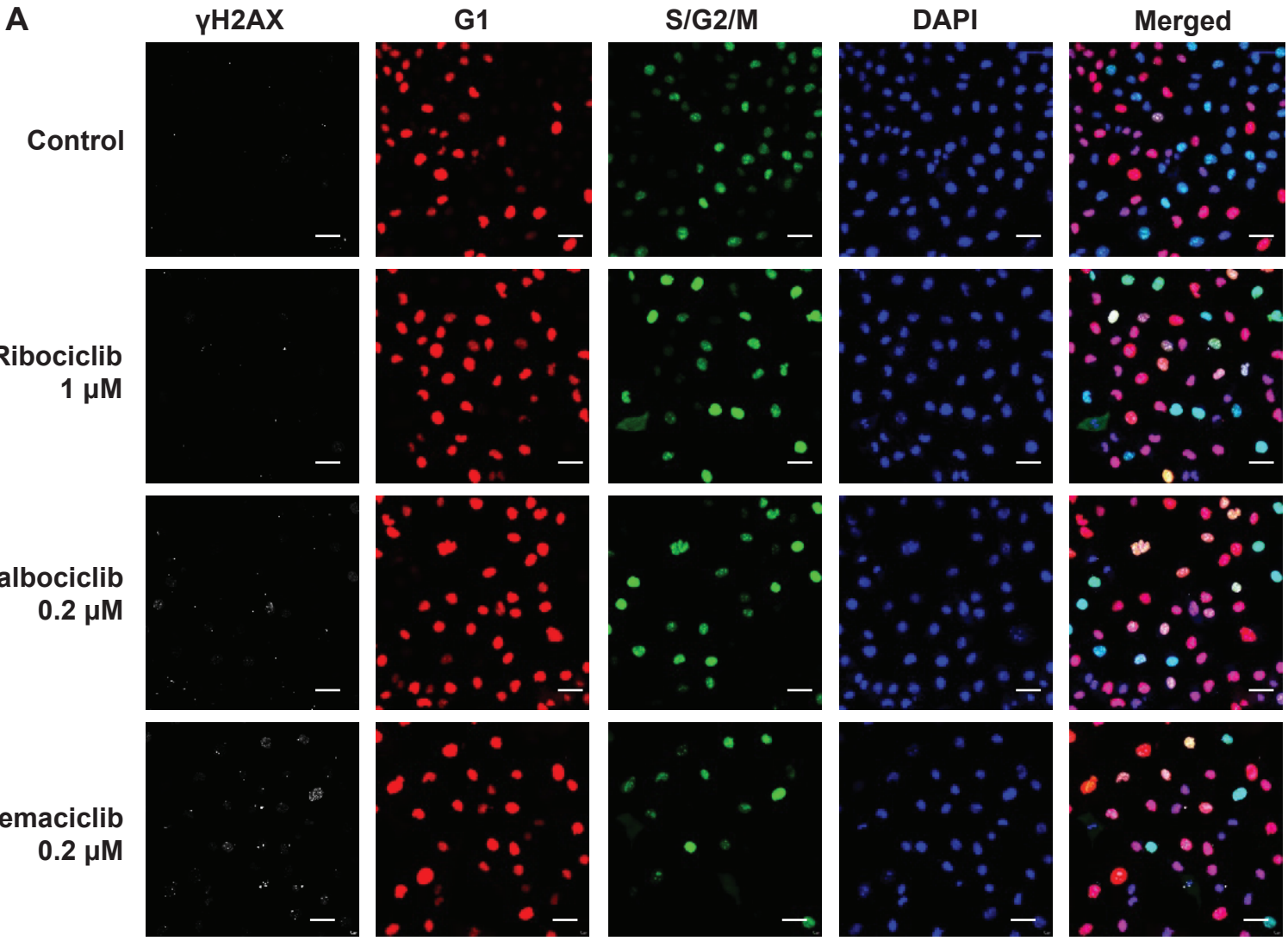
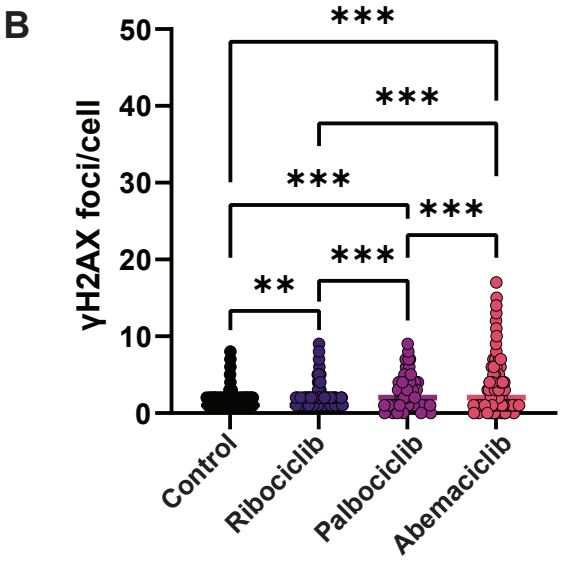

A
Control
Abemaciclib
Gemcitabine
Concomitant
Sequential
HT-1080
Rh30
Propidium Iodine
Annexin V
B
% HT-1080 apoptotic cells
% Rh30 apoptotic cells
Control
Abemaciclib
Gemcitabine
Concomitant
Sequential
C
Count
PI
D
sub G1
G1
S
G2/M
% Cells
HT-1080
Rh30

Figure S7 (related to Figure 4). Abemaciclib, but not other CDK4/6 inhibitors, induce significant DNA damage detected in all phases of the cell cycle in Rb(+ve) cell lines. (A) Representative confocal images of γH2AX staining in FUCCI labeled HT-1080 cells after 6 days of treatment with the indicated CDK4/6 inhibitors, all at IC50 concentrations (scale bar = 50 μm). **(B)** and **(C)** Effect of the indicated treatments on DNA damage, quantified by γH2AX foci per cell (panel B) and % γH2AX- positive cells (foci>5, panel C). Experiments are representative of three independent biological replicates. The statistical analyses were performed using one- or two-way ANOVA. *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.


A
γH2AX
G1
S/G2/M
DAPI
Merged
Control
Ribociclib
1 μM
Palbociclib
0.2 μM
Abemaciclib
0.2 μM


B
γH2AX foci/cell
50
40
30
20
10
0
***
***
***
***
***
**
Control
Ribociclib
Palbociclib
Abemaciclib

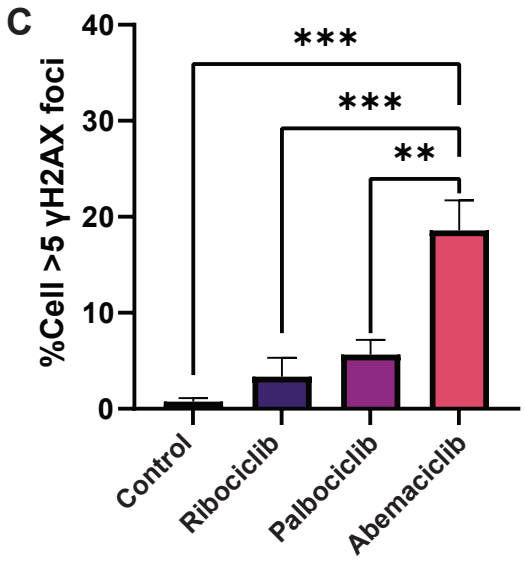
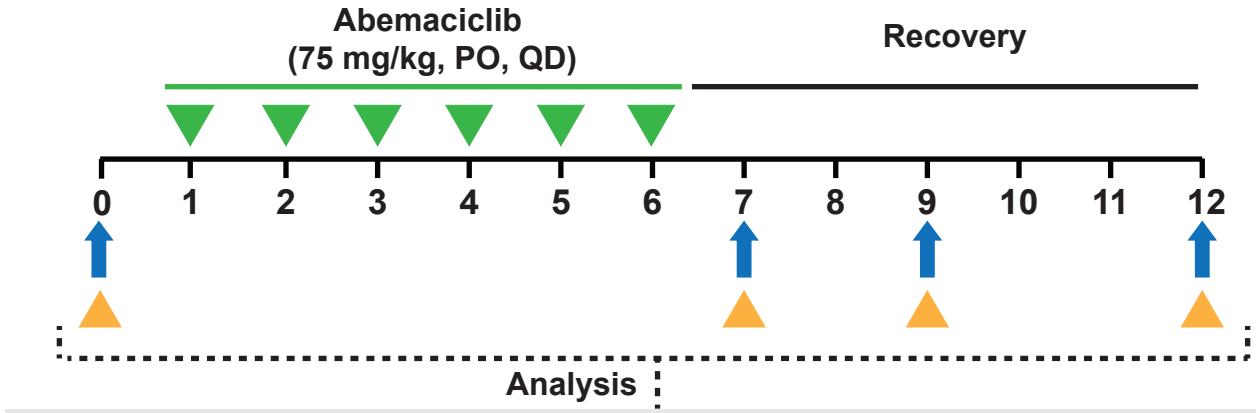

C
%Cell >5 γH2AX foci
40
30
20
10
0
***
***
**
Control
Ribociclib
Palbociclib
Abemaciclib

Figure S8 (related to Figure 5, 6, and 7). Abemaciclib induces reversible G1 arrest in *in vivo* cell line xenografts and patient-derived xenograft models. (A) Schematic illustrating the treatment and assessment schedule to evaluate the reversible G1 arrest induced by abemaciclib in *in vivo*. For the pilot experiments, 21 Rb(+ve) HT-1080 or Rh30 tumor bearing mice were randomly assigned to 6 arms (n = 3 mice/arm) and treated with vehicle or 75 mg/kg abemaciclib daily for 6 days, followed by 0, 3, or 6 days of recovery. Tumors and blood were collected on day 0 (baseline), day 7 (after 6 days of treatment), day 9 (after 3 days of recovery), and day 12 (after 6 days of recovery) for the *ex vivo* biomarker analysis. For longitudinal experiments, n = 3 per treatment arm per model were used to evaluate the level of circulating TKa and changes in [18]F-FLT tumor uptake. **(B)** Immunohistochemistry (IHC) of tumors for Rb and Ki67 reveal the concordance between IHC and western blot analysis.

Figure S8-related to Figure 5, 6 and 7

A



Pilot experiment (Figure 5 and 7A-B)
(n=3/time point/treatment arm/model)
(1) BrdU injection (100mg/kg)
(2) Blood collection (cardiac puncture)
(3) Ex vivo analysis for tumors (Ki67, BrdU, TK1)

Longitudinal experiment (Figure 7E-K)
(n=3/time point/treatment arm/model)
(1) [18]F-FLT injection and PET/CT imaging
(2) Blood collection (retro-orbital bleeding)
(3) Circulating TKa

B

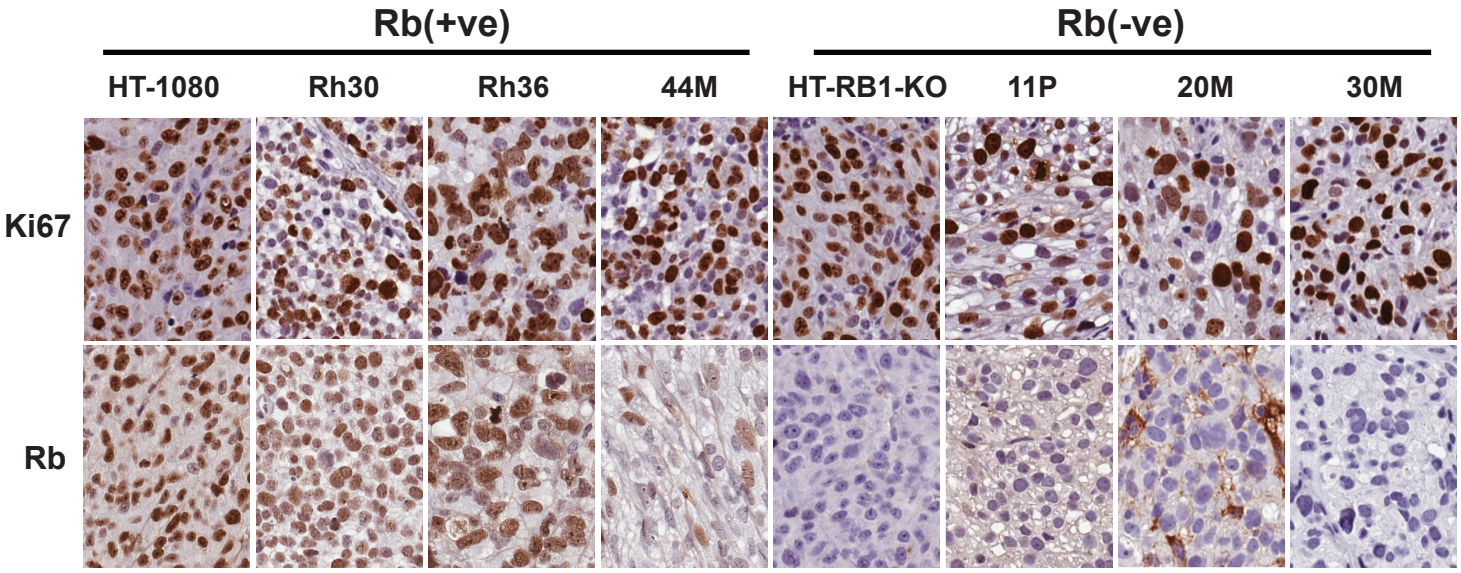


Figure S9 (related to Figure 6). Combination treatment with abemaciclib followed by gemcitabine inhibits tumor growth in Rb(+ve) chemo resistant PDX sarcoma models. (A) Schematic illustrating the dosing and scheduling of one cycle of treatment in PDX models to evaluate tumor growth inhibition and survival. **(B)** Growth curve showing relative tumor growth inhibition over time of the chemo resistant Rh36 PDX tumor model subjected to the indicated 5 treatments (n=4 or 5/treatment arm, 12 days/cycle). Mice treated sequentially completed 2-3 cycles of treatment while other treatments arms stopped at cycle 1 or early cycle 2 due to tumor burden. **(C)** Percent of tumor growth inhibition of the Rh36 tumor model subjected to the indicated 5 treatments for 1 cycle (day 12) as indicated by black arrow in (B) (n=4 or 5/treatment arm). **(D)** Survival curves of Rh36 tumor model showing that the sequential combination arm (orange) had the longest survival time compared to single agents or concomitant combination. (**E**) Densitometry depicting the relative protein expression of pATM, cleaved caspase 3/total caspase 3 and cleaved PARP/PARP normalized to corresponding loading control (vinculin) at the indicated time point following abemaciclib removal from western blot analysis, as shown in Figure 6I. Experiments are representative of 4 or 5 independent biological replicates. The statistical analyses were performed using one- or two-way ANOVA. *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

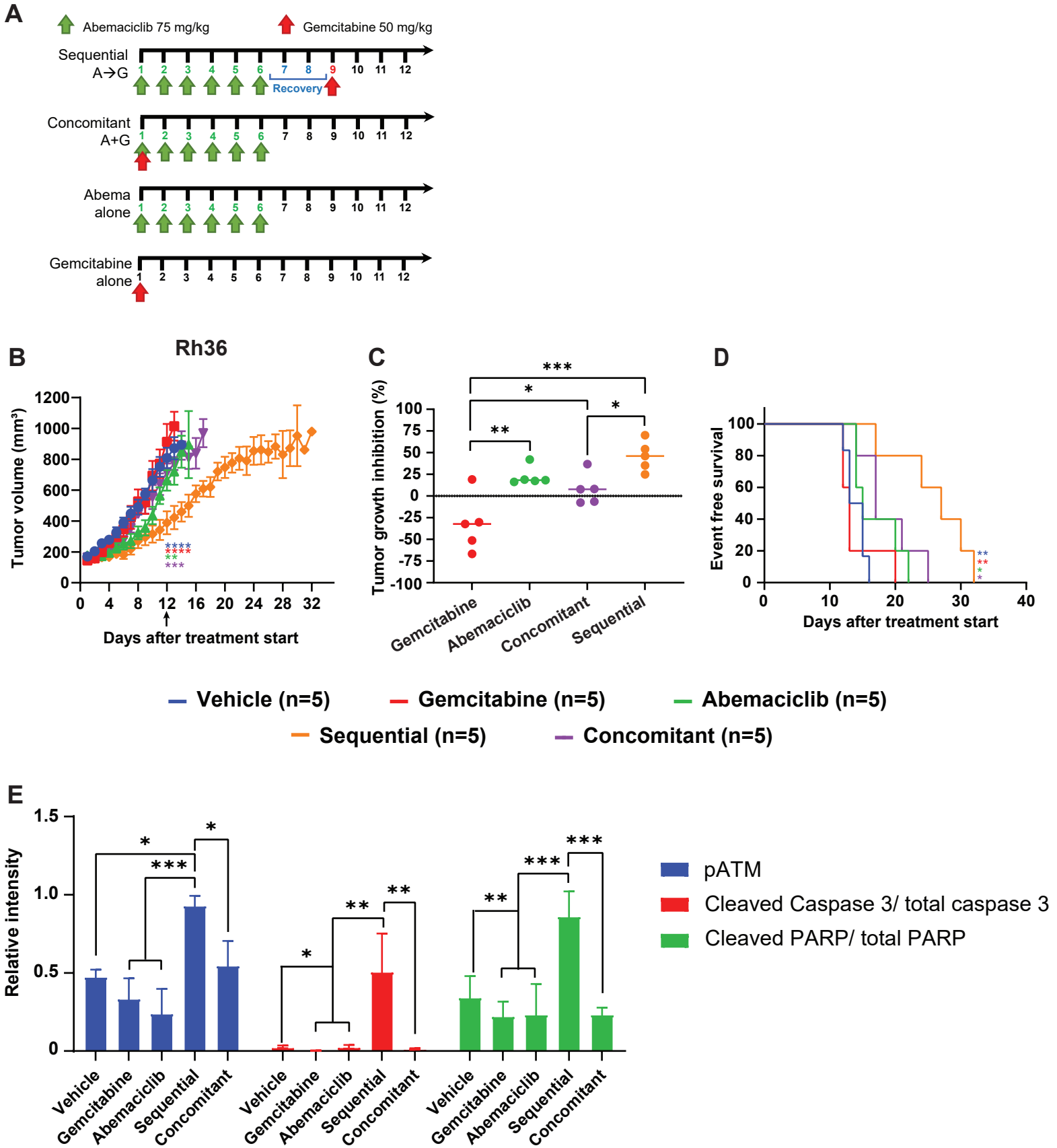

A
Abemaciclib 75 mg/kg
Gemcitabine 50 mg/kg
Sequential A→G
Recovery
Concomitant A+G
Abema alone
Gemcitabine alone
B
Rh36
Tumor volume (mm³)
Days after treatment start
C
Tumor growth inhibition (%)
Gemcitabine
Abemaciclib
Concomitant
Sequential
D
Event free survival
Days after treatment start
Vehicle (n=5)
Gemcitabine (n=5)
Abemaciclib (n=5)
Sequential (n=5)
Concomitant (n=5)
E
Relative intensity
pATM
Cleaved Caspase 3/ total caspase 3
Cleaved PARP/ total PARP
Vehicle
Gemcitabine
Abemaciclib
Sequential
Concomitant

Figure S10 (related to Figure 7). [18]F-FDG uptake fails to capture recovery from abemaciclib mediated arrest in Rb(+ve) sarcoma cells. (A) Western blot analysis showing HKII protein expression at basal level across sarcoma cell lines. **(B)** Western blot analysis showing that HKII protein expression remains unchanged following removal of abemaciclib treatment. **(C)** Schematic depicting the [18]F-FDG uptake mechanism. Similar to glucose, [18]F-FDG depends on hexokinase (HK) activity to convert FDG into its corresponding phosphorylated forms. The radio substitution (18F) at C2 prevents phosphorylated [18]F-FDG from further advancing into glycolysis. **(D)** Changes in [18]F-FDG uptake in Rb(+ve) and Rb(-ve) cells post abemaciclib removal. For both experiments, cells were treated with IC50 of abemaciclib for 6 days before undergoing the indicated recovery period in drug free media. Cellular tracer uptake was corrected with [18]F decay time and normalized with total protein in cell lysates. The percent change in [18]F-FDG uptake was calculated by subtracting normalized [18]F-FDG uptake in drug-treated cells from normalized [18]F-FDG uptake in untreated cells at each indicated recovery time. **(E)** Quantification of TKa in plasma samples collected longitudinally by retro-orbital bleeding in the Rb(-ve) tumor model (n = 3/arm/timepoint). Experiments are representative of three independent biological replicates. The statistical analyses were performed using one- or two-way ANOVA. *p <0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Figure S10-related to Figure 7