TABLES

Supplemental Table 1: Antibodies used in this study

Primary

β-actin (WB): Sigma, AC-74 (RRID:AB_476743)

phospho-AKT (S473, WB): CST 9271 (RRID:AB_329825)

total AKT (WB): CST 9272 (RRID:AB_329827)

phospho-ERK1/2 (WB): CST 9101 (RRID:AB_331646)

total ERK1/2 (WB): CST 9102 (RRID:AB_330744)

phospho-Histone H3 (WB): CST 9701 (RRID:AB_331535)

phospho-Histone H3 (IF): Thermo K.872.3 (RRID:AB_11008586)

phospho-MAPKAPK2 (T222, WB): CST 3316 (RRID:AB_2141311)

total MAPKAPK2 (WB): CST 3042 (RRID:AB_10694238)

phospho-MEK1/2 (S217/S221, WB): CST 9154 (RRID:AB_2138017)

phospho-MEK1 (T286, WB): CST 9127 (RRID:AB_331654)

phospho-MEK1 (T292, WB): CST 26975 (RRID:AB_2798935)

total MEK1 (WB): CST 2352 (RRID:AB_10693788)

phospho-MKK4 (WB): CST 4514 (RRID:AB_2140946)

phospho-p38 (WB): CST 9216 (RRID:AB_331296)

total p38 (WB): CST 9212 (RRID_330713)

cleaved PARP (WB): CST 5625 (RRID:AB_10699459)

pericentrin (IF): AbCam ab28144 (RRID:2160664)

acetylated-α-tubulin (WB): CST 5335 (RRID:AB_10544694)

α-tubulin (WB): CST 2125 (RRID:AB_2619646)

α-tubulin (IF): AbCam ab18251 (RRID:AB_2210057)

Secondary

Alexa Fluor 488 goat anti-rabbit IgG (H+L) Invitrogen #A-11008 (RRID:AB_143165)

Alexa Fluor 594 goat anti-mouse IgG (H+L) Invitrogen #A-11032 (RRID:AB_2534091)

Anti-rabbit IgG HRP: CST 7074 (RRID:AB_2099233)

Anti-mouse IgG HRP: CST 7076 (RRID:AB_330924)

Supplemental Table 2: RAS mutation status of the rhabdomyosarcoma and neuroblastoma cell lines used in this study

Cell line	Histology	RAS status
RD (RRID:CBCL_1649)	rhabdomyosarcoma	$NRAS^{Q61H}$
SMS-CTR (RRID:CVCL_A770)	rhabdomyosarcoma	$HRAS^{Q61K}$
BIRCH (RRID:CVCL_M599)	rhabdomyosarcoma	$HRAS^{Q61K}$
RH4 (RRID:CVCL_5916)	rhabdomyosarcoma	wild type
RH30 (RRID:CVCL_0041)	rhabdomyosarcoma	wild type
RMS-YM (RRID:CVCL_A792)	rhabdomyosarcoma	wild type
RH18 (RRID:CVCL_DG02)	rhabdomyosarcoma	wild type
SKNAS (RRID:CVCL_1700)	neuroblastoma	$NRAS^{Q61K}$
NBEB (RRID:CVCL_E218)	neuroblastoma	$KRAS^{G12D}$
CHP212 (RRID:CVCL_1125)	neuroblastoma	$NRAS^{Q61K}$
SHEP (RRID:CVCL_0524)	neuroblastoma	wild type
SY5Y (RRID_CVCL_0019)	neuroblastoma	wild type

Supplemental Table 3: Biotin pull-down mass spectrometry. Biotinylated versions of rigosertib and the inactive isomer of rigosertib, ON01911, were used to affinity purify rigosertib binders from whole cell lysates of the RMS cell line, RD. Interacting proteins were identified by LC MS/MS. Free biotin was used as a negative control. Results are sorted based on the number of peptide spectrum matches (PSM) in the rigosertib-affinity purified samples. TUBB2A/B is the specific binder for rigosertib and not ON01911 or free biotin that has the highest number of PSM.

Accession	Gene Symbol	Biotin	Rigosertib	ON01911
P60709, P63261	ACTB,ACTG1	3	30	22
Q14315	FLNC		21	22
P68366	TUBA4A		16	15
P11142	HSPA8	8	13	17
P68104	EEF1A1	6	12	9
P10809	HSPD1	3	12	16
P02461	COL3A1	1	10	6
Q13885, Q9BVA1	TUBB2A/B		10	
P11021	HSPA5	3	9	11
P14618	PKM	1	9	9
P55060	CSE1L	6	8	13
P06733	ENO1		8	10
P29508	SERPINB3		6	
P15924	DSP		5	
P49327	FASN		5	2
P21333	FLNA		5	6
Q9NZI8	IGF2BP1		5	3
P78371	CCT2	3	4	6
P17661	DES	1	4	6
P35579	MYH9	1	4	1
P19338	NCL	1	4	5
Q01469	FABP5		4	
P38646	HSPA9		4	4
P48594	SERPINB4		4	
P62987, P62979, P0CG47, P0CG48	UBA52, RPS27A, UBB/C		4	3
Q02413	DSG1		3	
Q5D862	FLG2		3	
Q14697	GANAB		3	1
O00425	IGF2BP3		3	3
P14923	JUP		3	
P06702	S100A9		3	
P07355	ANXA2		2	
P40227	CCT6A		2	4

SUPPLEMENTAL FIGURE LEGENDS

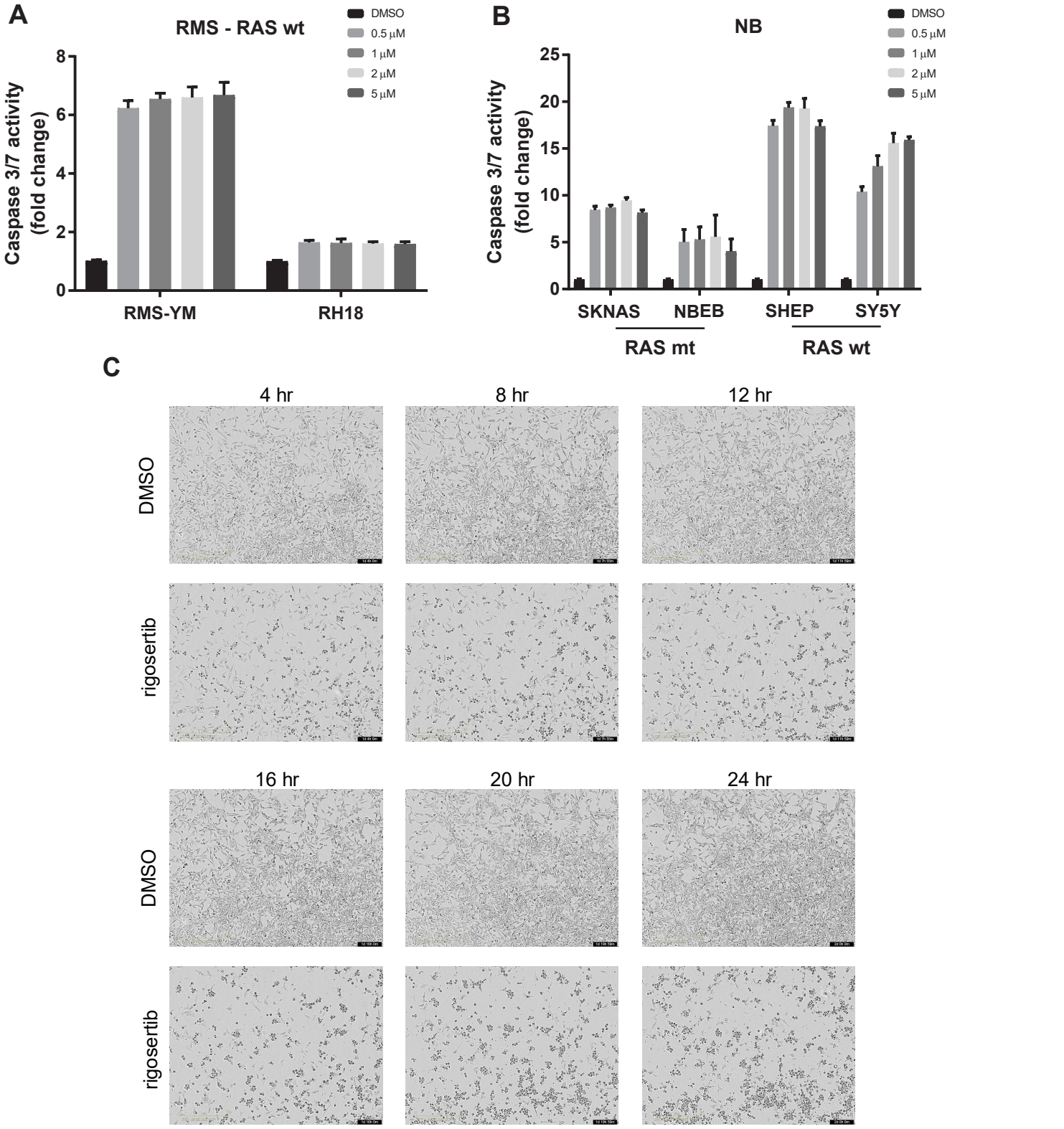
Supplemental Figure 1: Rigosertib induces apoptosis in rhabdomyosarcoma and neuroblastoma cells expressing wild type RAS. **(A)** Caspase 3/7 activity, determined by Caspase-Glo, 18 hours after rigosertib treatment of the indicated rhabdomyosarcoma (RMS-YM or RH18) cell lines. **(B)** Caspase 3/7 activity, determined by Caspase-Glo, 18 hours after rigosertib treatment of the indicated neuroblastoma cell lines. SKNAS (NRAS) and NBEB (KRAS) express mutant RAS isoforms while SHEP and SY5Y express wild type RAS. **(C)** Phase-contrast images of SMS-CTR cells treated with vehicle (DMSO) or 1 μM rigosertib for the indicated time periods. Images taken in IncuCyte live cell imaging system (Essen). **(D)** Percent confluence over time for RD (left) or SMS-CTR (right). Cells were plated at time = 0 and were treated with vehicle (DMSO, blue) or 1 μM rigosertib (red) at time = 24 hours. In the wash condition, at time = 48 hours the cell culture media containing the indicated treatment was replaced with fresh media that did not contain inhibitors (dashed lines).

Supplemental Figure 2: The cell cycle block induced by rigosertib treatment promotes generation of reactive oxygen species in RMS cells and activation of the stress MAP kinase cascades in NB cells. **(A)** Reactive oxygen species (ROS) production as measured by ROS-glo in media alone (no cells) or in the presence of rhabdomyosarcoma cells (RD or SMS-CTR) that are either untreated or treated with vehicle, 2 μM rigosertib, 10 mM NAC, rigosertib + NAC, or 50 μM menadione (positive control) for 18 hours. * denotes $p < 0.05$ and ** denotes $p < 0.001$ as determined by 2-way ANOVA with Tukey's correction for multiple comparisons. **(B)** Immunoblot analysis of p-p38, p-MAPKAPK2, p-MKK4, and cleaved PARP in SKNAS (left) or NBEB (right) cells treated with 2 μM rigosertib with or without co-treatment with 10 mM NAC or 20 μM SB-203580 for 18 hours. **(C)** Neither NAC (left) nor SB-203580 (right) co-treatment prevents rigosertib-induced G2/M arrest in SKNAS as determined by DNA content analysis after 24 hours of treatment. **(D)** NAC co-treatment does not prevent rigosertib-induced caspase 3/7 activity in SKNAS (left) or NBEB (right) as determined by Caspase-glo after 18 hours of treatment. ns denotes not significant as determined by 2-way ANOVA with Tukey's correction for multiple comparisons. **(E)** SB-203580 co-treatment does not prevent rigosertib-induced caspase 3/7 activity in SKNAS (left) or NBEB (right) as determined by Caspase-glo after 18 hours of treatment. ns denotes not significant as determined by 2-way ANOVA with Tukey's correction for multiple comparisons.

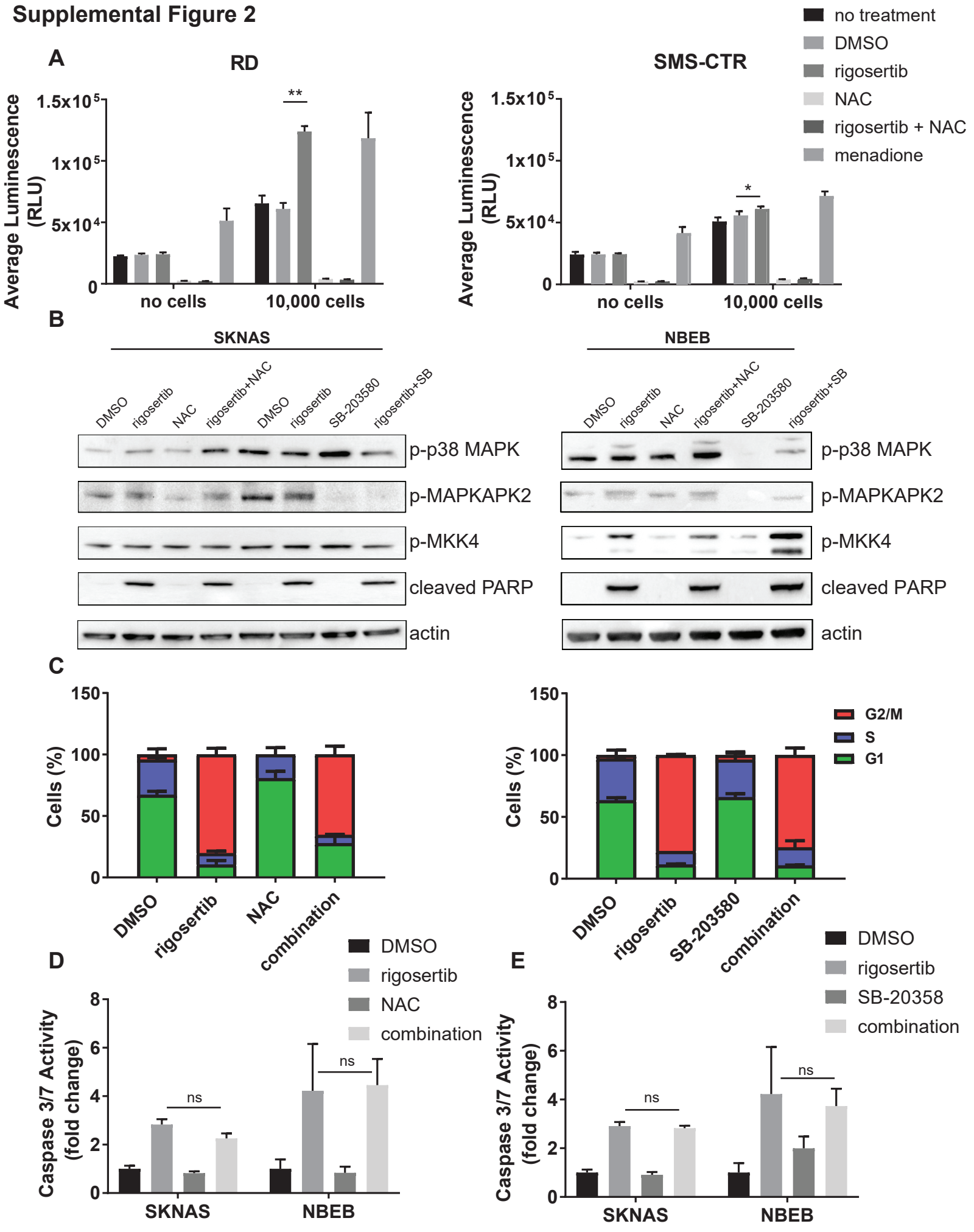
Supplemental Figure 3: Tubulin binding compounds decrease cell viability in RMS. **(A)** Treatment of SMS-CTR with additional tubulin binding compounds, nocodazole, albendazole and combretastatin A4, also decreases cell viability. Cell viability was determined by percent confluence after 72 hours of treatment. **(B)** Relative potency of combretastatin A4 in RAS wild-type and RAS-mutant cells. **(C)** Treatment of RD (top) or SKNAS (bottom) with 2.5 nM combretastatin A4 for 24 hours induces mitotic spindle defects in unsynchronized cells, as determined by immunofluorescence staining of α-tubulin and pericentrin. Representative images are shown. Boxed insets are shown as zoomed images at right.

Supplemental Figure 4: Rigosertib delays time to tumor progression in an RD xenograft model but not an SKNAS model. **(A)** Twice daily rigosertib delays tumor growth in an orthotopic RD cell line xenograft model. Error bars denote standard deviation. **(B)** The microtubule destabilizing agent, vincristine, given at a dose of 1 mg/kg IV once weekly, prevents tumor growth in an RD cell line xenograft model. **(C)** Twice daily rigosertib does not delay tumor growth in a subcutaneous SKNAS xenograft model.

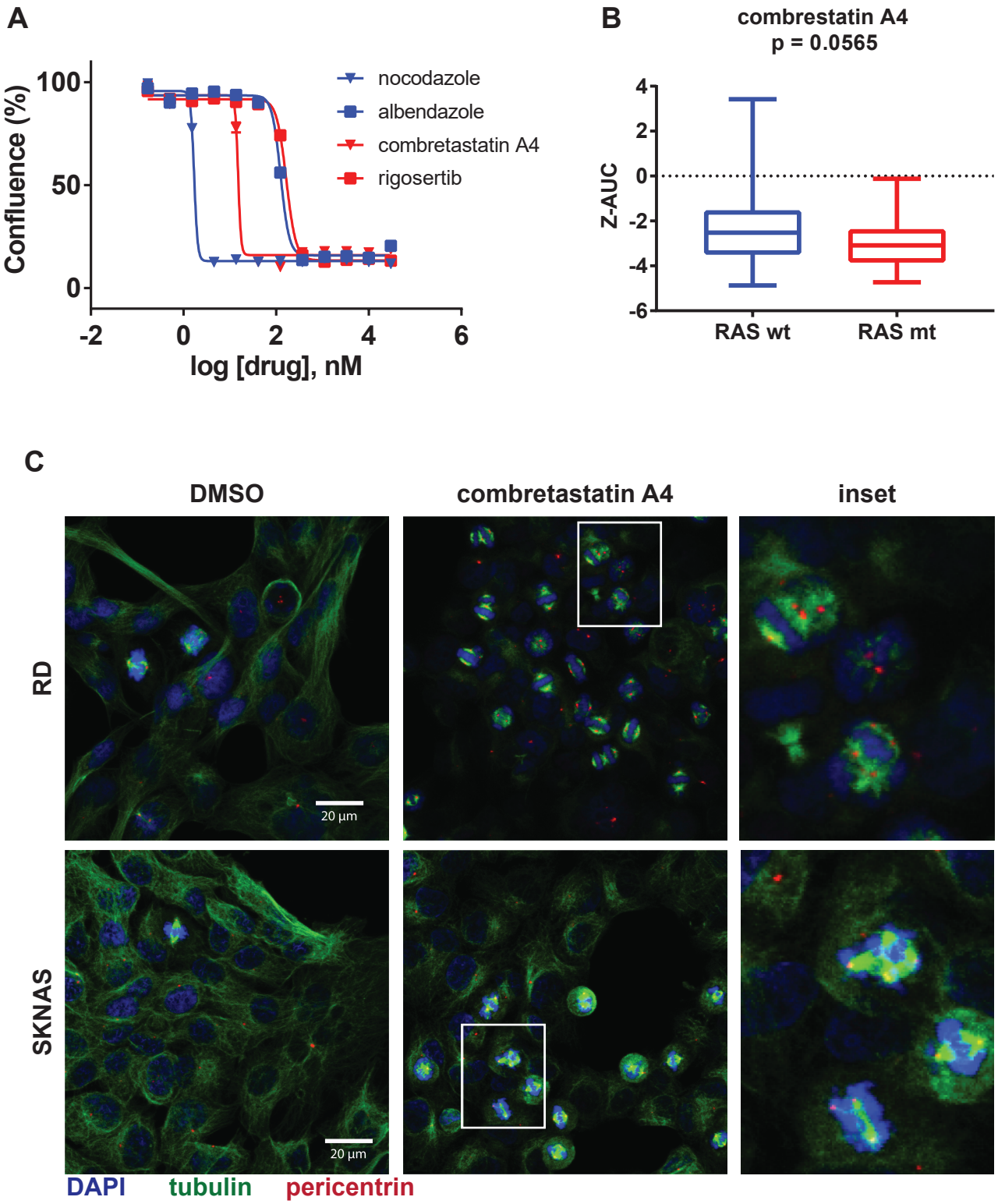
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4