Supporting Information

Chen et al. 10.1073/pnas.1317731111

SI Methods

T-Cell Factor/Lymphoid Enhancer Factor Reporter Experiments. RD and 381T cells were seeded in 96-well clear-bottom plates (Fisher) at a density of 5×10^3 cells per well and transfected with T-cell factor/lymphoid enhancer factor (TCF/LEF) dual-luciferase (firefly luciferase and Renilla) constructs essentially as described in the Qiagen TCF/LEF Cignal Report Assay kit protocol. Firefly luciferase is driven by tandem repeats of TCF/LEF promoter element, whereas Renilla is expressed constitutitvely by a ubiquitous CMV promoter. Six hours after transfection, the media (10% FBS in DMEM) was changed, and cells were treated with 0.1% DMSO, BIO (200–1,000 nM), or Wnt-3a (400 ng/mL) for 24 h. Cells were supplemented with luciferase assay substrate from the Dual Luciferase Assay kit (Promega) and processed per the manufacturer's protocol before the measurement of luciferase activity using a microplate reader (Spectramax). There was a 10-s measurement period for each reporter assay, with the firefly luciferase activity quenched by the Stop and Glo reagent (supplied with the kit) before reading the Renilla luciferase activity. Each measurement of firefly luciferase activity was normalized to Renilla activity.

Analysis of Zebrafish Embryonal Rhabdomyosarcoma. Immunohistochemical analysis of zebrafish sections was performed essentially as previously described (1, 2) except for β-catenin (CTNNB1) staining, which was performed after antigen retrieval in EDTA buffer at 95 °C. Antibody dilutions used for immunohistochemistry on paraffin-embedded sections of zebrafish embryonal rhabdomyosarcoma (ERMS) were β-catenin (Sigma), 1:250; phospho-H3 (Millipore), 1:1,000. BGAR, biotinylated goat secondary anti-rabbit (Vector #BA-1000) was at 1:250 dilution. 5-ethynyl-2′-deoxyuridine (EdU) and Annexin stains were performed essentially as previously described (3).

Isolation of Zebrafish Tumors for Quantitative RT-PCR. Tumor tissue was isolated from ERMS-bearing fish treated with DMSO, BIO, or CHIR99021 by fine dissection. The tissue was macerated using a tissue grinder pestle and subsequently lysed in TRIzol reagent (Life Technologies). RNA extraction was performed per the manufacturer's protocol. RNA (500–1,000 ng) was used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Life Technologies).

1. Chen EY, et al. (2013) Cross-species array comparative genomic hybridization identifies novel oncogenic events in zebrafish and human embryonal rhabdomyosarcoma. *PLoS Genet* 9(8):e1003727.
2. Langenau DM, et al. (2007) Effects of RAS on the genesis of embryonal rhabdomyosarcoma. *Genes Dev* 21(11):1382–1395.
3. Ignatius MS, et al. (2012) In vivo imaging of tumor-propagating cells, regional tumor heterogeneity, and dynamic cell movements in embryonal rhabdomyosarcoma. *Cancer Cell* 21(5):680–693.

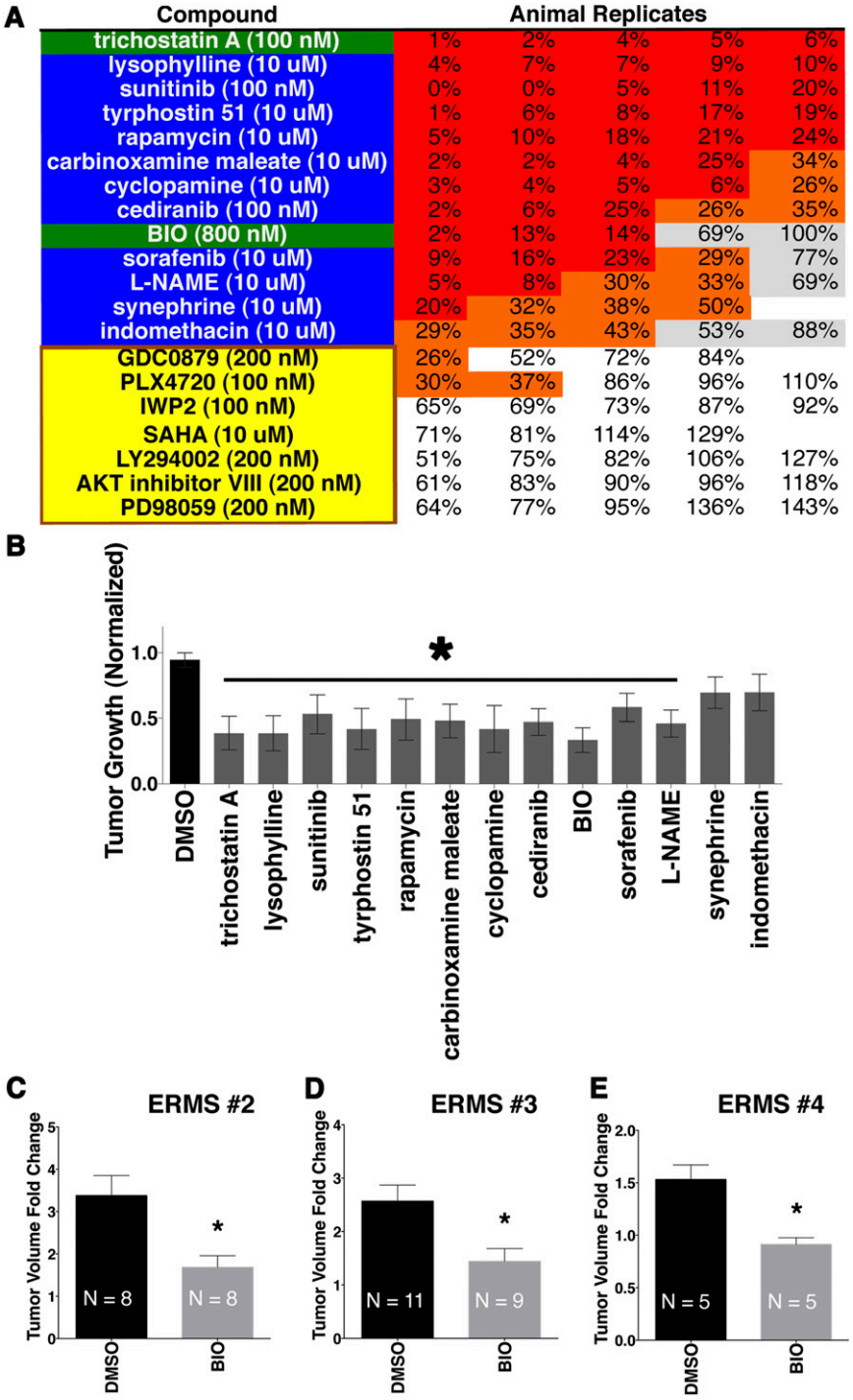

Fig. S1. Chemical hits that reduced tumor growth in zebrafish engrafted with ERMS. Summary of tumor volume changes within individual animals treated with each compound. Tumor volume of <25% when compared with DMSO-treated tumors indicated by red, 25–50% by orange, and >50% by gray. (*B*) The 13 lead compounds were revalidated in a separate transplantation experiment using a different fluorescent-labeled ERMS. Summary of tumor growth normalized to that of DMSO is shown. $n = 4$–8. Each error bar indicates SEM. *$P < 0.05$. (*C*–*E*) Three additional transplanted ERMS were assessed for BIO effects on tumor growth. N, number of animals treated per group. Each error bar denotes SEM. *$P < 0.05$, *t* test.

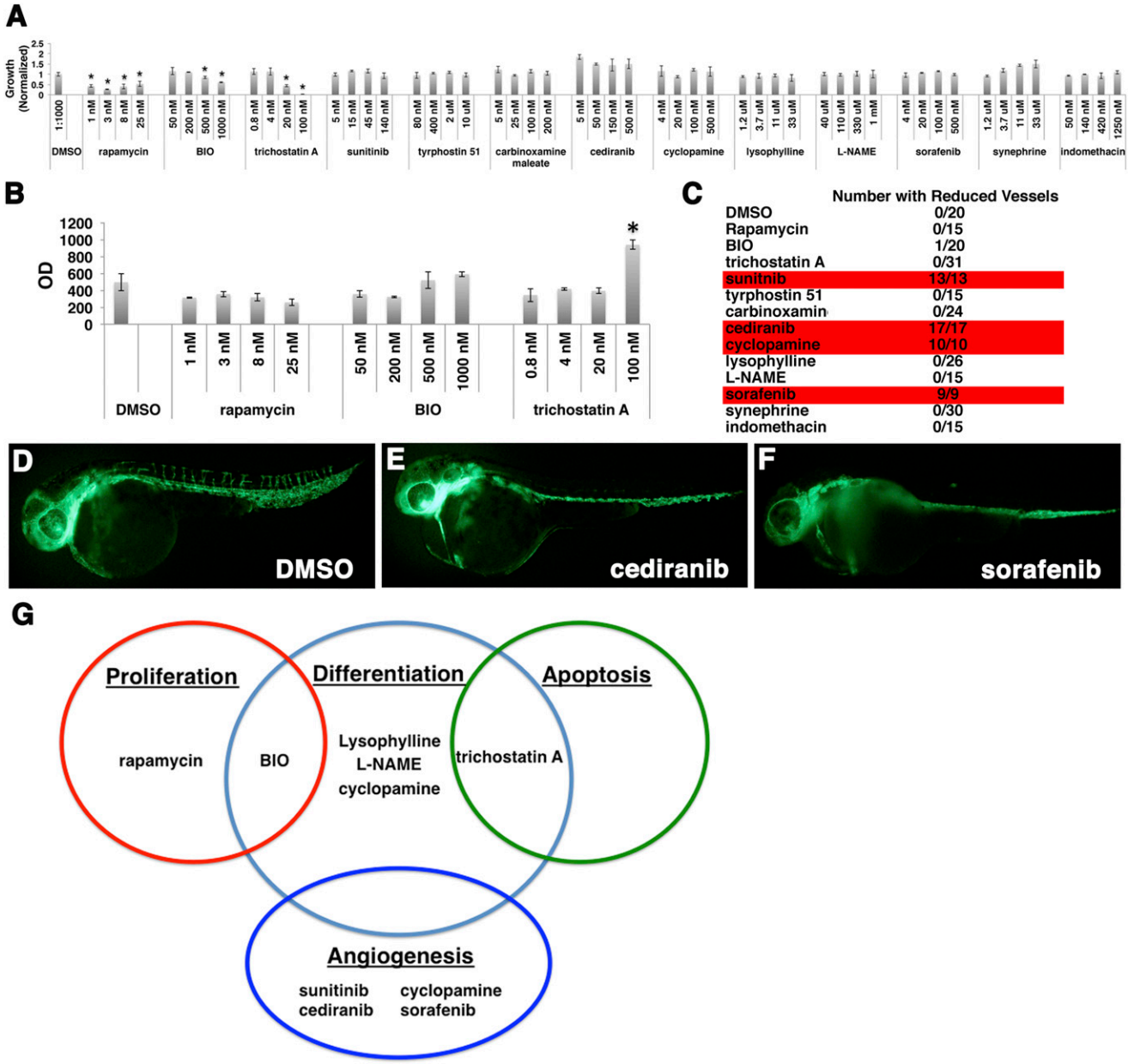

Fig. S2. Lead compounds functionally alter cancer-specific processes. (*A*) RD cells were treated with lead compounds from the zebrafish secondary screen at four concentrations in the range of IC_{50}. Cell growth/viability were assessed by cell titer glo after 4 d of drug treatment. Relative growth was normalized to cell numbers contained within each well at the beginning of the experiment. *Statistical significance with $P < 0.05$. (*B*) Lead compounds that affected growth (rapamycin, BIO, and trichostatin A) were further tested for their role in regulating apoptosis using a caspase glo assay. Average OD values from triplicate plating in an assay are shown. Each error bar denotes SD. *P value < 0.05, Student t test comparing test compound and DMSO. (*C*) Summary of angiogenesis assay. Tg(*fli1*:*GFP* embryos) were treated with DMSO and lead compounds (50 nM–10 μM) starting at early bud stage for 48 h. The effect of each compound on angiogenesis of intersegmental and tail vessels was assessed. Red indicates compounds that inhibit angiogenesis in *fli1*:*GFP* embryos. (*D–F*) Representative images of DMSO-treated (*D*), cediranib-treated (*E*), and sorafenib-treated (*F*) *fli1*:*GFP* embryos are shown. (*G*) Venn diagram summarizing functional categories of lead compounds in ERMS tumorigenesis.

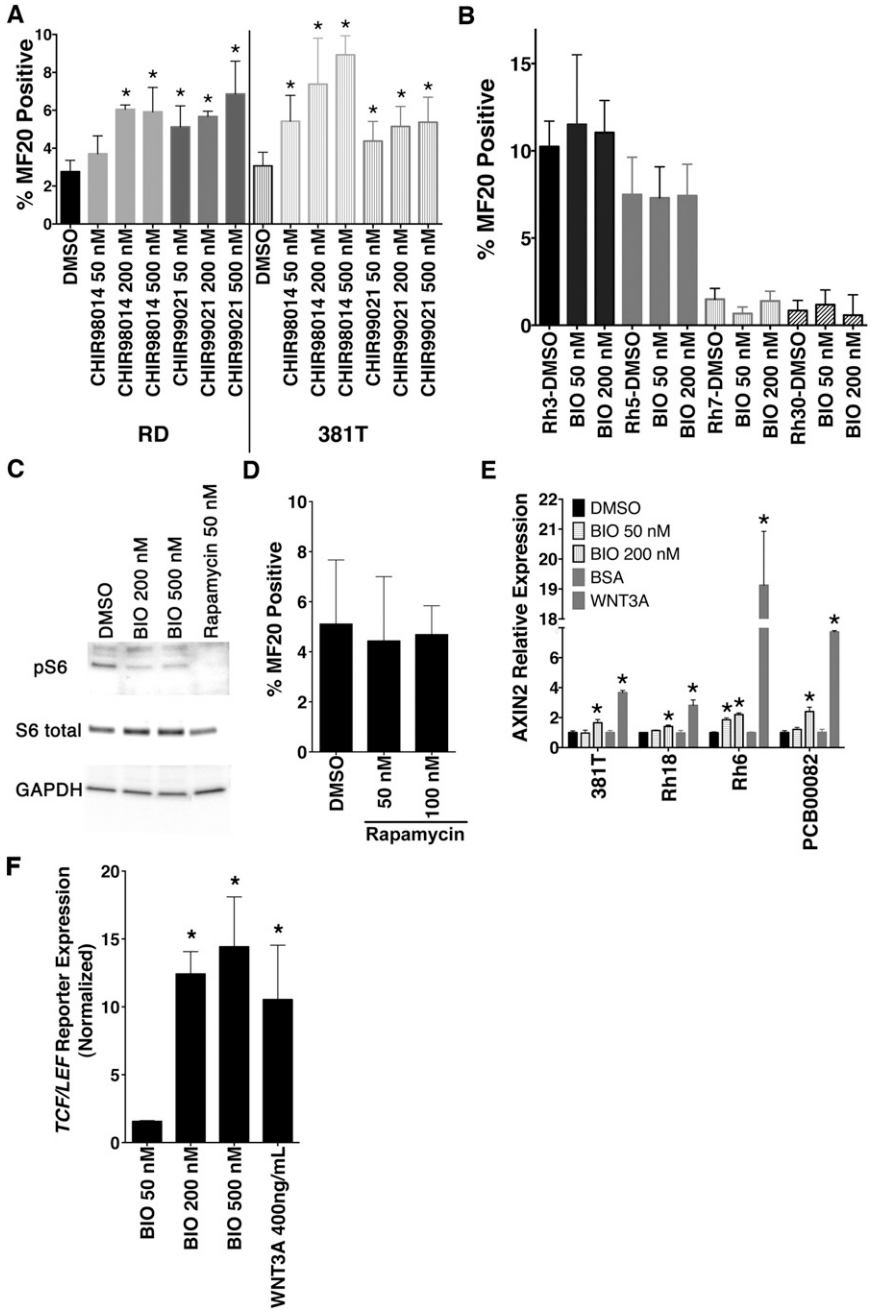

Fig. S3. The β-catenin/WNT pathway enhances ERMS differentiation, whereas activation of mammalian target of rapamycin-C1 (mTORC1) does not alter differentiation. (*A*) Summary of MF20 staining of RD and 381T cells treated with DMSO, CHIR98014, and CHIR99021. $*P < 0.05$. (*B*) Summary of MF20 staining of four alveolar rhabdomyosarcoma cell lines. (*C*) Western blot analysis of RD cells treated with BIO and rapamycin. (*D*) Quantization of MF20 staining in RD cells after treatment with rapamycin. (*E*) Real-time RT-PCR analysis of *AXIN2* expression in ERMS cell lines treated with BIO and WNT3A for 24 h. Expression level is normalized to that of DMSO-treated cells. $*P < 0.05$. (*F*) *TCF/LEF* reporter assay on RD cells treated with BIO and WNT3A. $*P < 0.05$.

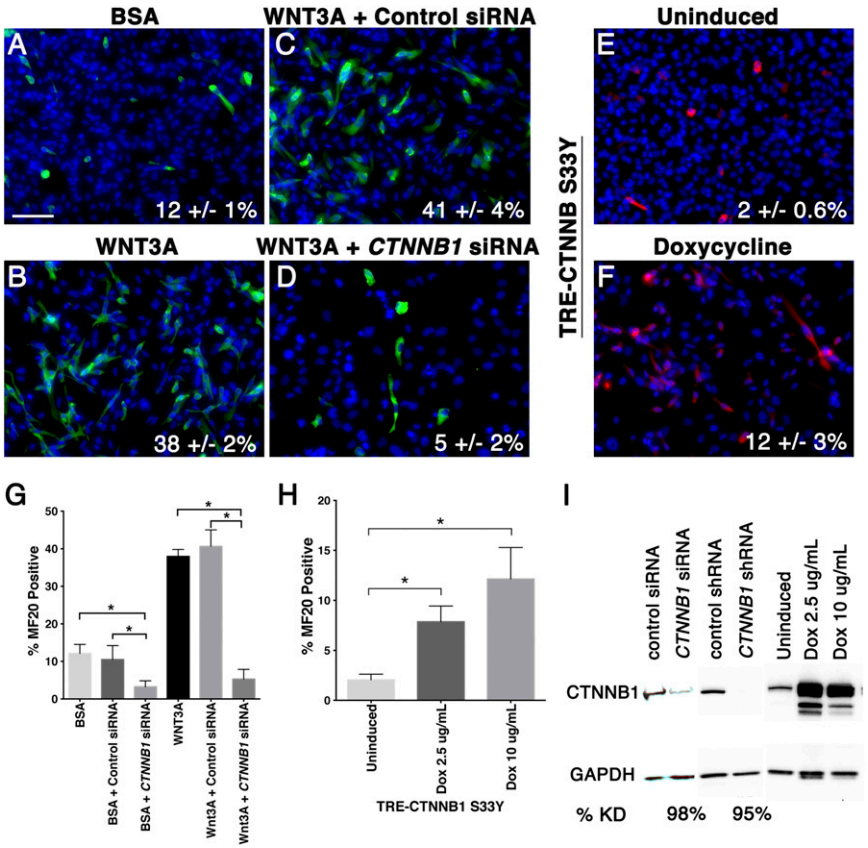

Fig. S4. Activation of canonical WNT pathway induces differentiation in 381T ERMS cells. (*A–D*) Immunofluorescent analysis for MF20 expression in human 381T ERMS cell line treated with BSA (*A*) and WNT3A (400 ng/mL) (*B*), transfected with control siRNA (*C*) and *CTNNB1* siRNA (*D*) in the presence of WNT3A. *Statistically significant differences between experimental and control treated cells ($P < 0.05$). (Scale bar, 100 μm.) (*E* and *F*) 381T cells were engineered to express a doxycycline-inducible CTNNB1S33Y that constitutively activates the canonical WNT/β-catenin pathway. Uninduced control (*E*) and induced (*F*) cells stained with MF20 (red) and DAPI (blue). (*G*) Summary of MF20 staining in cells treated with Wnt-3a, BSA (vehicle control), control siRNA, and *CTNNB1* siRNA. (*H*) Summary of MF20 staining in stable cell lines harboring doxycycline-inducible CTNNB1S33Y. (*I*) Western blot analysis. Percentage of knockdown is noted.

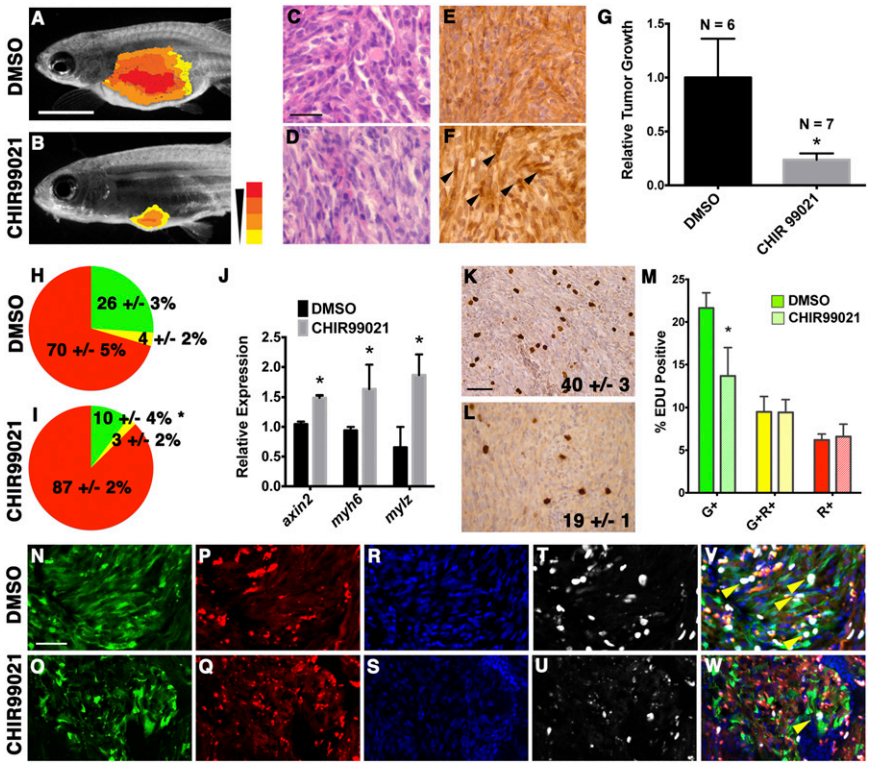

Fig. S5. CHIR99021 inhibits ERMS tumor growth and alters differentiation of tumor cells. (*A* and *B*) Representative images of ERMS-bearing fish after 6 d of drug treatment with DMSO (*A*) or 400 nM CHIR99021 (*B*). (Scale bar, 0.2 cm.) Heat map scale indicates increasing tumor volume intensity. (*C*–*F*) H&E-stained sections (*C* and *D*) and immunohistochemistry for β-catenin (*E* and *F*). (Scale bar, 20 μm.) (*G*) Summary of tumor volume changes. N, number of tumor-bearing fish per treatment group. Each error bar denotes SEM. (*H* and *I*) Summary of frequency of tumor cell subpopulations upon treatment with (*H*) DMSO (vehicle) and (*I*) CHIR99021 by FACs analysis. Each pie chart represents an average of three tumor-bearing fish analyzed, showing the relative percentage of *myf5*:GFP$^+$ /*mylz2*:mCherry$^-$ tumor-propagating cells (green), *myf5*:GFP$^+$/*mylz2*:mCherry$^+$ (yellow), and late-differentiating *myf5*:GFP$^-$/*mylz2*:mCherry$^+$ cells (red). SD for each fraction is indicated. *Significance of <0.05. (*J*) Quantitative RT-PCR analysis. *$P < 0.05$, Student *t* test. Each error bar indicates SEM. $n = 3$–4 tumors analyzed for each treatment group. (*K* and *L*) Immunohistochemistry for Phospho-H3 for DMSO- (*K*) and CHIR99021-treated (*L*) tumors. The number of Phospho-H3-positive cells was quantified in three independent fields at 400× magnification. The values (with SEM) indicate average of three tumors analyzed for each treatment group ($P < 0.05$). (Scale bar, 20 μm.) (*M*) Summary of EdU analysis. Larval fish engrafted with tumors expressing *myf5*:GFP and *mylz2*:mCherry were treated with DMSO or CHIR99021 and pulsed with EdU for IF analysis. Each error bar indicates SEM of three tumors from each treatment group. *$P < 0.005$, Student *t* test. (*N*–*W*) Representative images from EdU IF staining. GFP (*N* and *O*), mCherry (*P* and *Q*), DAPI (*R* and *S*), and EdU (*T* and *U*). (*V* and *W*) Merge image of all four channels. Yellow arrowheads, representative *myf5*:GFP$^+$/*mylz2*:mCherry$^-$ cells that have EDU incorporation. (Scale bar, 20 μm.)

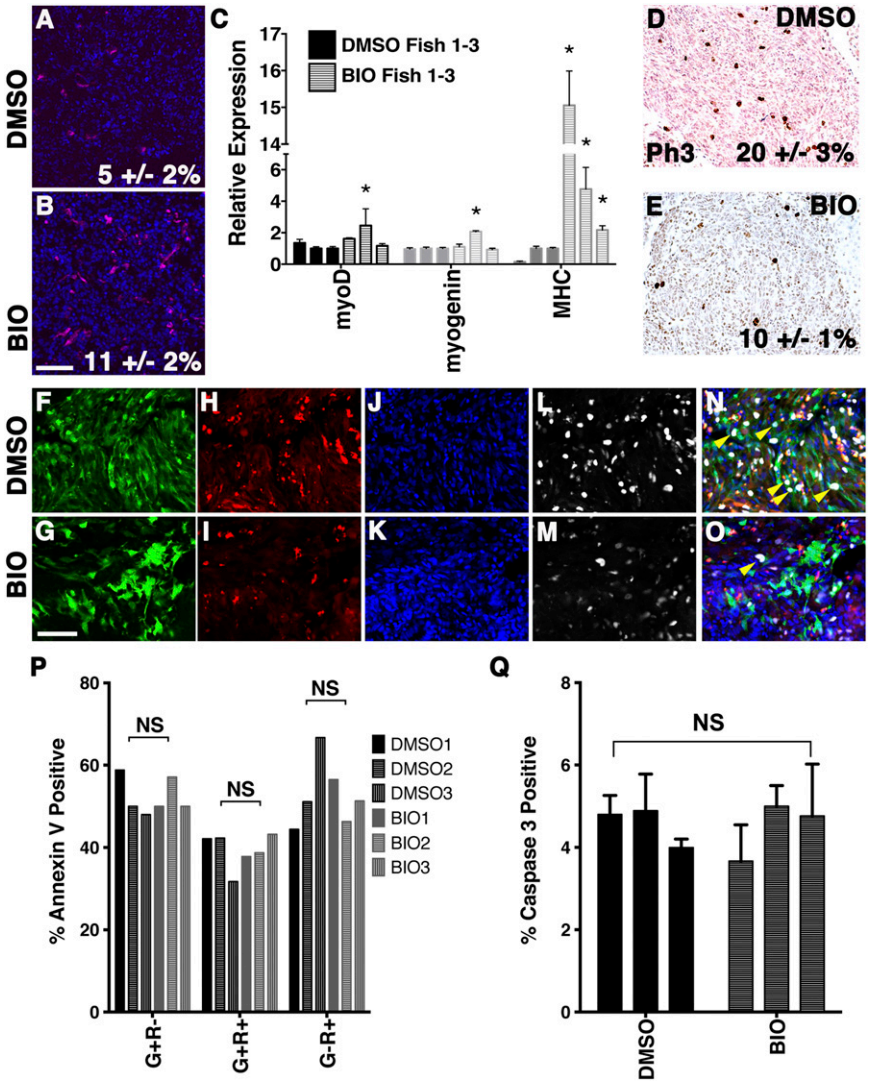

Fig. S6. BIO alters the differentiation status of zebrafish ERM tumor cells. (*A*–*C*) Transplanted ERMS tumor-bearing larval zebrafish were treated with DMSO and BIO (300 nM) for 5 d. MF20 staining was performed on cryosections of treated tumors to assess the extent of terminal differentiation. (*A*) DMSO-treated tumor. (*B*) BIO-treated tumor. Three independent tumors from each treatment group were analyzed. Average percentage of MF20-positive cells ±SD are noted. (Scale bar, 20 μM.) (*C*) Real-time RT-PCR assessing expression of myogenic markers in DMSO- and BIO-treated fish ($n = 3$). Error bar indicates SD across experimental triplicate. *$P < 0.05$ in comparison with the DMSO-treatment group. Myosin heavy chain-6 (*mhc*). (*D* and *E*) Immunohistochemistry for Phospho-H3. The number of Phospho-H3-positive cells was quantified in three independent fields at 400× magnification. The values (with SEM) indicate average of three tumors analyzed for each treatment group. (*F*–*O*) EdU analysis. Larval fish engrafted with tumors expressing *myf5*:GFP and *mylz2*:mCherry were treated with DMSO or BIO and pulsed with EdU for immunofluorescence (IF) analysis. GFP (*F* and *G*), mCherry (*H* and *I*), DAPI (*J* and *K*), and EdU (*L* and *M*). (*N* and *O*) Merge image of all four channels. Yellow arrowheads denote representative *myf5*:GFP$^+$/*mylz2*:mCherry$^-$ cells that have EdU incorporation. (Scale bar, 50 μM.) (*P*) Analysis of apoptosis by Annexin V staining after multiparameter FACS. (*Q*) Quantitation of caspase-3 immunohistochemical staining. Average of number of positive cells in three high-power (400× magnification) fields is shown for three independent tumors treated with either DMSO or BIO. Each error bar indicates SD. NS, not significant.

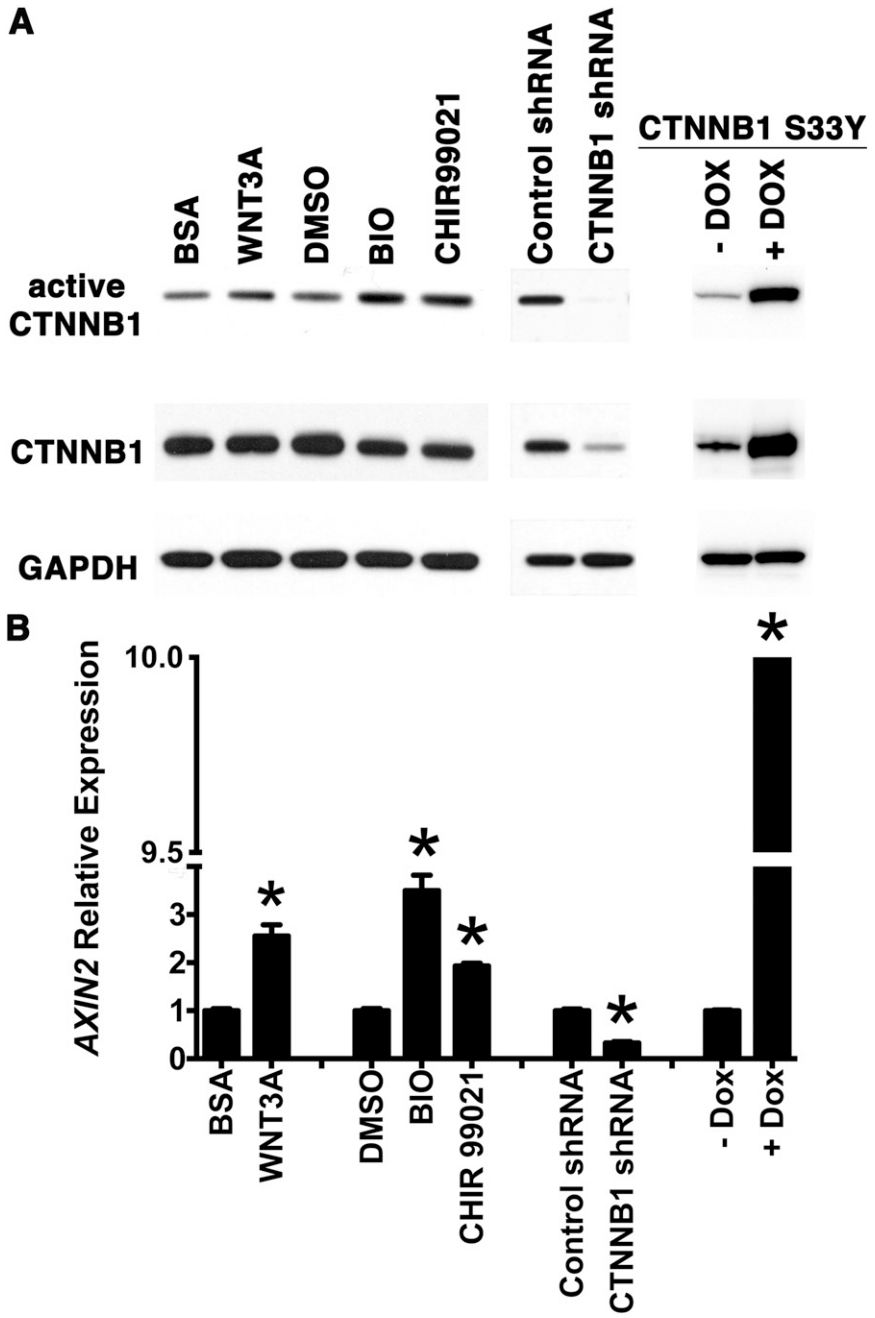

Fig. S7. Human ERMS spheres activate canonical WNT signaling upon stimulation. (*A*) Western blot analysis for active nonphosphorylated CTNNB1 (Ser33/37/Thr41) and total CTNNB1 in spheres derived from the RD cell line treated with BSA (carrier only control), WNT3A, DMSO (vehicle control), BIO, and CHIR 99021, as well as cells stably transduced with control shRNA, CTNNB1shRNA, and doxycycline (DOX)-inducible CTNNB1 S33Y. (*B*) Quantitative RT-PCR analysis of canonical WNT target gene, *AXIN2*, in treated spheres derived from the RD cell line. Each error bar indicates SD of experimental triplicate. *$P < 0.05$ in comparison with the control treatment group.

Table S1. Remaining nonhit compounds from the zebrafish tumor growth screen

Compound	(%)	Animal replicates (%)			
Valinomycin	0.2	53	63	92	109
Vincristine	17	34	57	119	125
Cyclophosphamide	60	80	97	119	
Ketotifen fumarate	45	72	85	96	
OBAA	47	73	152	85	107
Ronidazole	48	62	77	103	
5-Azacytidine	53	62	193	63	120
Pazopanib	65	76	119	85	118
Tropine	104	109	109	D	D
γ-Secretase I	120	222	231	234	D
RG-108	180	211	231	283	D
PI-103	178	231	211	327	D
Compound E	121	91	231	D	D
NS-398	71	99	99	D	D
N-NLAM	224	116	228	93	D
9-cis-retinoid a	79	169	D	D	D
XK-469	15	191	245	32	D
Mycophenolic Acid	110	140	256	454	D
Clidinium bromide	47	65	145	145	D
NMDA	65	90	99	160	365
Purpurogallin	57	85	D	D	D
Catechin	72	113	D	D	D
Fluorometholone	126	254	D	D	D
NAD	44	53	80	160	D
Hesperetin	91	120	D	D	D
Isoetharine mesylate	63	90	98	150	D
Levamisole HCl	25	76	166	200	D
Dipyrone	107	126	270	271	D
Clobetasol propionate	53	58	67	134	D
Trioxsalen	28	62	80	120	D
Tyrphostin AG-358	67	75	77	93	D
Pyrrolidine carbothionic	13	66	D	D	D
B581	32	52	92	D	D
Suprofen	33	57	65	83	128
PGE	65	86	87	105	D
Tamoxifen citrate	25	108	117	D	D
LiCl	131	226	303	D	D
Lidoflazine	54	61	80	103	
Ordansetron	103	D	D	D	D
Wortmannin	D	D	D	D	D
Lovastatin	D	D	D	D	D
N-P-TPCK	D	D	D	D	D
Bortezomib	D	D	D	D	D
Cinngel	D	D	D	D	D
Antimycin A	D	D	D	D	D
Tyrphostin A9	D	D	D	D	D
Oligomycin	D	D	D	D	D
Simvastatin	D	D	D	D	D

Table S1. Cont.

Compound	(%)		Animal replicates (%)		
Menandione	D	D	D	D	D
Benzyl-isothiocyanate	D	D	D	D	D
Eodin	D	D	D	D	D
Aceclofenac	D	D	D	D	D
5,7-dichloro-8-quinolinol	D	D	D	D	D
Celastrol	D	D	D	D	D
Clofibrate	D	D	D	D	D
Ciclopirox olamine	D	D	D	D	D
2,2 thiobis (4,6 dichlorophenol)	D	D	D	D	D
Promazine hydrochloride	D	D	D	D	D
Benzbromarone	D	D	D	D	D
Tracozolate hydrochloride	D	D	D	D	D
Haloperidol	D	D	D	D	D
Riluzole	D	D	D	D	D
Loperamide hydrochloride	D	D	D	D	D
Rosuvastatin	D	D	D	D	D
6-methoxyharmalan	D	D	D	D	D
SFK-95282	D	D	D	D	D
Echinomycin	D	D	D	D	D
H-89 dihydrochloride	D	D	D	D	D
(+)-bicuculline	D	D	D	D	D
		0–20% relative growth to DMSO			
		21–50%			
		>50%			
		Death from drug toxicity			

Table S2. RT-PCR primer sequences

Zebrafish		
18S	Forward	TCGCTAGTTGGCATCGTTTATG
	Reverse	CGGAGGTTCGAAGACGATCA
myca	Forward	TATGCTGCAAGTGACCGGAG
	Reverse	TCACCGGCATTTTGACACTTG
ccnd1	Forward	TGCGACAGACGTCAACTTCA
	Reverse	CAGTCAACAGTTTGGGCGTG
axin2	Forward	GATAGCCAGACTGGAGCG
	Reverse	CGCTCTTCCTCCTCCTGATC
mylz2	Forward	ACCGCAGAGGAGATGAAGAA
	Reverse	TCCGTGTGTGATGACGTAGC
myh6	Forward	CAGCTGGAGGAGAAGGAATG
	Reverse	CCTCAAGTTGCCTTCTCAGG
Human		
AXIN2	Forward	AAACGCAATGGGAAAGGCAC
	Reverse	TGTGCTTTGGGCACTATGGG
CCND1	Forward	ACACGGACTACAGGGGAGTT
	Reverse	GTTCCTCGCAGACCTCCAG
cMYC	Forward	CAGCGACTCTGAGGAGGAAC
	Reverse	GCTGGTGCATTTTCGGTTGT
GAPDH	Forward	GGTGGTCTCCTCTGACTTCAACA
	Reverse	GTTGCTGTAGCCAAATTCGTTGT