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Supplementary Materials for

The HDAC3–SMARCA4–miR-27a axis promotes expression of the *PAX3:FOXO1* fusion oncogene in rhabdomyosarcoma

Narendra Bharathy, Noah E. Berlow, Eric Wang, Jinu Abraham, Teagan P. Settelmeyer, Jody E. Hooper, Matthew N. Svalina, Yoshihiro Ishikawa, Keith Zientek, Zia Bajwa, Martin W. Goros, Brian S. Hernandez, Johannes E. Wolff, Michelle A. Rudek, Linping Xu, Nicole M. Anders, Ranadip Pal, Alexandria P. Harrold, Angela M. Davies, Arya Ashok, Darnell Bushby, Maria Mancini, Christopher Noakes, Neal C. Goodwin, Peter Ordentlich, James Keck, Douglas S. Hawkins, Erin R. Rudzinski, Bishwanath Chatterjee, Hans Peter Bächinger, Frederic G. Barr, Jennifer Liddle, Benjamin A. Garcia, Atiya Mansoor, Theodore J. Perkins, Christopher R. Vakoc, Joel E. Michalek, Charles Keller*

*Corresponding author. Email: charles@cc-tdi.org

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This PDF file includes:

Table S13. Statistical analysis of CTG-1008 data.

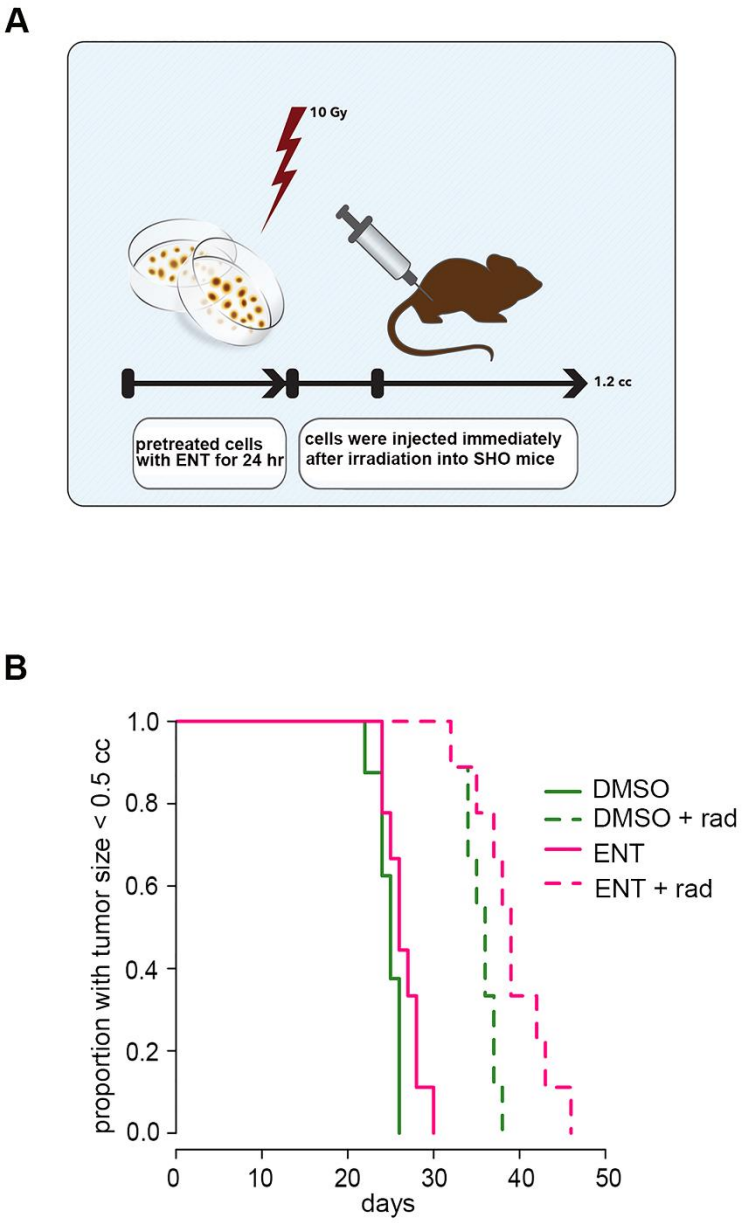

Fig. S1. ENT treatment of aRMS in vivo. (A) Experimental design schematic showing pretreatment of U23674 with entinostat (2 μM) for 24 hours followed by irradiation (10 Gy) and injection into SHO mice. **(B)** Kaplan-Meier plot showing mice injected with irradiated and entinostat pretreated cells. Data are means ± SEM (N=9 mice per cohort, except in DMSO where N=8). **P < 0.01 by log-rank test.

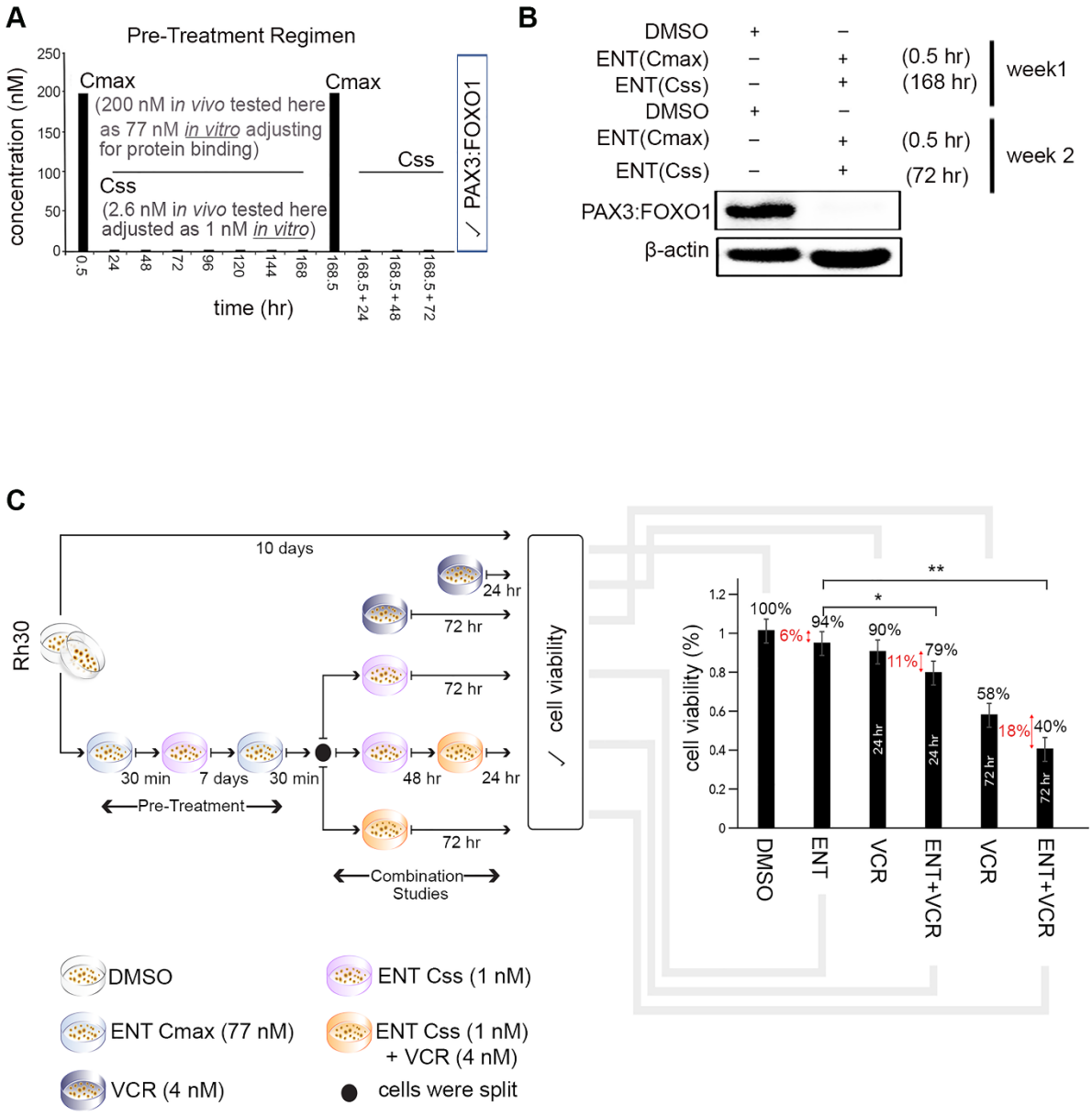

Fig. S2. ENT reduces PAX3:FOXO1 abundance at a clinically relevant dose and time of exposure and reduces cell viability synergistically with VCR. (A) Graphical representation of 10-day treatment with ENT at its clinical Cmax and Css doses (indicated; values adjusted for protein binding). **(B)** Western blot of PAX3:FOXO1 protein abundance upon treatment with a clinically relevant dose of ENT. Blot represents N=3 independent experiments. **(C)** Diagrammatic representation of Rh30 cells pulsed for 30 min with ENT at its C_{max} followed by Css for one week followed by second dose of ENT at its C_{max} and last three days' treatment at its C_{ss} with or without VCR (left panel). Graph showing cell viability for Rh30 cells pretreated with ENT, ENT plus VCR (24 hours) or ENT plus VCR (72 hours) relative to the DMSO control. Data are means ± SD. N=3 independent experiments, each in triplicate (N represents cell cultures). * $P < 0.05$, ***$P < 0.001$ by a two-sided Student's *t* test.

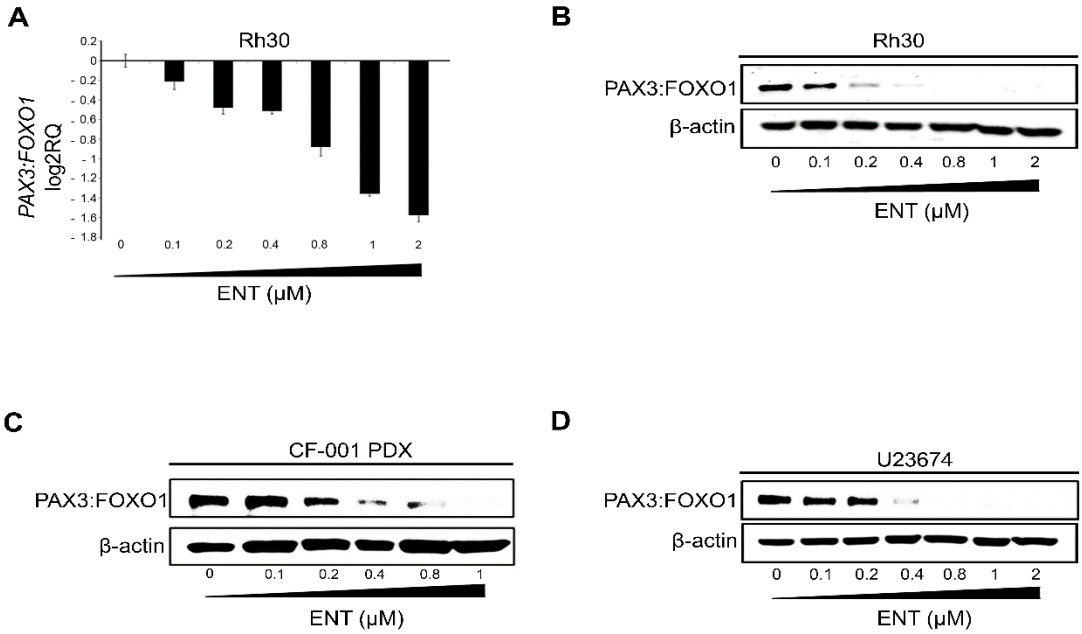

Fig. S3. ***PAX3:FOXO1*** **mRNA expression is reduced by ENT in aRMS cell lines. (A)** qPCR analysis of mRNA expression of *PAX3:FOXO1* upon 24 hours treatment with increasing amount of entinostat (0, 0.1, 0.2, 0.4, 0.8 and 1 μM) in Rh30 cells. Data are means ± SEM (N=3 independent experiments). **(B to D)** Western blot assays showing PAX3:FOXO1 protein expression upon 72 hours treatment with entinostat in Rh30, CF-1 and U23674 in a dose-dependent manner (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, and 2 μM). Blots are representative of N=3 independent experiments.

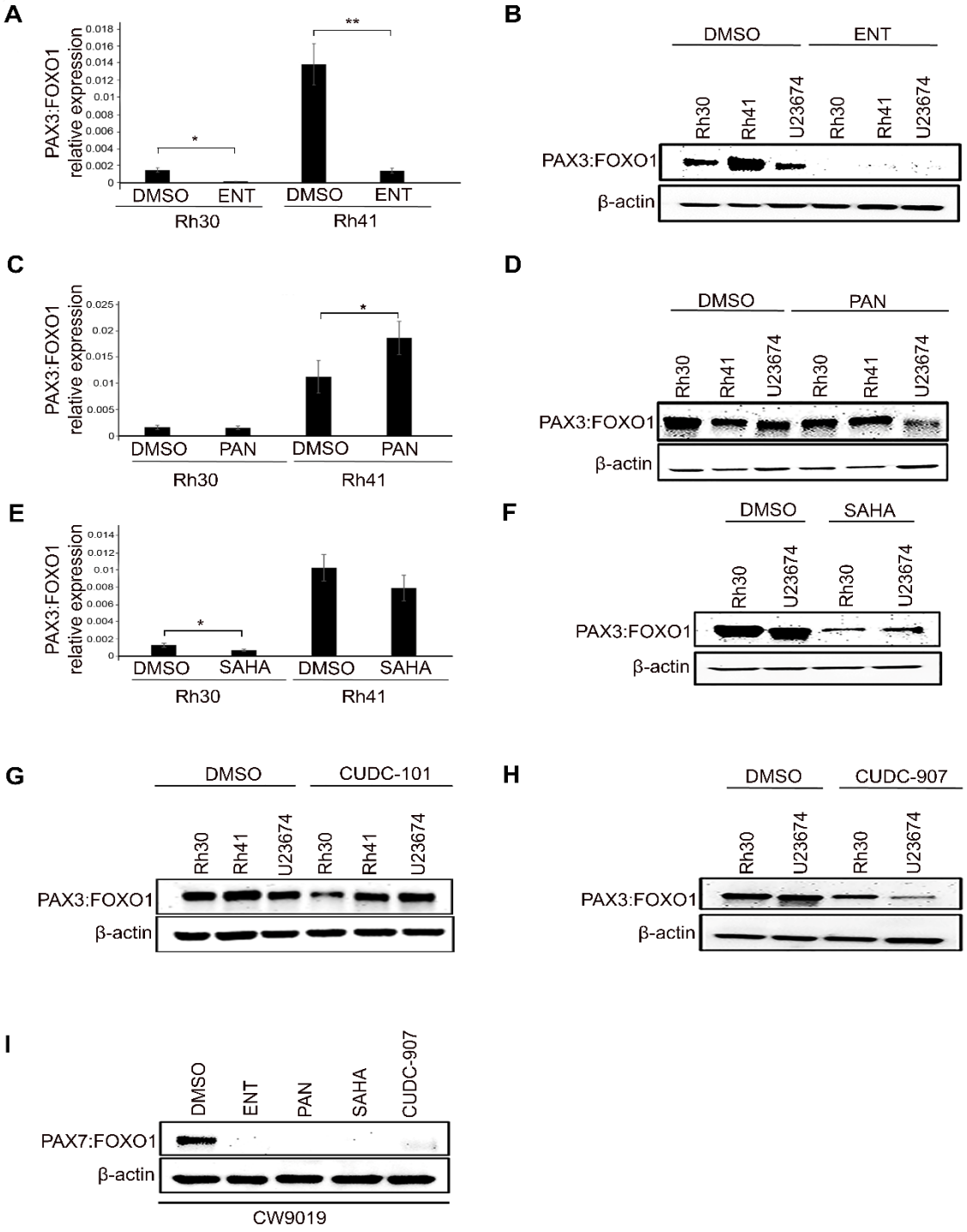

Fig. S4. Better than other HDACis, ENT reduces PAX3:FOXO1 abundance in murine and human aRMS cells. (A) PAX3:FOXO1 mRNA expression was analyzed by RT-PCR in entinostat (1μM)-treated human aRMS cell lines (Rh30 and Rh41) at the end of 24 hours. Data are means ± SD; N=3 biological replicates; *P < 0.05, **P < 0.01 by two-sided Student's *t* test. **(B)** Western blots showing relative PAX3:FOXO1 abundance upon 72 hours treatment with entinostat (1 μM). Blots are representative of N=3 independent experiments. **(C)** *PAX3:FOXO1* mRNA expression was analyzed by RT-PCR in

panobinostat (45 nM) treated human aRMS cell lines (Rh30 and Rh41) at the end of 24 hours. Data are presented as means ± SD. N=3 biological replicates, each in triplicate; * $P < 0.05$ by two-sided Student's t test. **(D)** Western blots showing relative expression of PAX3:FOXO1 upon 72 hours treatment with panobinostat (45 nM). N=3 independent Blots are representative of N=3 independent experiments. **(E)** *PAX3:FOXO1* mRNA expression was analyzed by RT-PCR in SAHA (1μM) treated human aRMS cell lines (Rh30 and Rh41) at the end of 24 hours. Data are presented as means ± SD. N=3 biological replicates; * $P < 0.05$ by a two-sided Student's *t* test. **(F to H)** Western blots showing relative decrease in the protein abundance of PAX3:FOXO1 upon 72 hours treatment with SAHA (1 μM), CUDC-101 (150 nM) and CUDC-907 (150 nM) respectively. Blots are representative of N=3 independent experiments. **(I)** PAX7:FOXO1 protein expression upon treatment with different HDACIs in CW9019 cells. Blots are representative of N=3 independent experiments.

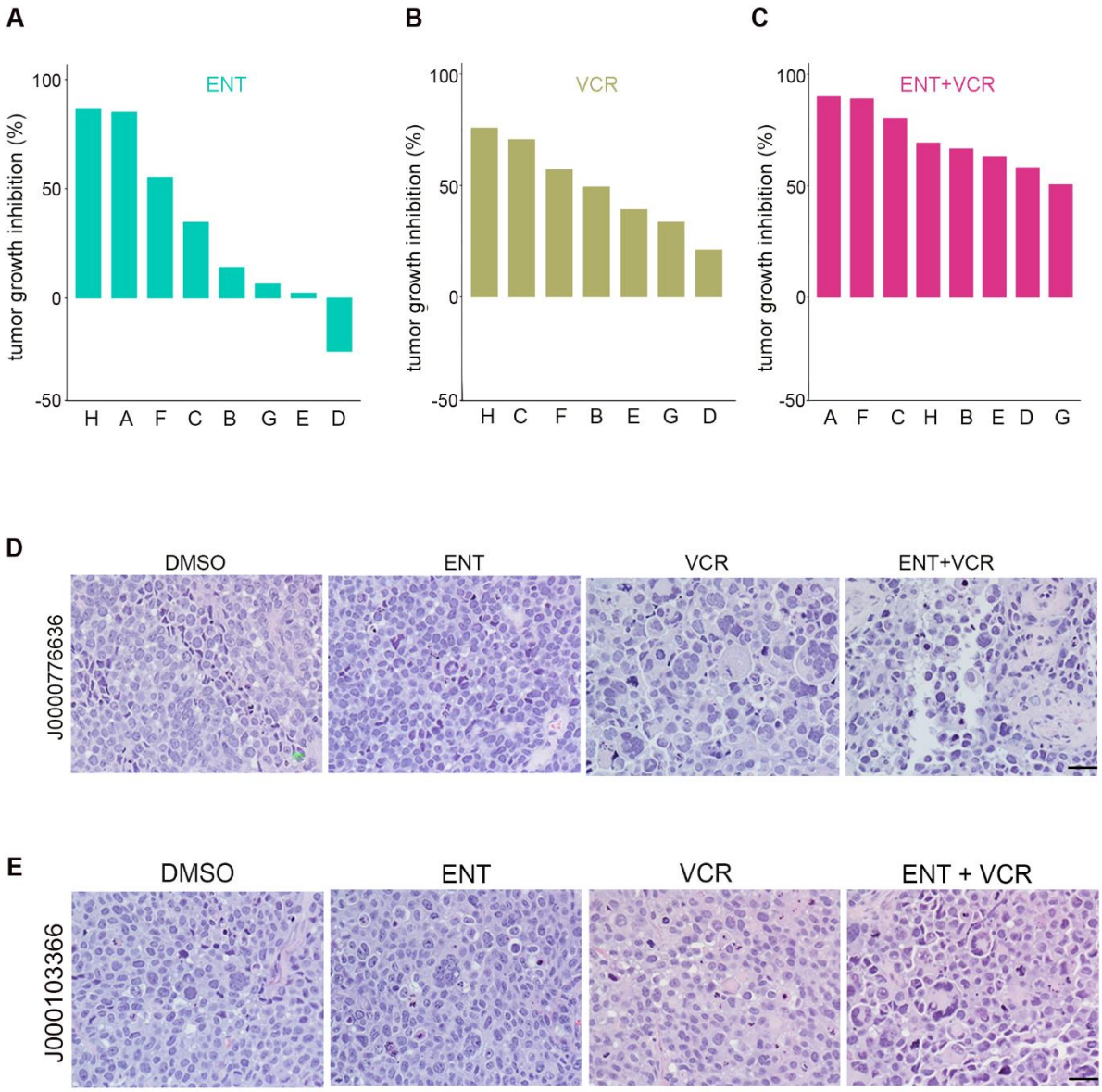

Fig. S5. Representative histology of PDX mouse aRMS tissue. (A to C) Waterfall plot showing the tumor growth inhibition (%) by either drug alone (ENT or VCR) and in combination (ENT+VCR). X-axis labeled from "A-H" corresponds to the 8 models shown in Fig. 2, A to H, respectively. **(D and E)** J000077636/PCB-481 (D) and J000103366/CF-13 (E) PDX tumors were stained by hematoxylin and eosin and scored blindly. Rhabdomyoblasts were not detected in any treatment group. Scale bar, 100 µM.

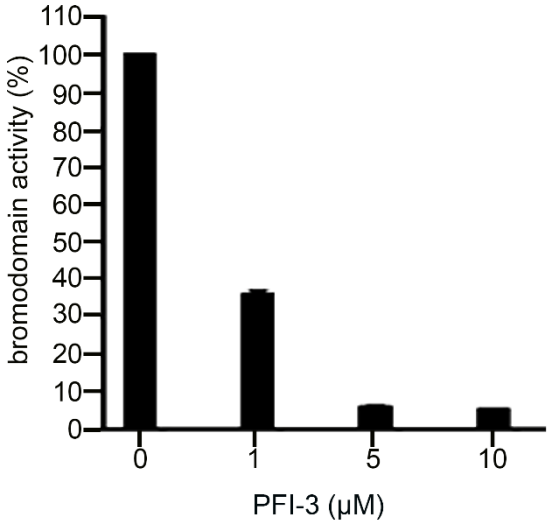

Fig. S6. PFI-3–mediated inhibition of SMARCA4 bromodomain activity. TR-FRET assay with different concentration of PFI-3 (1, 5, and 10 μM) to find the % inhibition. The TR-FRET data were analyzed using the computer software, GraphPad Prism (GraphPad Software Inc.).

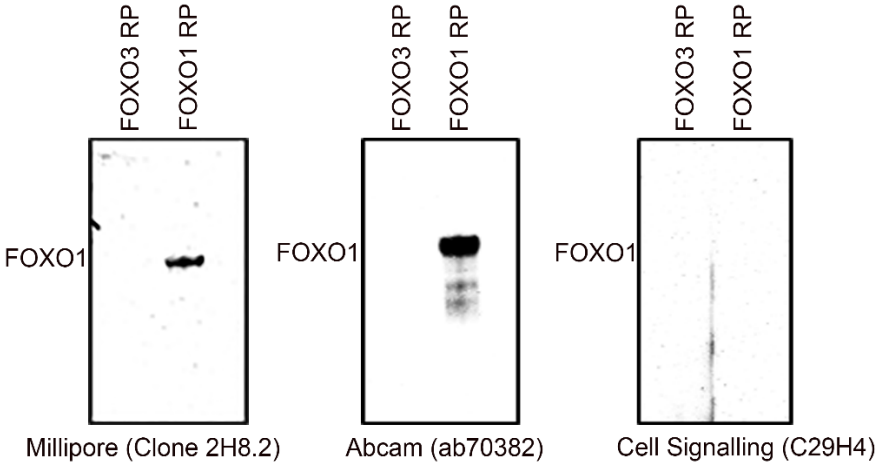

Fig. S7. Validation of commercially available antibodies for detecting endogenous FOXO1 abundance in aRMS cell lines. Recombinant FOXO1 and FOXO3 were tested with antibodies from Millipore, Abcam and Cell Signaling. For analyzing endogenous wild type FOXO1 expression, recombinant FOXO11 (NM_002015; Origene) was used. FOXO3 (NM_001455) was used as negative control to choose the appropriate antibody. Anti-FOXO1 antibody (clone 2H8.2, 1:500, Millipore) detects recombinant FOXO1 without background compared to other antibodies and subsequently used for analyzing FOXO1.

Table S1. Histological markers of differentiation in aRMS orthotopic allograft PDX mice. Scoring of % of rhabdomyoblasts in samples from mice across treatment groups for CTG-1604. N=5 mice per cohort. ENT, entinostat; VCR, vincristine.

unique identifier of each aRMS animal	% rhabdomyoblasts	Treatment
67840	0 (necrotic tumor)	DMSO
67858	2	DMSO
67841	0 (scant tumor with abundant necrosis)	DMSO
67855	0 (necrotic tumor)	DMSO
67853	0 (necrotic tumor)	DMSO
67849	5	ENT+VCR
67850	0	ENT+VCR
67859	0	ENT+VCR
67852	0	ENT+VCR
67847	2	ENT+VCR
67846	0	ENT+VCR
67838	10	VCR
67839	1	VCR
67843	2	VCR
67854	30	VCR
67845	0	VCR
67842	5	ENT
67851	0	ENT
67856	2	ENT
67857	15	ENT

Table S2. Treatment schedules for the CF-4/PCB-513 PDX mice. (**A and B)** Treatment schedules for the Champions Oncology (A) and Jackson Laboratory (B) PDX model mice, including the group of mice, number of mice, treatment agent used, doses and drug administration schedule given. p.o., oral; i.p., intraperitoneal; qd, daily; bid, twice a day; q7d, every 7 days.

A.

group	n	agent	dose (mg/kg/dose)	schedule
1	5	control		
2	5	ENT VCR	25 1	p.o./qd x21 i.p./q7d x3
3	5	vandetanib everolimus	50 20	p.o./qd x21 p.o./qd x21
4	5	pazobanib CB-839	100 200	p.o./qd x21 p.o./bid x21
5	5	ENT	25	p.o./qd x21

B.

group	n	agent	dose (mg/kg/dose)	Schedule
1	3	control		
2	3	ENT	4	p.o./qd x21
3	3	VCR	0.75	i.p./q7d x3
4	3	ENT VCR	4 0.75	p.o./qd x21 i.p./q7d x3

Table S3. Patient history of the Champions Oncology PDX aRMS models. The patient history of 3 different PDX aRMS models used in the study (Fig. 2) such as RMS model, histology, harvest site, disease stage, diagnosis and age are given. All models below express the PAX3:FOXO1 oncogene.

RMS Model	alias	histology	harvest site	genetic features	disease stage	age	sex
CTG-1409	POS-14107	alveolar	abdomen	PAX3:FOXO1	IV; recurrent	14	M
CTG-1604	POS-14175	alveolar	lymph node	PAX3:FOXO1	IV; recurrent	36	M
CTG-1008	POS-11038B	alveolar	uterus	PAX3:FOXO1	IV; recurrent	11	F

Table S4. Patient history of The Jackson Laboratory PDX aRMS models. The patient history of 5 different PDX aRMS models used in the study (Fig. 2) such as RMS model, histology, harvest site, disease stage, diagnosis, age and se are given. All models below except CF-2 express the PAX3:FOXO1 oncogene; CF-2 expresses PAX7:FOXO1.

RMS Model	alias	histology	harvest site	genetic features	disease stage	age	sex
CF-1	J000099761	alveolar	disseminated met (autopsy)	PAX3:FOXO1	IV	1.8	M
PCB481	J000077636	alveolar	brain met (autopsy)	PAX3:FOXO1	IV	13	M
CF-4	J000101220	alveolar	disseminated met (autopsy)	PAX3:FOXO1	IV	36	M
CF-13A	J0000103366	alveolar	lung	PAX3:FOXO1	IV	24	M
CF-2	J000099873	alveolar	disseminated met (autopsy)	PAX7:FOXO1	IV	6	F

Table S5. Histological scoring of markers of differentiation in PDX aRMS mice. Tables below show scoring of % of rhabdomyoblasts in samples from PDX models (J000103366, top; J000077636, bottom) across different treatment groups (Fig. 2). N=3 mice per cohort.

unique identifier of each PDX animal (J000103366)	% rhabdomyoblasts	Treatment
366-18	1	DMSO
366-06	< 1	DMSO
366-19	< 1	DMSO
366-20	0	ENT
366-09	< 1	ENT
366-17	< 1	ENT
366-01	1	VCR
366-05	1	VCR
366-14	1	VCR
366-13	< 1	ENT+VCR
366-07	1	ENT+VCR
366-12	< 1	ENT+VCR

unique identifier of each PDX animal (J000077636)	% rhabdomyoblasts	Treatment
219-04	< 1	DMSO
219-07	< 1	DMSO
219-11	1	DMSO
219-01	< 1	ENT
219-08	0	ENT
219-12	< 1	ENT
219-02	< 1	VCR
219-15	< 1	VCR
219-16	< 1	VCR
219-13	< 1	ENT+VCR
219-14	1	ENT+VCR
219-18	< 1	ENT+VCR

Table S6. Statistical analysis of CTG-1604/POS-14175 data. Statistical summary for PDX model CTG-1604 is given below. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=5 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
20	control	ENT	0.83±0.12	(0.33, 1.33)	<0.001
20	control	ENT/VCR	1±0.12	(0.5, 1.49)	<0.001
20	control	pazobanib / CB839	0.61±0.11	(0.17, 1.05)	<0.001
20	control	vandetanib/ everolimus	0.66±0.12	(0.16, 1.16)	<0.001
20	entinostat	ENT/VCR	0.17±0.14	(-0.41, 0.74)	1
20	entinostat	pazobanib / CB839	-0.22±0.13	(-0.74, 0.31)	1
20	entinostat	vandetanib/ everolimus	-0.17±0.14	(-0.74, 0.41)	1
20	ENT/VCR	pazobanib / CB839	-0.39±0.13	(-0.91, 0.14)	0.48
20	ENT/VCR	vandetanib/ everolimus	-0.34±0.14	(-0.91, 0.24)	0.88
20	Pazopanib / CB839	vandetanib/ everolimus	0.05±0.13	(-0.47, 0.57)	1

Table S7. Statistical analysis of J101220/CF-4 data. PDX model (J101220) statistical summary is given below. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=3 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
18	control	ENT	0.06±0.1	(-0.36, 0.48)	1
18	control	ENT/VCR	0.51±0.1	(0.08, 0.93)	0.004
18	control	ENT/VCR	0.29±0.1	(-0.13, 0.72)	0.67
18	ENT	ENT/VCR	0.45±0.1	(0.02, 0.87)	0.03
18	ENT	VCR	0.23±0.1	(-0.19, 0.66)	0.96
18	ENT/VCR	VCR	-0.21±0.1	(-0.64, 0.21)	0.99

Table S8. Statistical analysis of J77636/PCB-481 data. Statistical summary for PDX model J77636 is given below. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=3 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
20	control	ENT	0.16±0.18	(-0.55, 0.87)	1
20	control	ENT/VCR	0.83±0.18	(0.14, 1.52)	0.006
20	control	VCR	0.72±0.18	(0.03, 1.42)	0.03
20	ENT	ENT/VCR	0.67±0.16	(0.02, 1.31)	0.03
20	ENT	VCR	0.56±0.16	(-0.09, 1.2)	0.18
20	ENT/VCR	VCR	-0.11±0.16	(-0.74, 0.52)	1

Table S9. Statistical analysis of J0103366/CF-13A data. Statistical summary for PDX model J0103366. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=3 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
27	control	ENT	-0.14±0.11	(-0.6, 0.31)	1
27	control	ENT/VCR	0.35±0.11	(-0.11, 0.8)	0.46
27	control	VCR	-0.06±0.12	(-0.53, 0.42)	1
27	ENT	ENT/VCR	0.49±0.11	(0.03, 0.95)	0.02
27	ENT	VCR	0.09±0.12	(-0.39, 0.56)	1
27	ENT/VCR	VCR	-0.4±0.12	(-0.88, 0.07)	0.23

Table S10. Statistical analysis of J099761/CF-1 data. Statistical summary for PDX model J099761. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing, all two-sided. Data are means ± SEM, N=3 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
28	control	ENT	-0.07±0.09	(-0.44, 0.31)	1
28	control	ENT/VCR	0.44±0.09	(0.08, 0.8)	0.003
28	control	VCR	0.22±0.09	(-0.14, 0.58)	0.84
28	ENT	ENT/VCR	0.51±0.09	(0.13, 0.88)	<0.001
28	ENT	VCR	0.29±0.09	(-0.08, 0.66)	0.41
28	ENT/VCR	VCR	-0.22±0.09	(-0.58, 0.14)	0.86

Table S11. Statistical analysis of CTG-1409/POS 14107 data. Statistical summary for PDX model CTG-1409 is given above. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=2 mice per cohort).

day	*group*	*comparator*	*difference*	*CI*	*p value*
27	control	ENT	-0.04±0.34	(-1.49, 1.4)	1
27	control	ENT/VCR	0.59±0.34	(-0.86, 2.03)	1
27	control	VCR	-0.01±0.34	(-1.45, 1.44)	1
27	ENT	ENT/VCR	0.63±0.34	(-0.82, 2.07)	0.99
27	ENT	VCR	0.03±0.34	(-1.41, 1.48)	1
27	ENT/VCR	VCR	-0.59±0.34	(-2.04, 0.85)	1

Table S12. Statistical analysis of J099873/CF-2 data. Statistical summary for PDX model J099873 is given above. The significance (P values) of pairwise treatment comparisons were based on a repeated measures linear model with a Tukey correction for multiple testing. All testing was two-sided. Data are means ± SEM, N=3 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
17	control	ENT	0.01±0.08	(-0.29, 0.32)	1
17	control	ENT/VCR	0.29±0.08	(-0.04, 0.63)	0.16
17	control	VCR	0.17±0.08	(-0.16, 0.51)	0.9
17	ENT	ENT/VCR	0.28±0.08	(-0.06, 0.61)	0.2
17	ENT	VCR	0.16±0.08	(-0.17, 0.5)	0.94
17	ENT/VCR	VCR	-0.12±0.09	(-0.48, 0.24)	1

Table S13. Statistical analysis of CTG-1008 data. Statistical summary for PDX model CTG-1008. The significance of variation (P value) in tumor volume with treatment was assessed with a repeated measures linear model with an autoregressive order 1 autocorrelation matrix and a Tukey correction for multiple comparisons of treatment, day, and treatment-by-day interaction. Data are means ± SEM, N=2 mice per cohort.

day	*group*	*comparator*	*difference*	*CI*	*p value*
53	control	ENT	0.87±0.1	(0.42, 1.32)	<0.001
53	control	ENT/VCR	0.55±0.12	(0.01, 1.09)	0.04
53	control	VCR	0.62±0.1	(0.17, 1.06)	<0.001
53	ENT	ENT/VCR	-0.32±0.12	(-0.86, 0.23)	0.94
53	ENT	VCR	-0.25±0.1	(-0.7, 0.19)	0.96
53	ENT/VCR	VCR	0.06±0.12	(-0.48, 0.6)	1