Overexpression of FoxM1 offers a promising therapeutic target in diffuse large B-cell lymphoma

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Online Supplementary Appendix

Cell culture

The human DLBCL cell lines, SUDHL4, SUDHL5, SUDHL8 and OCI-LY19 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and HBL-1 and OCI-LY3 were a kind gift from Dr Laura Pasqualucci, Institute for Cancer Genetics and the Herbert Irving Comprehensive Cancer Center, Columbia University, New York, USA. SUDHL5, OCI-LY3 and OCI-LY19 cell lines have wild-type p53 while SUDHL4, SUDHL8 and HBL-1 express mutant p53. The cell lines were cultured in RPMI 1640 medium (SUDHL4, SUDHL5, SUDHL8 and OCI-LY19) and IMDM (HBL-1 and OCI-LY3) supplemented with 20% (v/v) fetal bovine serum (FBS), 100 caspases μ/mL penicillin, 100 caspases μ/mL streptomycin at 37°μC in a humidified atmosphere containing 5% CO_2. All the experiments were performed in either RPMI-1640 or IMDM containing 5% serum.

Reagents and antibodies

Thiazole antibiotic (thiostrepton) and Bax 6A7 monoclonal antibodies were purchased from Sigma Aldrich (St Louis MO, USA). Antibodies against SKP-2, caspase-9, p27 and cleaved caspase-3, were purchased from Cell Signaling Technologies (Beverly, MA, USA). FoxM1, Cytochrome c, cIAP1, cIAP2, caspase-3 and poly-(ADP) ribose polymerase (PARP), cyclin A, cyclin B1 and pRb antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). XIAP, survivin and caspase-8 antibodies were purchased from R&D (Minneapolis, MN, USA). MMP-9 and MMP-2 antibodies were purchased from Anespec (San Jose, CA, USA). Beta-actin and Aurora B antibodies were purchased from Abcam (Cambridge, MA, USA). Annexin V kit was purchased from Molecular Probes (Eugene, OR, USA). DNA laddering kit was purchased from Roche Pharmaceuticals (Indianapolis, IN, USA).

Cell growth studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays

Initially, DLBCL cells were incubated at a concentration of 10^4 cells in triplicates in a 96-well format. Cells were then treated with different doses of thiostrepton and bortezomib for 48 h in a final volume of 0.2 mL for 48 h. Cell viability was measured by MTT cell viability assay, as previously described.[1,2] Six wells for each dosage including vehicle control were analyzed for each experiment.

Live dead assay

To measure cell death, Live-Dead assay (Invitrogen, Eugene, OR, USA) was used as described by the manufacturer. Briefly, following treatment, 1×10^6 DLBCL cells were incubated in 1 mL of PBS containing 50 μM calcein AM and 8 μM ethidium homodimer and visualized under an Olympus fluorescent microscope using a longpass filter.

Cell cycle analysis, annexin V staining, and DNA laddering

DLBCL cell lines were treated with different concentrations of thiostrepton as described in the figure legends. For cell cycle analysis, cells were washed once with PBS and re-suspended in 500 μL hypotonic staining buffer and analyzed by flow cytometry as described previously.[3] For detection of apoptosis, cells were harvested and percentage apoptosis was measured by flow cytometry after staining with flouresceine-conjugated annexin-V and propidium iodide and DNA laddering using a 1.5% agarose gel as described previously.[4]

Cell lysis and immunoblotting

Cells were treated with thiostrepton as described in the figure legends and lysed as previously described.[5] We separated 15-20 μg of proteins by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) (Immobilion, Millipore). Proteins were immunoblotted with different antibodies and visualized by the enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) method.

Soft agar colony assays

Soft agar colony experiments were performed according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). Briefly, following treatment with thiostrepton for 24 h, 2500 cells were plated in 0.5mL culture medium containing 0.4% (v/v) top agar and 20% FBS layered over a basal layer of 0.8% (v/v) agar and 20% FBS with culture medium and allowed to grow for four weeks.[6] Following four weeks of incubation, cells were stained at a final concentration of the 1 mg/mL cell stain solution that was supplied with the kit and counted manually.

Cell invasion and migration assays

Cell invasion and migration assay were performed using 24-well Transwell Permeable Supports with 8-lM pores (Corning, Lowell, MA, USA). Cells were suspended in serum-free medium and seeded into Transwell inserts either uncoated (for migration assay) or coated (for invasion assay) with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA). Bottom wells were filled with complete media and after 24 h cells were stained with Diff-Quick stain set (Fisher Scientific, Pittsburg, PA, USA) and photographed under a fluorescent microscope.

ELISA assay for MMP-2 secretion

DLBCL cells were appropriately treated with and without 5 and 10 μM thiostrepton for 48 h following which the culture media were collected and centrifuged to remove cellular debris. The assay was performed using MMP-2 ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instruction.

Measurement of mitochondrial potential and cytochrome c release

After treatment of DLBCL cell lines with thiostrepton for 48 h, mitochondrial membrane potential was measured using JC1 dye and release of cytochrome c was analyzed using immunoblotting of cytosolic protein fractions as described previously.[3]

Detection of Bax conformational changes

Cells were treated with 10 μM thiostrepton for various time periods and lysed with Chaps lysis buffer (10mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps), immunoprecipitated with anti-Bax-6A7 monoclonal antibody and Bax conformation was detected as described earlier.[4]

Gene silencing using siRNA

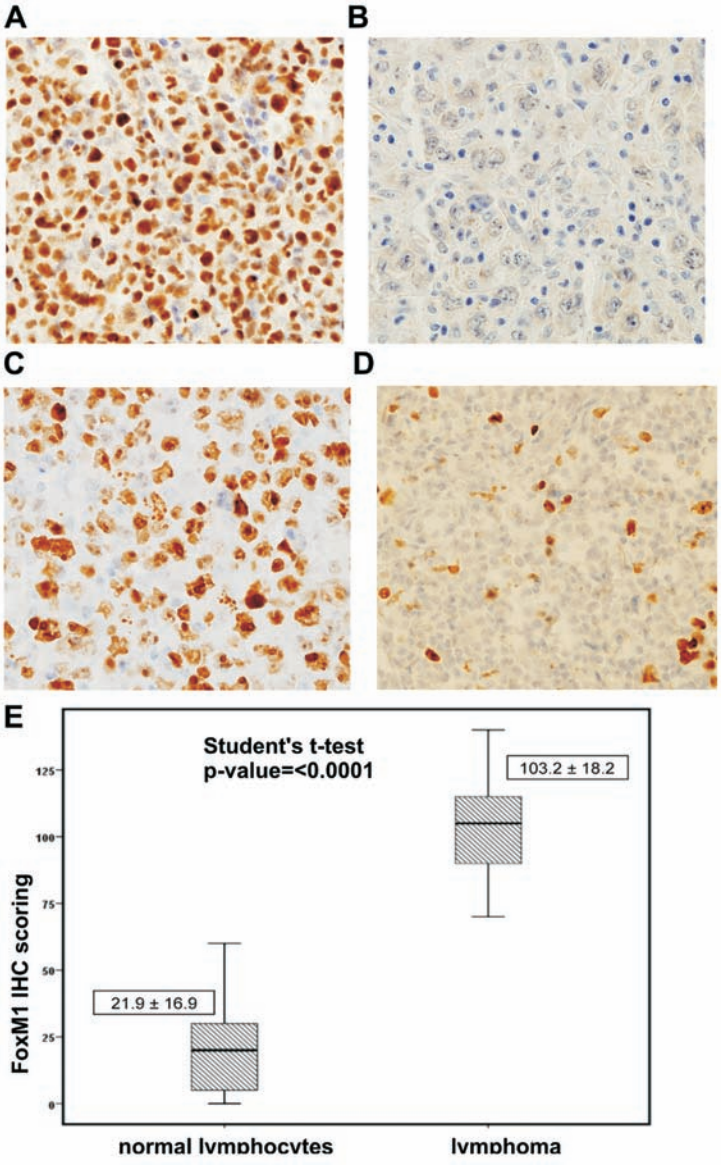
FoxM1 siRNA, SKP-2 siRNA and scrambled control siRNA were purchased from Qiagen (Valencia, CA, USA). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 6 h following which the lipid and siRNA complex was removed and fresh growth medium was added. Cells were either lysed 48 h after transfection or treated with thiostrepton and specific protein levels were determined by Western blot analysis with specific antibodies. The sequences of the siRNA used in the study were:

FoxM1 (1): hp Genomewide siRNA: cat n. SI00421036
Sequence: CCGCCGGAACATGACCATCAA

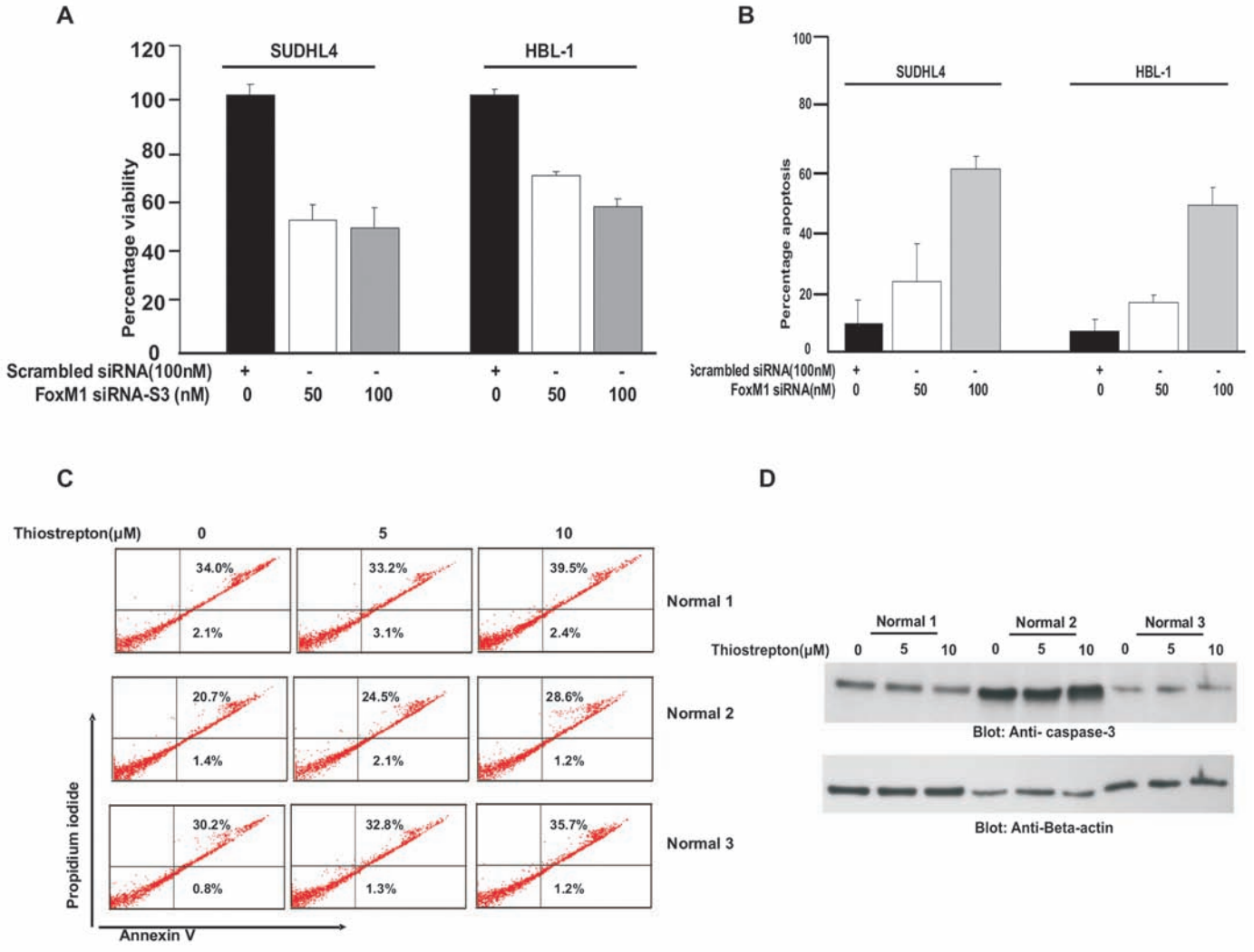
FoxM1 (3): hp Genomewide siRNA: cat n. SI00421050
Sequence: TCGGAAATGCTTGTGATTCAA

SKP2: HP validated siRNA (1): Cat n. SI02659692
Sequence: ACCCTTCAACTGTTAAAGGAA

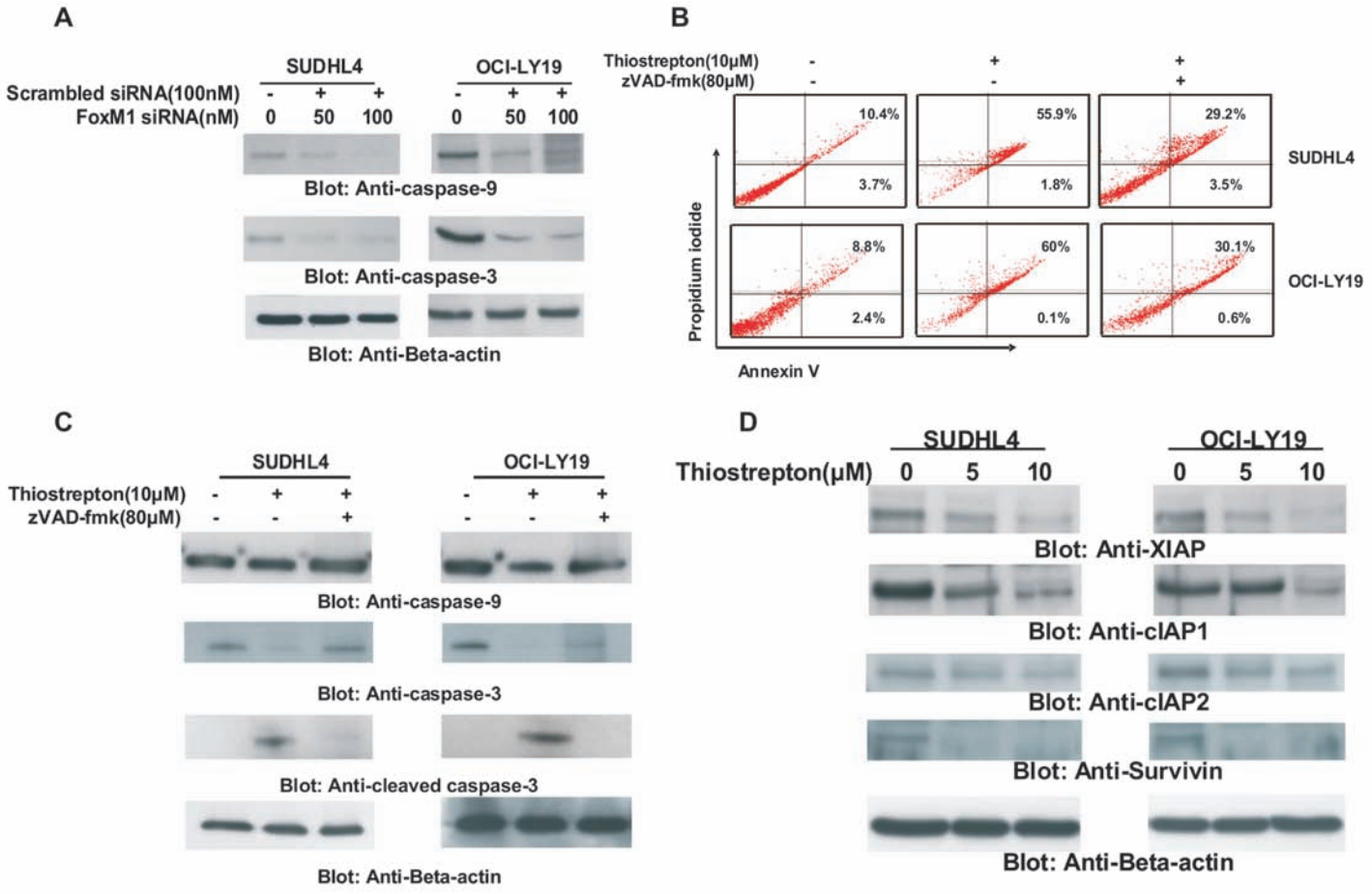
SKP2: HP validated siRNA (2): Cat n. SI00287819
Sequence: AAGTGATAGTGTCATGCTAAA



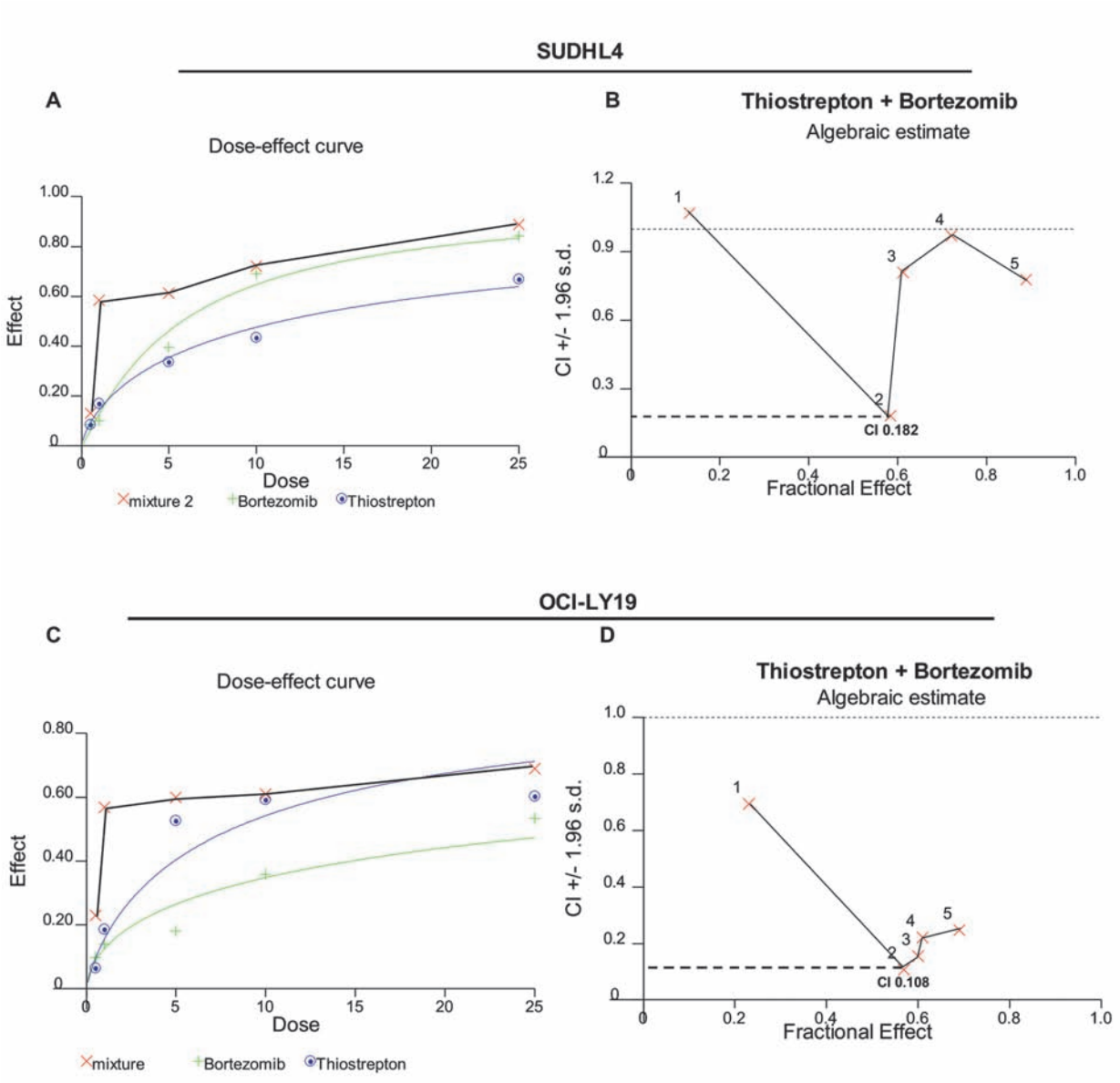
Online Supplementary Figure S1. Tissue microarray based immunohistochemical analysis of FoxM1 in DLBCL patients. DLBCL array spot showing high expression (A) and low expression of FoxM1 (B), high expression of Ki67 (C) and low expression of Ki67 (D). 20 x 0.70 objective on an Olympus BX 51 microscope (Olympus America Inc, Center Valley, PA, USA). (E) Box plot of FoxM1 expression in DLBCL and non-neoplastic lymphoid cells. Using Student's t-test the mean±SD of FoxM1 expression in lymphoma (103.2±18.2) was significantly higher as compared to FoxM1 expression in normal lymphoid cells (21.9±16.9; $P<0.0001$).



Online Supplementary Figure S2. (A) SUDHL4 and HBL-1 cells were transfected with scrambled siRNA or FoxM1 specific siRNA (3) for 48 h. Following treatment, cell viability was measured by MTT. The graph displays the mean±SD (standard deviation) of 3 independent experiments with replicates of 6 wells for all the doses and vehicle control for each experiment * $P<0.05$, statistically significant (Student's t-test). (B) SUDHL4 and HBL-1 cells were transfected with scrambled siRNA or FoxM1 specific siRNA for 48 h. Following treatment, cells were subsequently stained with flourescein-conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry. Bar graph denotes standard deviation of 3 independent transfections. (C) Peripheral blood mononuclear cells (PBMNC) were isolated from 3 healthy individual donors and the cells were treated with 5 and 10 μM thiostrepton for 48 h. Following treatment, cells were either analyzed for apoptosis after staining with annexin V/PI dual staining by flow cytometry (C) or proteins were isolated and immunoblotted with antibodies against caspase-3 and beta-actin (D).



Online Supplementary Figure S3. (A) SUDHL4 and OCI-LY19 cells were transfected with either 100 nM scrambled siRNA or 50 and 100 nM specific siRNA targeted against FoxM1 for 48 h. After incubation, cells were lysed and immunoblotted with antibodies against caspase-9, caspase-3 and Beta-actin. (B) Effect of z-VAD-fmk on thiostrepton-induced apoptosis in DLBCL. SUDHL4 and OCI-LY19 cells were pre-treated with 80 μM of z-VAD for 2 h and subsequently treated with 10 μM thiostrepton for 48 h. Following treatment, cells were stained with fluorescein conjugated annexinV/PI and apoptosis was measured by flow cytometry. (C) Effect of z-VAD-fmk on thiostrepton-induced activation of caspases in DLBCL. SUDHL4 and OCI-LY19 cells were pre-treated with 80 μM of z-VAD for 2 h and subsequently treated with 10 μM thiostrepton for 48 h. Following treatment, cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against caspase-9, caspase-3, cleaved caspase-3 and beta-actin. (D) SUDHL4 and OCI-LY19 cells were treated with 5 and 10 μM thiostrepton for 48 h. Following treatment cells were lysed and proteins were applied directly to SDS–PAGE, transferred to immobilon membrane and immunoblotted with specific antibodies against XIAP, cIAP1, cIAP2, survivin and beta-actin.



Online Supplementary Figure S4. Synergistic apoptotic response of thiostrepton and bortezomib in DLBCL cells. SUDHL4 and OCI-LY19 cells were treated with various combinations of thiostrepton and bortezomib for 48 h and dose effect (A and C) and fractional effect (B and D) graphs were generated using Calcusyn software. Apoptotic response analysis was performed as mean±SD values normalized to control. Combination indices were calculated using the Chou-Talalay method.

Online Supplementary Table S1. Correlation of FOX-M1 expression with clinico-pathological parameters in DLBCL.

	Total		HIGH FOX-M1		LOW FOX-M1		*P*
	N.	%	N.	%	N.	%	
N. of patients[§]	214		181	84.6	33	15.4	
Age (all pts)							
<60	141	65.9	120	85.1	21	14.9	0.7677
>60	73	34.1	61	83.6	12	16.4	
Sex (all pts)							
Female	91	42.5	79	86.8	12	13.2	0.4335
Male	123	57.5	102	82.9	21	17.1	
IPI Group[@]							
Low-Low Inter	98	65.8	85	86.7	13	13.3	0.1983
High Inter-High	51	34.2	40	78.4	11	21.6	
GC *vs*. ABC[^]							
ABC	168	78.9	138	82.1	30	17.9	0.0126
GC	45	21.1	43	95.6	2	4.4	
p-AKT							
High (2-3)	91	42.7	82	90.1	9	9.9	0.0649
Low (0-1)	122	57.3	99	81.1	23	18.9	
SKP2							
High	97	45.7	92	94.8	5	5.15	< 0.0001
Low	115	54.3	88	76.5	27	23.5	
p27Kip1							
High	114	53.5	91	79.8	23	20.2	0.0215
Low	99	46.5	90	90.9	9	9.1	
Ki-67							
High	137	64.3	133	97.1	4	2.9	< 0.0001
Low	76	35.7	48	63.2	28	36.8	
MMP-2							
High	112	60.9	104	92.9	8	7.1	0.0008
Low	72	39.1	54	75.0	18	25.0	
MMP-9							
High	101	55.8	95	94.1	6	5.9	0.0002
Low	80	44.2	60	75.0	20	25.0	
Aurora B Kinase							
High	184	90.2	165	89.7	19	10.3	0.0061
Low	20	9.8	13	65.0	7	35.0	

[@]IPI information was only available in 142 patients. [^]GCvs ABC Germinal Center (GC) versus *Activated B Cell (ABC) phenotype. [§]Of the 231 diffuse large B-cell lymphomas, FoxM-1 results were available in 214 cases and the remaining 17 spots were non-informative. Analysis of failure for these IHC markers was attributed to missing or non-represntative spots. Of these 214 TMA spots with available FoxM1 data, range of non-available spots for rest of the IHC markers ranged from 1 spot for p-AKT to 33 spots for MMP9. The remaining cases were considered for correlation analysis.*

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