Supplemental Material to

Histone Deacetylase Inhibitors Interrupt HSP90•RASGRP1 and HSP90•CRAF Interactions to Upregulate BIM and Circumvent Drug Resistance in Lymphoma Cells

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Supplemental Method

Immunoprecipitation. To assess changes in binding of proteins to HSP90, Jurkat cells were treated with diluent or 20 nM romidepsin for 6 h in the presence of 5 μM Q-VD-OPh. At the completion of the incubation, cells were washed in ice cold RPMI-HEPES and solubilized for 30 min at 4° C in lysis buffer consisting of 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% (v/v) glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate and 20 nM microcystin. After sedimentation of insoluble material at 14000 x *g* for 15 min, aliquots of supernatant containing 1000 μg protein were incubated overnight at 4° C with anti-HSP90 that was precoupled to protein G-agarose beads using dimethyl pimelimidate. Beads were sedimented at 400 x *g* for 5 min, washed five times in lysis buffer, and heated for 20 min at 65° C in SDS sample buffer consisting of 4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA and 5% (v/v) 2-mercaptoethanol to release bound polypeptides.

Table S1 Sources of Commercial Antibodies		
Antibody	**Product number**	**Vendor**
Phospho-Thr^{202}/Tyr^{204}: -ERK1/2	9101S	Cell Signaling Technology (Beverly, MA)
ERK1/2	9102S	
Phospho-Ser^{217}/Ser^{221}-MEK1/2	9121S	
MEK1/2	4694S	
BAX	2772S	
BIM	2819S	
BID	2002S	
MCL1	4572S	
$BCLX_L$	2764S	
Cleaved caspase 3	9661S	
PARP1	9532S	
HSP70	ADI-SPA-810D	Enzo Life Sciences (Farmington, NY)
NOXA	ALX-804-408-C100	
BAK	06-536	Millipore (Billerica, MA)
CRAF	sc-133	Santa Cruz Biotechnology (Santa Cruz, CA)
PUMA	sc-374223	
BCL2 (rabbit polyclonal)	sc-492	
BCL2 (murine monoclonal)	Clone 124	Dako (Carpenteria, CA)
Acetylated tubulin	T6793	Sigma-Aldrich (St. Louis, MO)
α-tubulin	T9026	
Acetylated lysine	200-301-A97	Rockland (Limerick, PA)
Cytochrome c	556433	BD Biosciences (Franklin Lakes, NJ)
Cytochrome oxidase subunit IV	20E8-C12	Thermo (Waltham, MA)

Table S2

Lymphoid Cell Lines Used in This Study

Cell line	Dx[a]	Age[b]	Sex	*BCL2* Status[c]
DoHH2	Centroblastic/centrocytic non-Hodgkin's Lymphoma	60	M	Wild Type
Daudi	Burkitt's lymphoma	16	M	Wild Type
HS445	Hodgkin's Lymphoma	56	F	Wild Type
HT	Diffuse mixed B cell lymphoma	70	M	Wild Type
Jeko	Mantle Cell Lymphoma	78	F	Wild Type
Karpas 422	DLBCL	71	M	Mutant
Nalm6	pre-B cell (ALL)	19	M	Wild Type
SKW6.4	MZL	65	F	Wild Type
SuDHL-4	DLBCL	38	M	Mutant
SuDHL-6	DLBCL	60	M	Mutant
RL	B cell-Non Hodgkin's	52	M	Mutant
WSU-DLCL2	DLBCL	46	F	Mutant
Jurkat	Acute T-cell Leukemia	14	M	Wild Type

[a]Abbreviations used: ALL, acute lymphoblastic leukemia; DLBCL, diffuse large B-cell lymphoma; MZL, splenic marginal zone lymphoma.

[b]Age and sex of patient from whom the line was established.

[c]From COSMIC database (http://www.sanger.ac.uk/genetics/CGPF/cosmic/), confirmed by Sanger sequencing.

Table S3

Clinical Samples of Lymphoid Malignancies Used in This Study

Pt #[a]	Dx[b]	Age	Sex	Stage	% Lymphoma cells	Prior treatment[c]	Tissue source
#1	MZL	82	M	IV	50%	None	Splenectomy
#2	BL, low grade	64	M	IV	75%	R-CHOP, BR	Splenectomy
#3a[d]	FL, grade 1	60	F	II	60%	None	Lymph node
#3b	FL, grade 1	62	F	II	66%	Rituximab	Lymph node
#4	FL, grade 1	62	M	IV	38%	None	Splenectomy
#5	CLL/SLL	66	F	IV	73%	PCR	Lymph node
#6	DLBCL	71	M	II	80%	None	Splenectomy
#7	MZL	65	F	IV	54%	None	Splenectomy
#8	FL	61	F	II	60%	CHOP x 3, RT	Lymph node
#9	MZL	60	F	IV	88%	None, initial dx	Splenectomy
#10	PTLD	64	F			None	Splenectomy
#11	T LBL	25	M	III	98%	Hyper-CVAD	Lymph node

[a]The same patient designations are used in this table, **Fig. 7a** and **Fig. S7**.

[b]Abbreviations used: BL, low grade; low grade B-cell lymphoma; BL-NOS, B-cell lymphoma, not otherwise classifiable; CLL/SLL, chronic lymphocytic leukemia, small lymphocytic lymphoma; FL, follicular lymphoma; MZL, splenic marginal zone lymphoma; T LBL, T-cell lymphoblastic lymphoma; PTLD, post-transplant lymphoproliferative disorder.

[c]Abbreviations for treatments are: BR, bendamustine/rituximab; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; HyperCVAD, hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone alternating with high dose methotrexate and cytarabine; PCR, pentostatin, cyclophosphamide and rituximab; RT, radiation therapy.

[d]Samples 3a and 3b are two samples from the same patient at two time points with intervening treatment (see **Fig. S7**).

SUPPLEMENTAL FIGURES AND LEGENDS

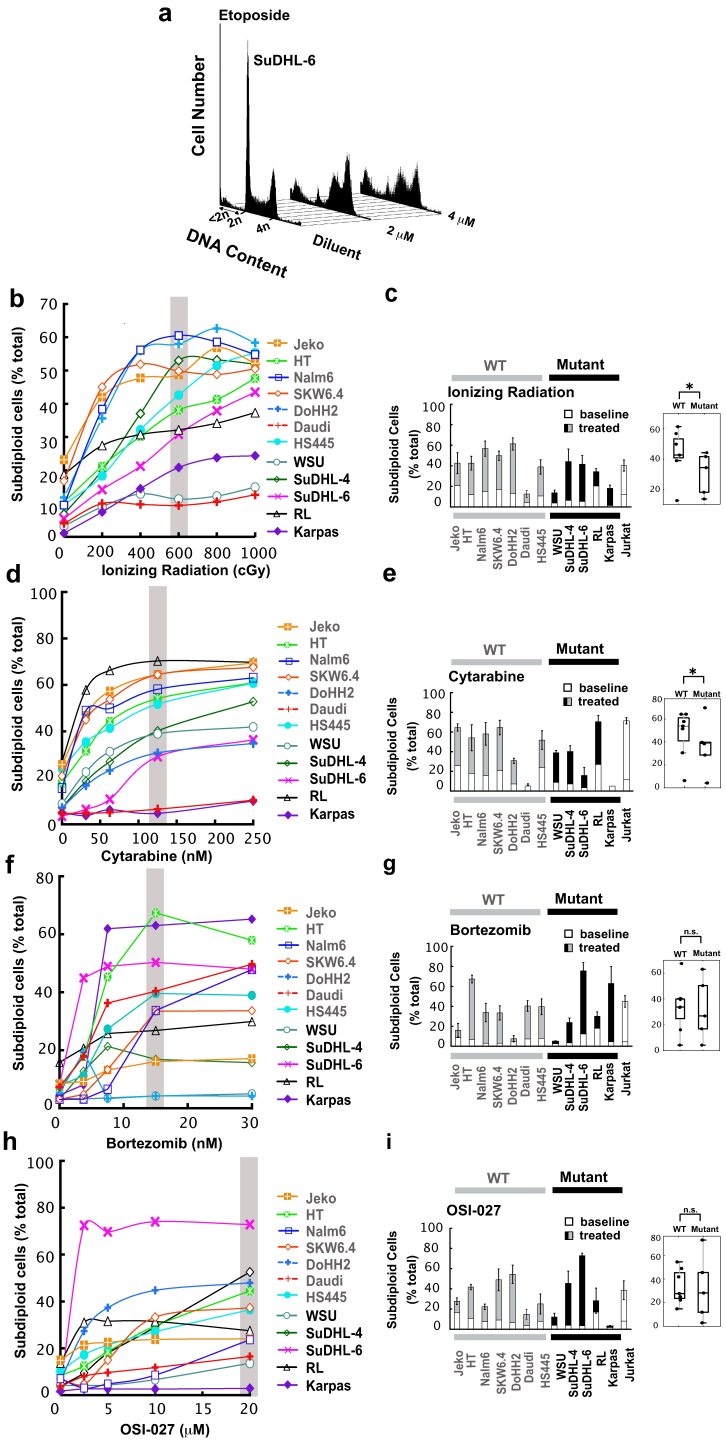

Figure S1. Sensitivity of malignant lymphoid cell lines to etoposide, ionizing radiation, cytarabine, bortezomib and OSI-027 (related to **Fig. 1**). **(a)** SuDHL-6 cells were treated for 24 h with diluent, 2 μM etoposide, or 4 μM etoposide, then stained with propidium iodide and subjected to flow microfluorimetry. Results are representative of data used to generate summary graphs in **Figs. 1-3** as well as subsequent supplemental figures. **(b, d, f, h)** Cells were treated with the indicated concentrations of ionizing radiation **(b),** cytarabine **(d)**, bortezomib **(f),**

or OSI-027 **(h)** and then harvested after 24 h **(b, f)**, 48 h **(h)** of 72 h **(d)**, timepoints chosen based on preliminary time course experiments. Curves shown are representative of 3 independent experiments. Grey bars indicate the drug concentration used for the box plots in panels **c, e, g,** and **i. (c, e, g, i)** Effects of ionizing radiation (600 cGy, 24 h before assay), cytarabine (125 nM, 72 h), bortezomib (15 nM, 24 h), or OSI-027 (20 μM, 48 h) on the number of subdiploid cells. Errors bars indicate mean ± SD of three independent experiments. *, $p < 0.05$. n.s., not significant.

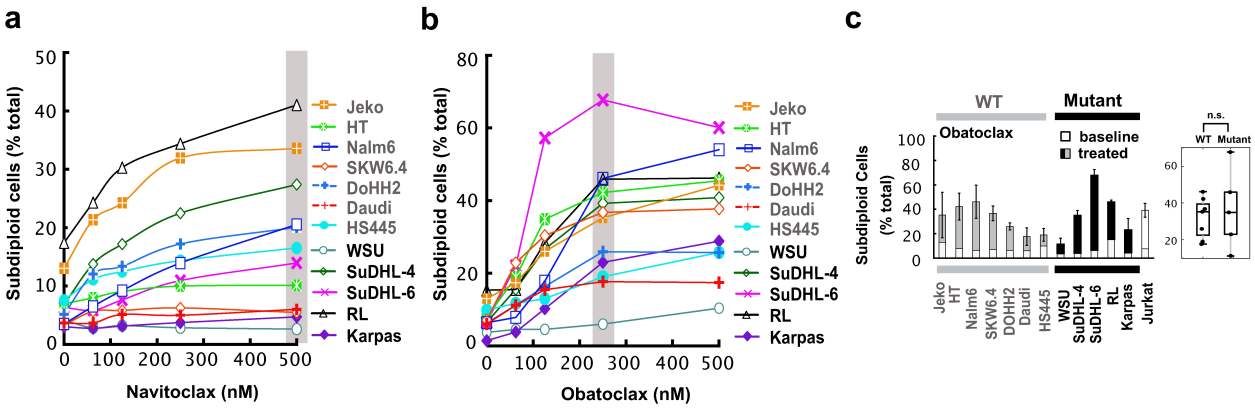

Figure S2. Sensitivity of malignant lymphoid cell lines to navitoclax and obatoclax (related to **Fig. 2**). Cells were treated for 48 h with the indicated concentrations of navitoclax **(a)** or obatoclax **(c)** and analyzed for subdiploid events by flow cytometry as illustrated in **Fig. S1a**. Curves shown are representative of 3 independent experiments. Grey bars indicate the concentration of navitoclax (500 nM) or obatoclax (250 nM) used for the box plots in panel **c** and **Fig. 2c.** Error bars in panel c indicate mean ± SD of three independent experiments, n.s., not significant.

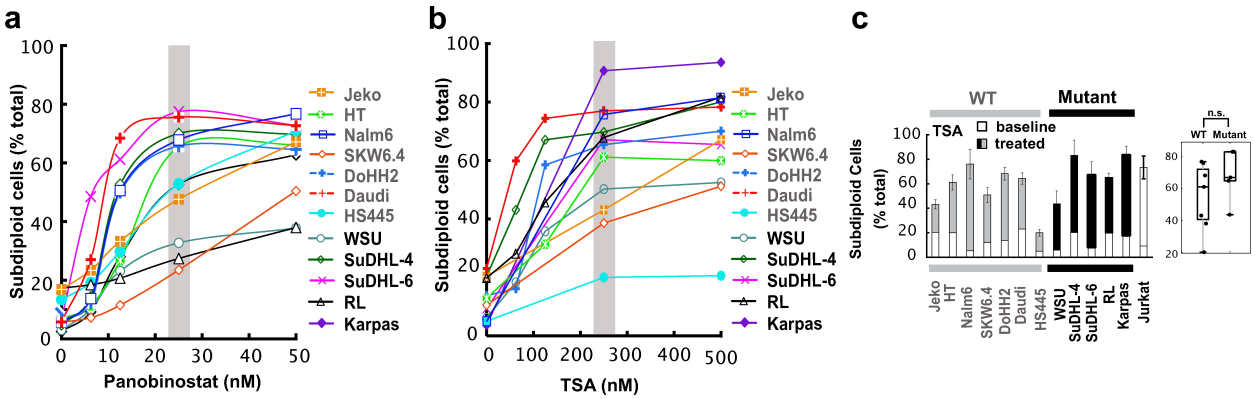

Figure S3. Sensitivity of malignant lymphoid cell lines to panobinostat and TSA (related to **Fig. 3**). Cells were treated for 48 h with the indicated concentrations of panobinostat **(a)** and TSA **(b)** and analyzed for subdiploid events by flow cytometry as illustrated in **Fig. S1a**. Curves shown are representative of 3 independent experiments. Grey bars indicate the drug concentrations (panobinostat 25 nM, TSA 250 nM) used for the box plots in **panel c** and **Fig. 3c**. Error bars in panel c indicate mean ± SD of three independent experiments, n.s., not significant.

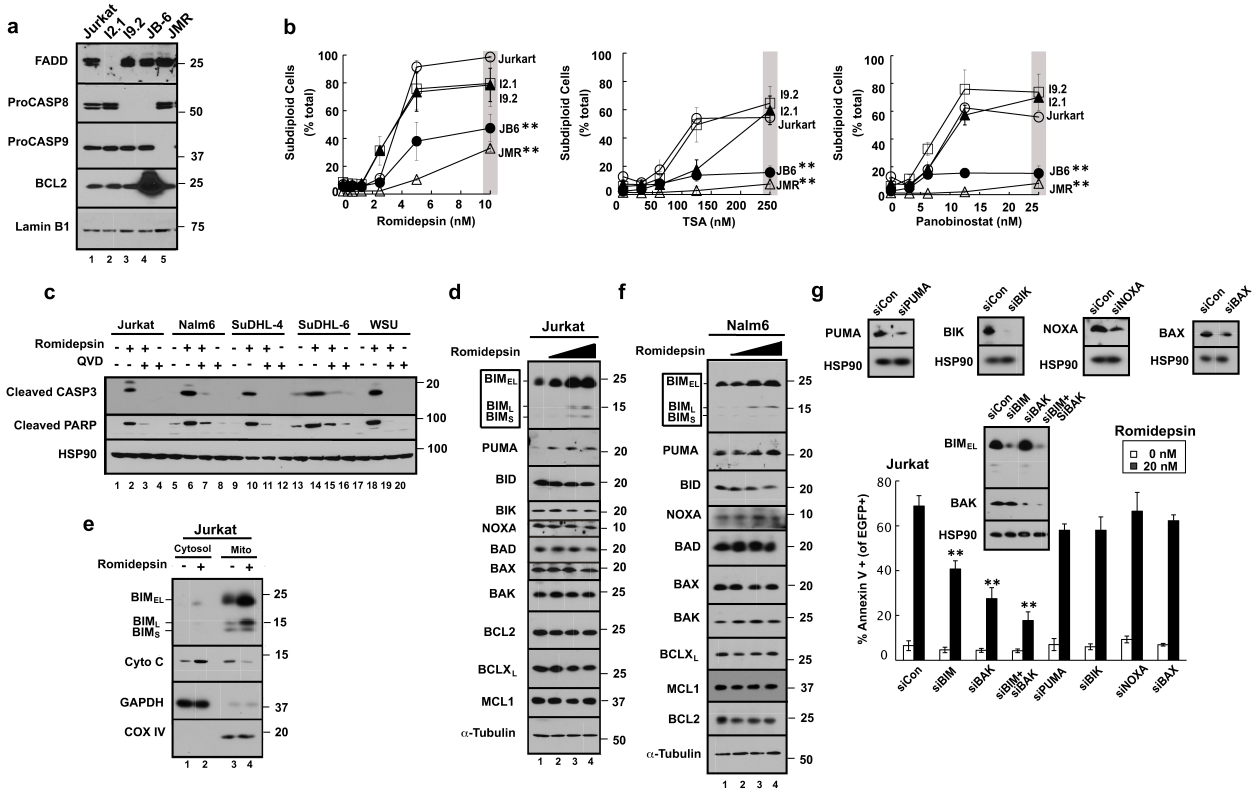

Figure S4. HDAC inhibitor-induced killing depends on BIM (related to **Fig. 4**). (**a**) Whole cell lysates from Jurkat cells or their derivatives were subjected to immunoblotting for the indicated antigens. Lamin B1 served as a loading control. (**b**) Jurkat and Jurkat variants were treated with the indicated concentrations of romidepsin for 72 h, TSA for 48 h or panobinostat for 48 h before staining with propidium iodide and flow microfluorimetry. Grey bars indicate the drug concentration used for *t*-test. (**c**) The indicated cells were treated with diluent (0.1% DMSO) or 20 nM romidepsin in the presence (+) or absence (-) of 5 μM Q-VD-OPh for 24 h and subjected to immunoblotting. HSP90 served as a loading control. (**d** and **f**) Western blots showing protein levels in Jurkat (**d**) and Nalm6 (**f**) cells upon treatment with romidepsin (0, 5, 10, 20 nM) in the presence 5 μM Q-VD-OPh for 24 h. (**e**) After Jurkat cells were treated with diluent (0.1% DMSO) or 20 nM romidepsin (+) in the presence of 5 μM Q-VD-OPh for 24 h, the indicated subcellular fractions were isolated and subjected to immunoblotting. GAPDH and cytochrome c oxidase subunit IV (COX IV) served as markers for cytosol and mitochondria, respectively. (**g**) 24 hours after transfection of the indicated siRNAs along with plasmid encoding EGFP-histone H2B (to mark successfully transfected cells), Jurkat cells were treated for 24 h with 20 nM romidepsin before staining with APC-conjugated annexin V and analysis by 2-color flow cytometry. Shown are the percentages of $EGFP^+$ (successfully transfected) cells that are annexin V^+. Additional results can be found in **Fig. S5**. Above the graph are immunoblots of whole cell lysates prepared from siRNA-treated cells incubated in drug-free medium in parallel with samples harvested for flow cytometry. HSP90 served as loading control. Error bars in panels **b** and **g** indicate mean ± SD of three independent experiments. ** in panels b and g, $p<0.01$ relative to parental Jurkat cells (b) or siRNA control (g).

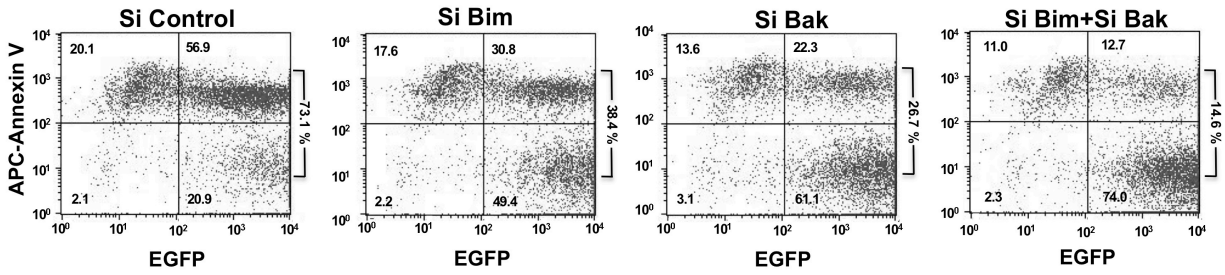

Figure S5. Effect of knockdown of BIM, BAK and BIM + BAK on romidepsin sensitivity (related to **Fig. S4g**). Beginning 24 h after transfection with the indicated siRNA + 5 μg of plasmid encoding EGFP-Histone H2B, Jurkat cells were treated for 24 h with 20 nM romidepsin, stained with APC-conjugate annexin V and analyzed by two-color flow cytometry. Numbers in each quadrant indicated % of events. Numbers at right indicate % of $EGFP^+$ cells that are annexin V-positive after romidepsin treatment.

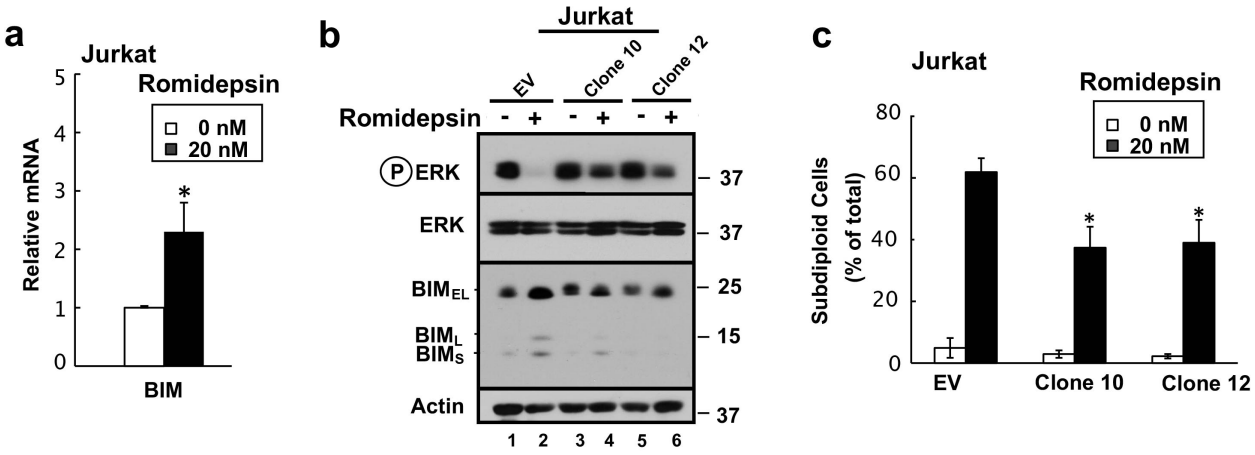

Figure S6. HDAC inhibitor-induced killing is diminished by activated MEK (related to **Figs. 4** and **S4**). **(a)** After Jurkat cells were treated with diluent or 20 nM romidepsin for 24 h, BIM mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. **(b)** After Jurkat cells were transfected with empty vector (EV) or constitutively active MEK1 (clones 10 and 12) and treated with diluent (0.1% DMSO, -) or 20 nM romidepsin (+) in the presence of 5 μM Q-VD-OPh for 24 h, whole cell lysates were subjected to immunoblotting with the indicated antibodies. **(c)** After the indicated clones were treated 24 h with diluent or 20 nM romidepsin, samples were stained with propidium iodide and subjected to flow microfluorimetry. Error bars in panels a and c indicate mean ± SD of three independent experiments. *, $p < 0.05$ relative to empty vector.

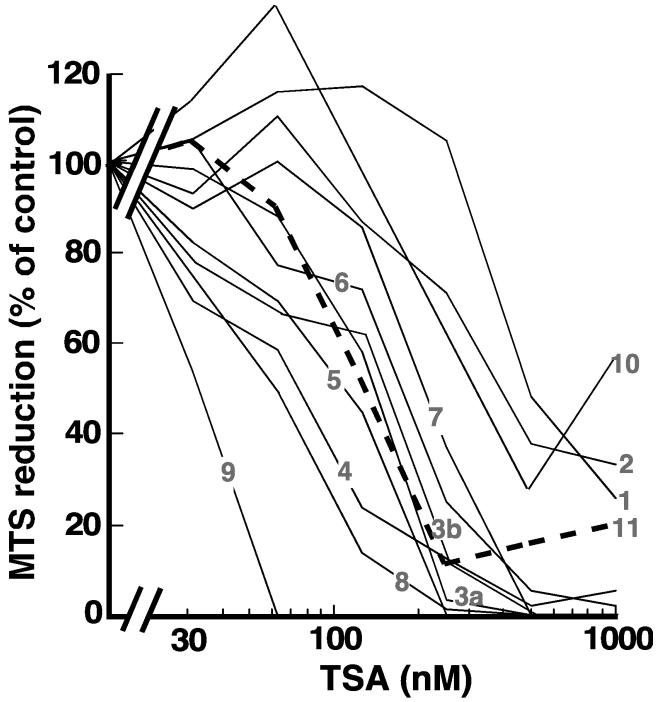

Figure S7. Effect of TSA on survival of clinical B-cell lymphoma samples *ex vivo* (related to **Fig. 7**). B-cell lymphoma specimens (solid lines) and T cell lymphoma specimens (dashed lines) were treated with the indicated concentrations of TSA for 5 days *ex vivo* and assayed for relative cell survival using MTS assays. Numbers in grey correspond to patient numbers in **Table S3**. Samples 3a and 3b came from the same patient at two points in time.