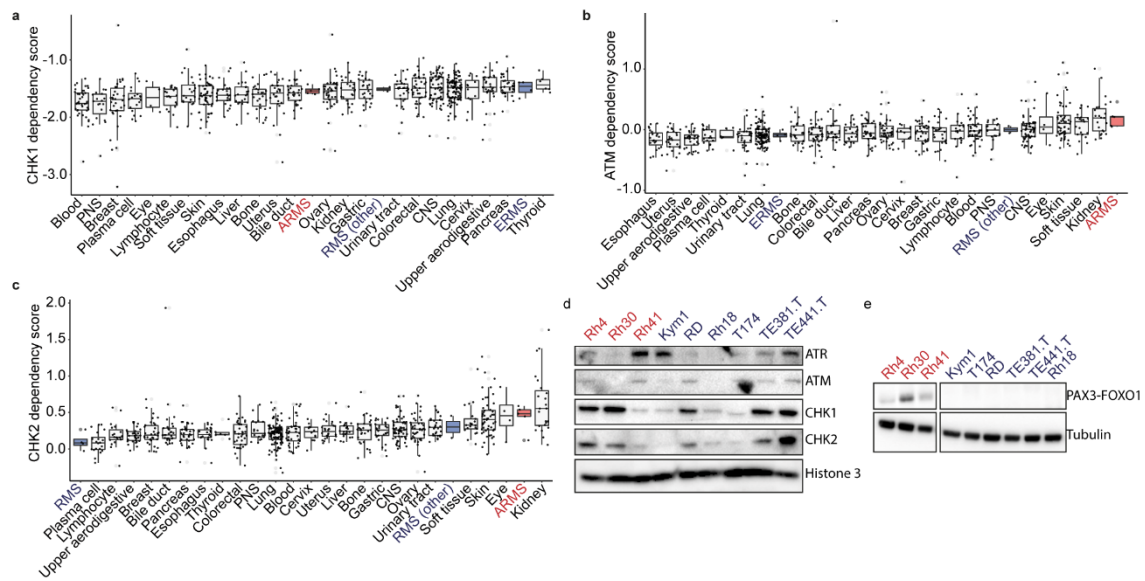


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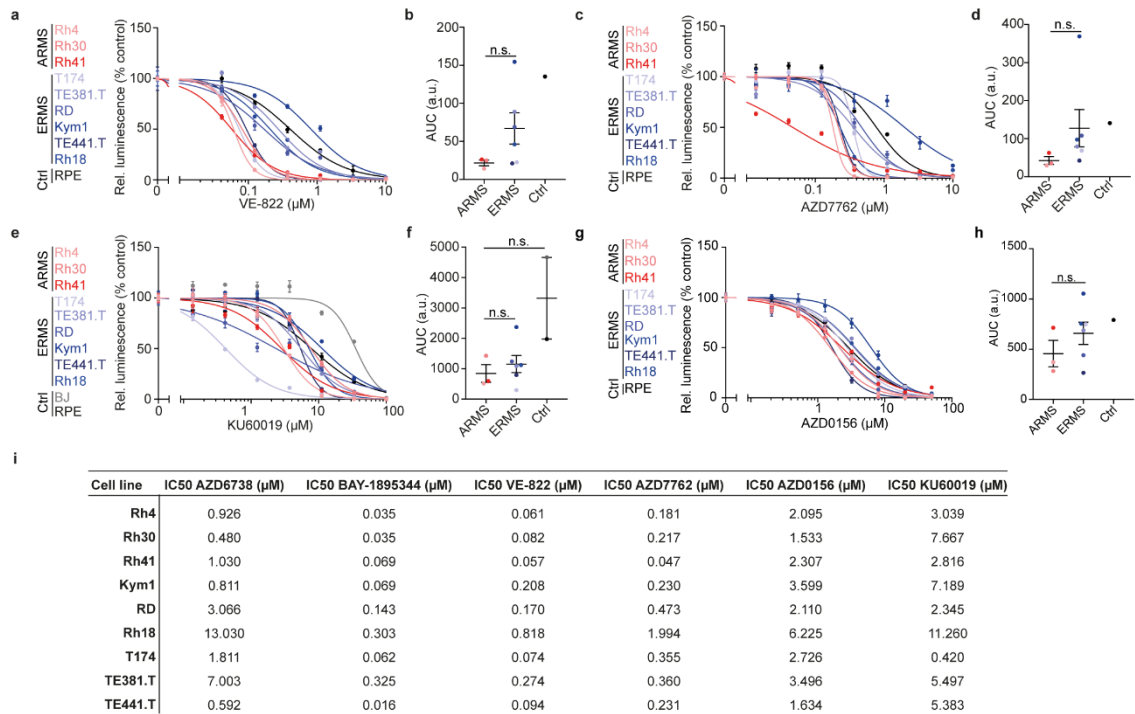
**PAX3-FOXO1-expressing alveolar rhabdomyosarcomas are hypersensitive to
ATR inhibition**

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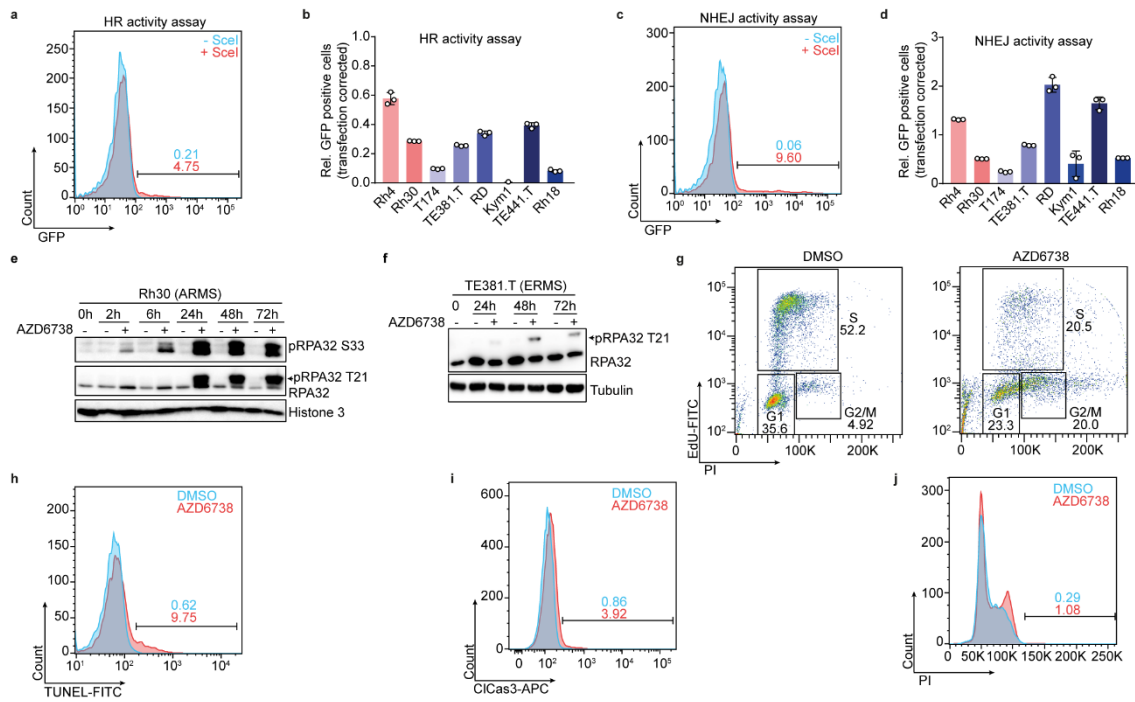
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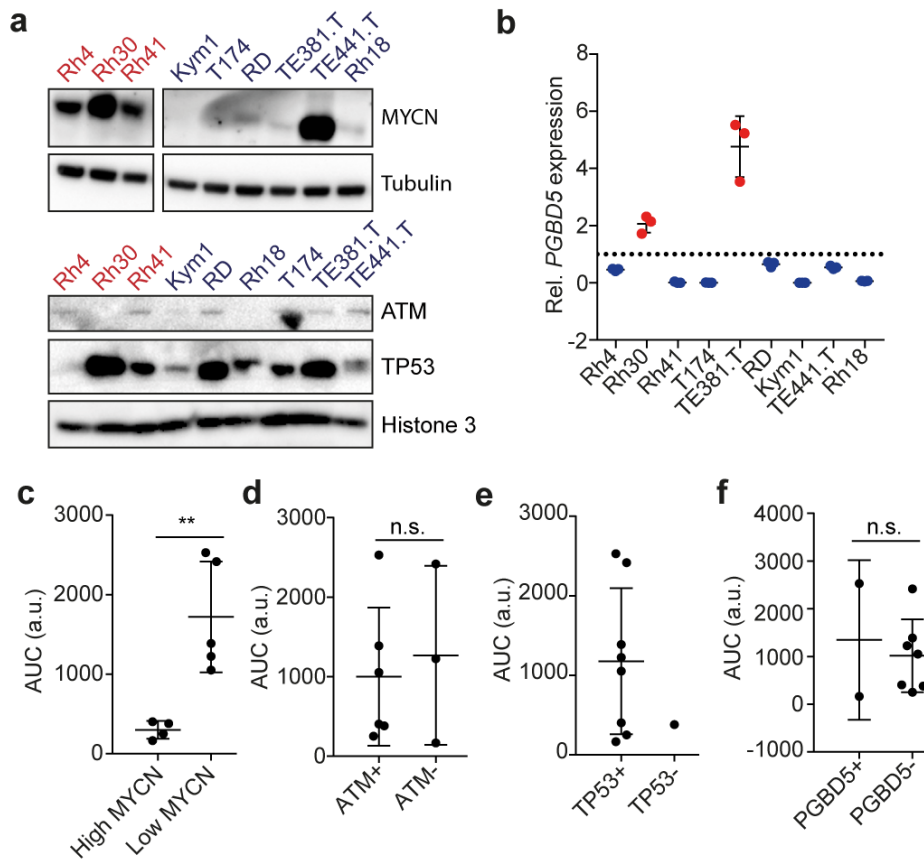
Extended Data Fig. 1. Alveolar rhabdomyosarcoma cells express ATR and depend on expression of ATR/CHK1 pathway members. (a-c) Dependency scores of several cancer types obtained from CRISPR (Avana) 20Q2 for CHK1 (a), ATM (b) and CHK2 (c). (d) Western immunoblot of ATR, ATM, CHK1 and CHK2 in rhabdomyosarcoma cell lines (Histone 3 as a loading control). (e) Western immunoblot of PAX3-FOXO1 in rhabdomyosarcoma cell lines. (Tubulin as a loading control). In red, alveolar rhabdomyosarcoma; in blue, other rhabdomyosarcomas.



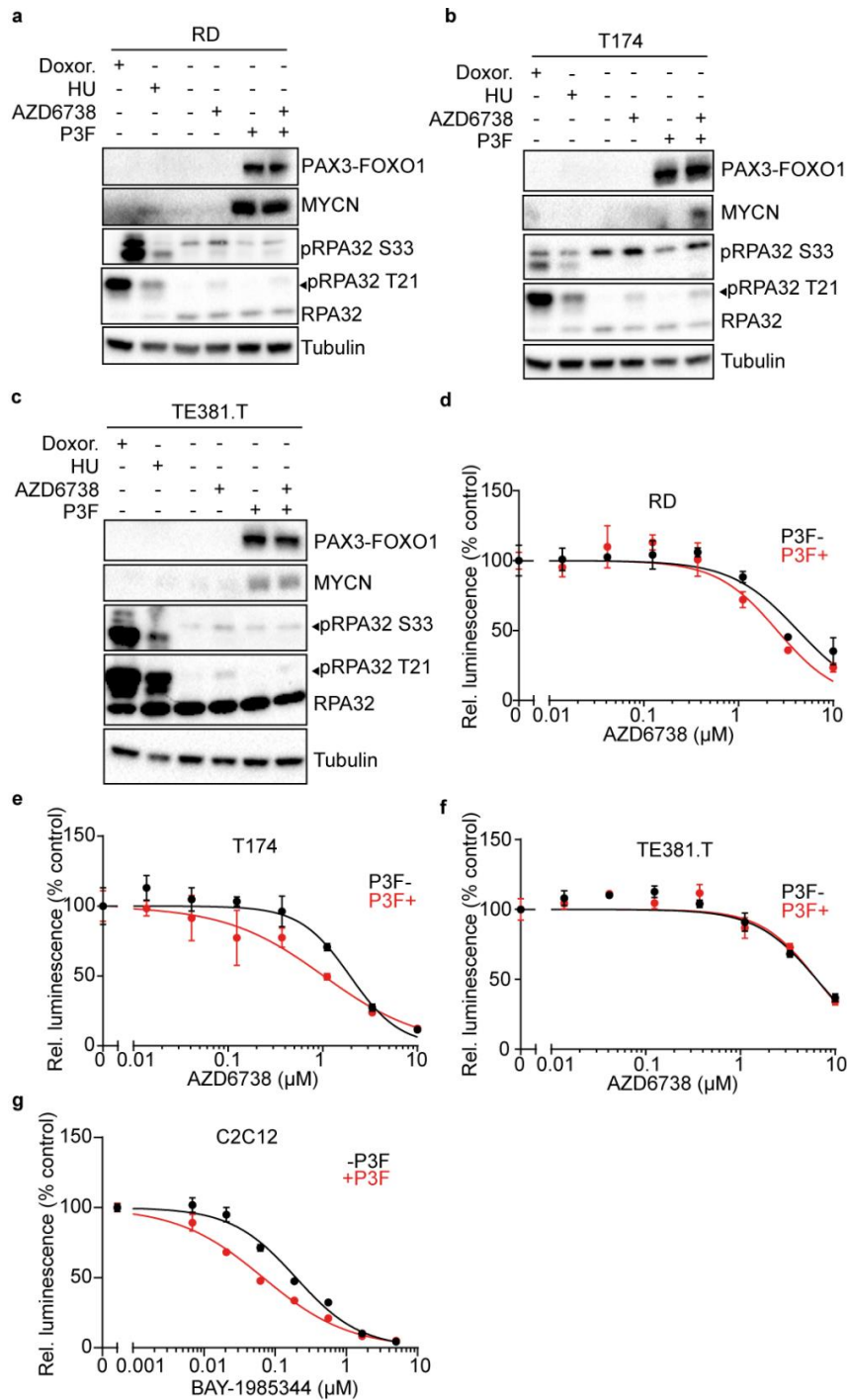
Extended Data Fig. 2. PAX3-FOXO1-expressing alveolar rhabdomyosarcoma cells are hypersensitive to pharmacological ATR pathway inhibition. (a) Dose-response curves rhabdomyosarcoma cell lines treated with ATR inhibitor VE-822 ($n=3$). (b) AUC of the previous curves ($P=0.178$ for ARMS vs ERMS). (c) Dose-response curves rhabdomyosarcoma cell lines treated with CHK1 inhibitor AZD7762 ($n=3$). (d) AUC of the previous curves ($P=0.279$ for ARMS vs ERMS). (e) Dose-response curves rhabdomyosarcoma cell lines treated with ATM inhibitor KU60019 ($n=3$). (f) AUC of the previous curves ($P=0.519$ for ARMS vs ERMS, $P=0.117$ for ARMS vs Ctrl). (g) Dose-response cell viability curves for ARMS cell lines (red) and ERMS cell lines (blue) treated with the ATM inhibitor AZD0156 ($n=3$). (h) AUC of the previous curves ($P=0.311$ for ARMS vs ERMS). (i) Summary of calculated IC50 from the dose-response curves (two-sided student's t-test; error bars represent standard error of the mean).



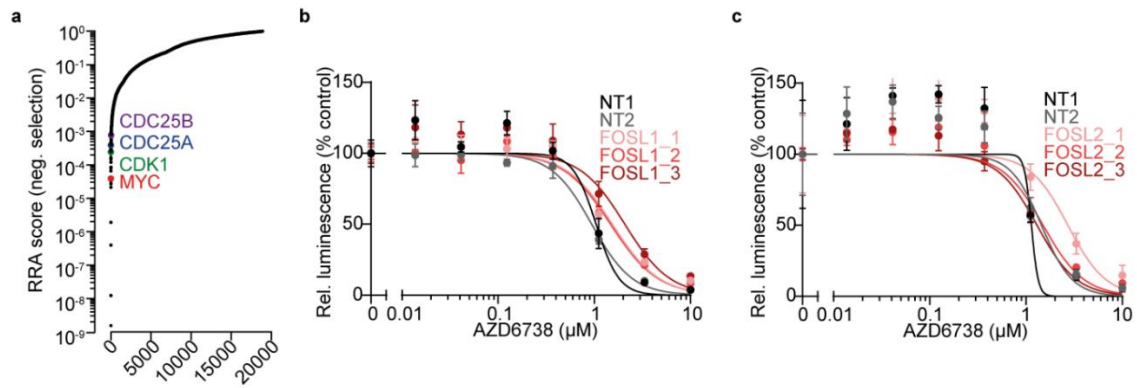
Extended Data Fig. 3. Pharmacological ATR inhibition exacerbates replication stress and leads to genomic instability, apoptosis and cell cycle disruption. (a) Representative histograms showing relative GFP signal in a rhabdomyosarcoma cell line co-transfected with a HR-GFP plasmid and a plasmid expressing ScelI (red) or an empty backbone (blue) as measured using FACS. **(b)** Homologous recombination activity of several rhabdomyosarcoma cell lines normalized to a non-cutting control and corrected for transfection efficacy ($n=3$). **(c)** Representative histograms showing relative measure GFP signal in a rhabdomyosarcoma cell line co-transfected with a NHEJ-GFP plasmid and a plasmid expressing ScelI (red) or an empty backbone (blue) as measured using FACS. **(d)** Non-homologous end joining activity of several rhabdomyosarcoma cell lines normalized to a non-cutting control and corrected for transfection efficacy ($n=3$; error bars represent standard error of the mean). **(e-f)** Western immunoblot of RPA32 phosphorylation in cells treated with ATR inhibitor AZD6738 (750 nM) over time for Rh30 (e) and TE381.T (f). **(g)** Representative histograms showing relative EdU and PI co-staining in the absence (left) and presence (right) of AZD6738 (750 nM) as measured using FACS. **(h)** Representative histograms showing relative TUNEL staining as measured using FACS. **(i)** Representative histograms showing relative cleaved Caspase3 staining as measured using FACS. **(j)** Representative histograms showing relative DNA content labeling (with propidium iodide, PI) as measured using FACS.



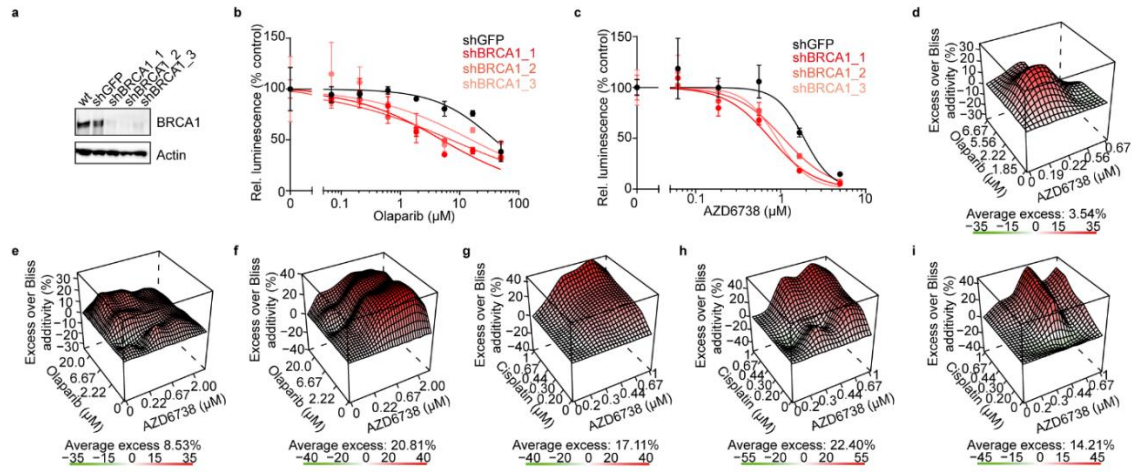
Extended Data Fig. 4. Molecular factors associated with ATR inhibitor sensitivity in rhabdomyosarcoma cells. (a) Western Immunoblot of MYCN, ATM and TP53 in rhabdomyosarcoma cell lines. (b) mRNA expression levels of *PGBD5* in rhabdomyosarcoma cell lines ($n=3$). (c-f) Comparison of AUC from dose response curves shown in Fig. 1b for MYCN ($P=0.005$; c), ATM ($P=0.702$; d), TP53 (e) and *PGBD5* ($P=0.677$; f).



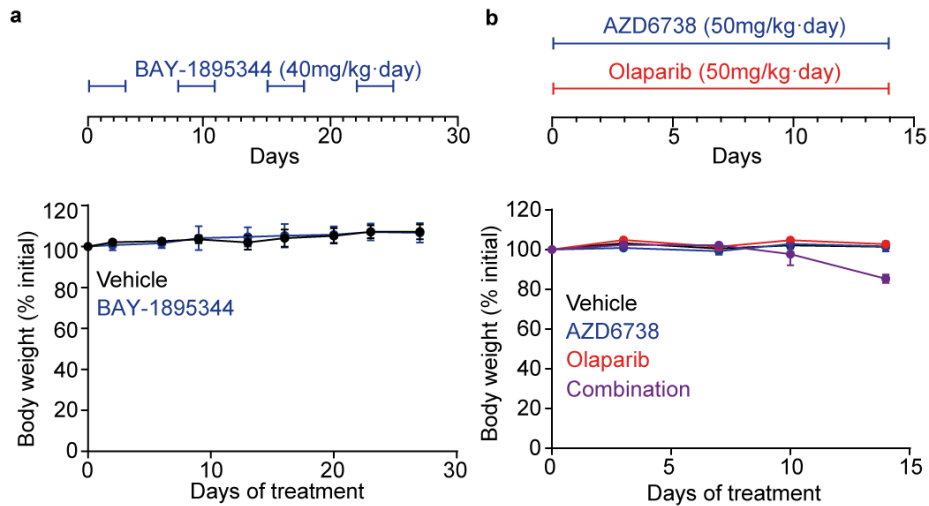
Extended Data Fig. 5. Ectopic expression of PAX3-FOXO1 increases sensitivity to ATR inhibition in rhabdomyosarcoma cells. (a-c) RD (a), T174 (b) and TE381.T (c) cells after induction of PAX3-FOXO1 expression with doxycycline (1000 ng/ml for 48 hours; 750 nM AZD6738 for 48 hours; doxorubicin and hydroxyurea treated cells, positive controls; Tubulin serves as a loading control). **(d-f)** Dose-response curves in RD (d), T174 (e) and TE381.T (f) cells treated with ATR inhibitor AZD6738 after doxycycline-induced ectopic expression of PAX3-FOXO1 ($n=3$). **(g)** Dose-response curves in C2C12 cells treated with ATR inhibitor BAY-1895344 after doxycycline-induced ectopic expression of PAX3-FOXO1 ($n=3$; error bars represent standard error of the mean).



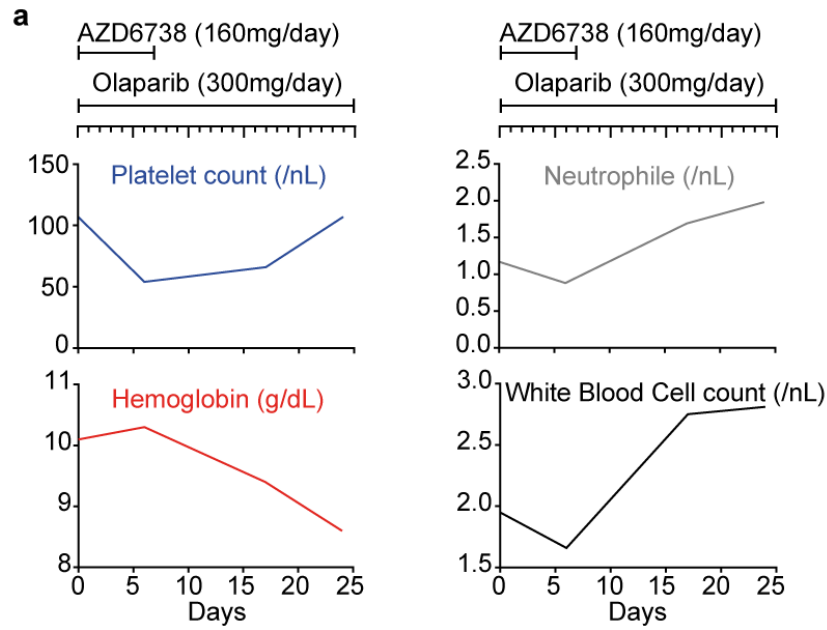
Extended Data Fig. 6. *FOSB*, *FOSL1* and *FOSL2* expression induces resistance to ATR inhibition in rhabdomyosarcoma cells. (a) Waterfall plot showing the negative robust rank aggregation (RRA) score of sgRNAs in Rh4 cells incubated in the presence of AZD6738 for 9 days compared to DMSO treated cells as analyzed using MaGECK. (b-c) Relative cell viability of cells stably expressing dCas9, lentiMPH and sgRNAs targeting *FOSL1* (b) and *FOSL2* (c) in the presence and absence of AZD6738 ($n=3$; two-sided student's t-test; error bars represent standard error of the mean).



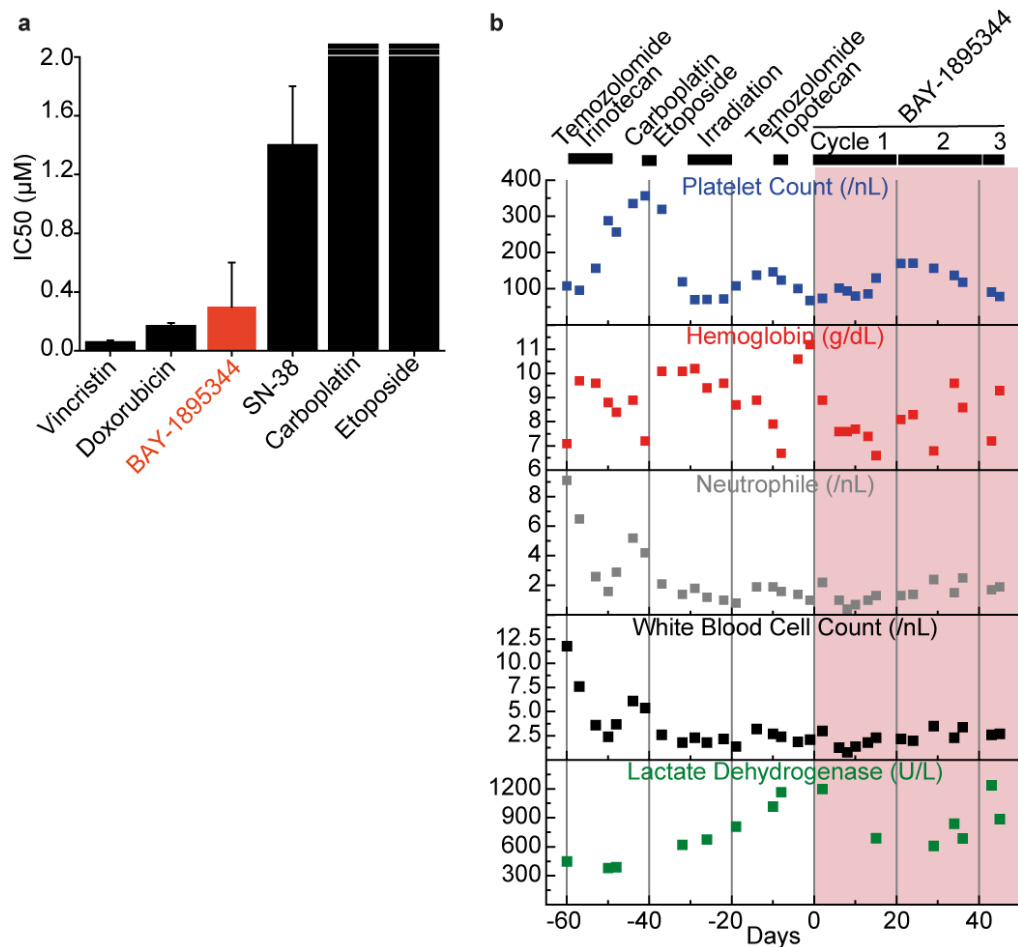
Extended Data Fig. 7. ATR inhibition synergizes with cisplatin and olaparib in rhabdomyosarcoma cell lines. (a) Western immunoblot of BRCA1 in Rh4 cells of stably expressing shRNAs targeting BRCA1 (shRNA targeting GFP was used as a control). (b) Dose-response curves for Rh4 stably expressing different shRNAs targeting BRCA1 and treated with PARP inhibitor olaparib (shRNA targeting GFP was used as a control) ($n=3$). (c) Dose-response curves for Rh4 transduced with different shRNA targeting BRCA1 treated with ATR inhibitor AZD6738 (shRNA targeting GFP was used as a control) ($n=3$; error bars represent standard error of the mean). (d-f) Excess over Bliss analysis of combined treatment with olaparib and AZD6738 in Rh30 (d), T174 (e) and TE381.T (f) cells ($n=3$). (g-i) Excess over Bliss analysis of combined treatment with cisplatin and AZD6738 in Rh30 (g), T174 (h) and TE381.T (i) cells ($n=3$).



Extended Data Fig. 8. Combined treatment of mice with AZD6738 and olaparib has no remarkable toxicity in mice harboring alveolar rhabdomyosarcoma PDX models. (a) Timeline of the drug schedule. BAY-1895344 was given twice daily by oral gavage at 40mg/kg body weight per day on a 3 days on/4 days off schedule. Body weight over time of mice harboring the rhabdomyosarcoma PDX model subcutaneously xenografted and treated with BAY1895344 or a vehicle control. (n=7). (b) Timeline of the drug schedule. AZD6738 and olaparib are given by oral gavage at 50 mg/kg body weight per day. Body weight over time of mice harboring the rhabdomyosarcoma PDX model subcutaneously xenografted and treated with AZD6738, olaparib, both or a vehicle control (n=4; error bars represent standard deviations of 4 individual xenografted mice per group).



Extended Data Fig. 9. Compassionate use of AZD6738 and olaparib in a patient suffering from relapsed metastasized PAX3-FOXO1-expressing alveolar rhabdomyosarcomas. (a) Patient blood counts over the course of treatment with AZD6738 and olaparib.



Extended Data Fig. 10. Compassionate use of BAY-1895344 in a patient suffering from relapsed metastasized PAX3-FOXO1-expressing alveolar rhabdomyosarcomas. (a) Half maximal inhibitory concentration (IC₅₀) for the anti-tumor effects of drugs against patient-derived rhabdomyosarcoma cells ($n=3$). Error bars represent standard error of the mean. **(b)** Key laboratory values in the second case report patient treated with BAY 1895344.

Supplementary Table 1. Oligonucleotide primers.

Primer	Sequence (5'-3')
FOSB_Fwd	GTG AGA GAT TTG CCG GGC TC
FOSB_Rv	AGA GAG AAG CCG TCA GGT TG
FOSL1_Fwd	GCC CAC TGT TTC TCT TGA GC
FOSL1_Rv	GAT GGA GAG TGT GGC AGT GA
FOSL2_Fwd	GCC CAG TGT GCA AGA TTA GC
FOSL2_Rv	GGG CTC CTG TTT CAC CAC TA
HPRT1_Fwd	TGA CAC TGG CAA AAC AAT GCA
HPRT1_Rv	GGT CCT TTT CAC CAG CAA GCT
PGBD5_Fwd	CAG CCT CTG GGT CAG ACA AT
PGBD5_Rv	GCT TAT TCT TCA GCG CAT CC

Supplementary Table 2. Antibodies.

Antibody	Company	Catalog number
FOXO1	(And Santa Cruz Biotechnology	sc-374427
PAX3-FOXO1)		
Tubulin	Cell Signaling Technology	3873
Actin	Cell Signaling Technology	3700
Histone 3	Cell Signaling Technology	4499
RPA32 T21	Abcam	ab61065
RPA32 S33	Bethyl Laboratories	A300-246A
MYCN	Santa Cruz Biotechnology	sc-53993
ATR	Cell Signaling Technology	13934
ATM	Cell Signaling Technology	92356
CHK1	Cell Signaling Technology	2360
CHK2	Cell Signaling Technology	2662
TP53	Santa Cruz Biotechnology	sc-98
BRCA1	Merck Millipore	OP92-100UG
BRCA1 S1524	Bethyl Laboratories	A300-001A