Supplemental Information

Cytotoxic effects of Bortezomib in Myelodysplastic syndrome/Acute Myeloid Leukemia depend on autophagy-mediated lysosomal degradation of TRAF6 and repression of PSMA1

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Material and Methods

Culture conditions

THP-1 and HL60 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (S12450; Atlanta Biologicals). TF-1 cells were maintained in the presence of 10ng/mL human interleukin 3 (hIL-3) (Stem Cell Technologies). The myelodysplastic cell line, MDS-L, was maintained in 10ng/mL hIL-3. $CD34^+$ cells were positively selected from cryopreserved marrow or peripheral blood cells by immunomagnetic separation (CCHMC Core) and maintained in StemSpan SFEM (StemCell Technologies) containing 10ng/mL of human stem-cell factor (hSCF) (Stem Cell Technologies), human Flt3 ligand (hFL), human thrombopoietin (hTPO), hIL-3 and hIL-6 (Stem Cell Technologies).

Methylcellulose

Cells were treated with Bort at the concentration of 10nM and DMSO for 24 hours, and were then plated in methylcellulose (04434; StemCell Technologies) at the number of 5×10^3 THP-1 cells/plate, 1×10^4 MDSL cells/plate and 1×10^4 bone marrow cells per plate from patients. The colonies were evaluated after 10 days. For the shTRAF6 knockdown purpose, cells were transduced, GFP positive cells were sorted with flow cytometry, and 3×10^3 transduced cells/plate were plated in methylcellulose for 10 days.

qPCR

RNA was extracted with Quick-RNA MiniPrep Kit (Zymo research), reverse transcribed into cDNA with High Capacity RNA-to-cDNA Kit (Invitrogen). Quantitative PCR was performed with Taqman Master Mix (Applied Biosystems) for TRAF6 (Hs00371512-g1; Applied Biosystems) and PSMA1 (Hs00267631_m1; Applied Biosystems). GAPDH (Hs02758991; Applied Biosystems) was used as an internal control.

Lentiviral Infections

Lentiviruses were pseudotyped with VSV-G, produced by 293-FT cells, and concentrated by ultracentrifugation at 20,000rpm for 2 hours at 4°C. Cells at 1×10^5/mL were transduced lentivirus at multiplicity of infection (MOI) of 0.5~1 and in the presence of 8μg/mL of polybrene (No. TR-1003-G; Millipore). At 48 hours post-transduction, GFP positive cells were isolated by fluorescence-activated cell sorting (FACS). The pLKO.1 (OpenBiosystems) constructs were obtained from the Lentiviral core at CCHMC and used to express shCTL, shTRAF6, and shPSMA1. Puromycin resistance gene was replaced by green fluorescent protein (GFP). Two independent and validated pLKO.1-shT6 constructs were obtained: TRCN0000007348 (#48) and TRCN0000007351 (#3). Two independent pLKO.1-shPSMA1 constructs were obtained: TRCN0000003870 and TRCN0000003872.

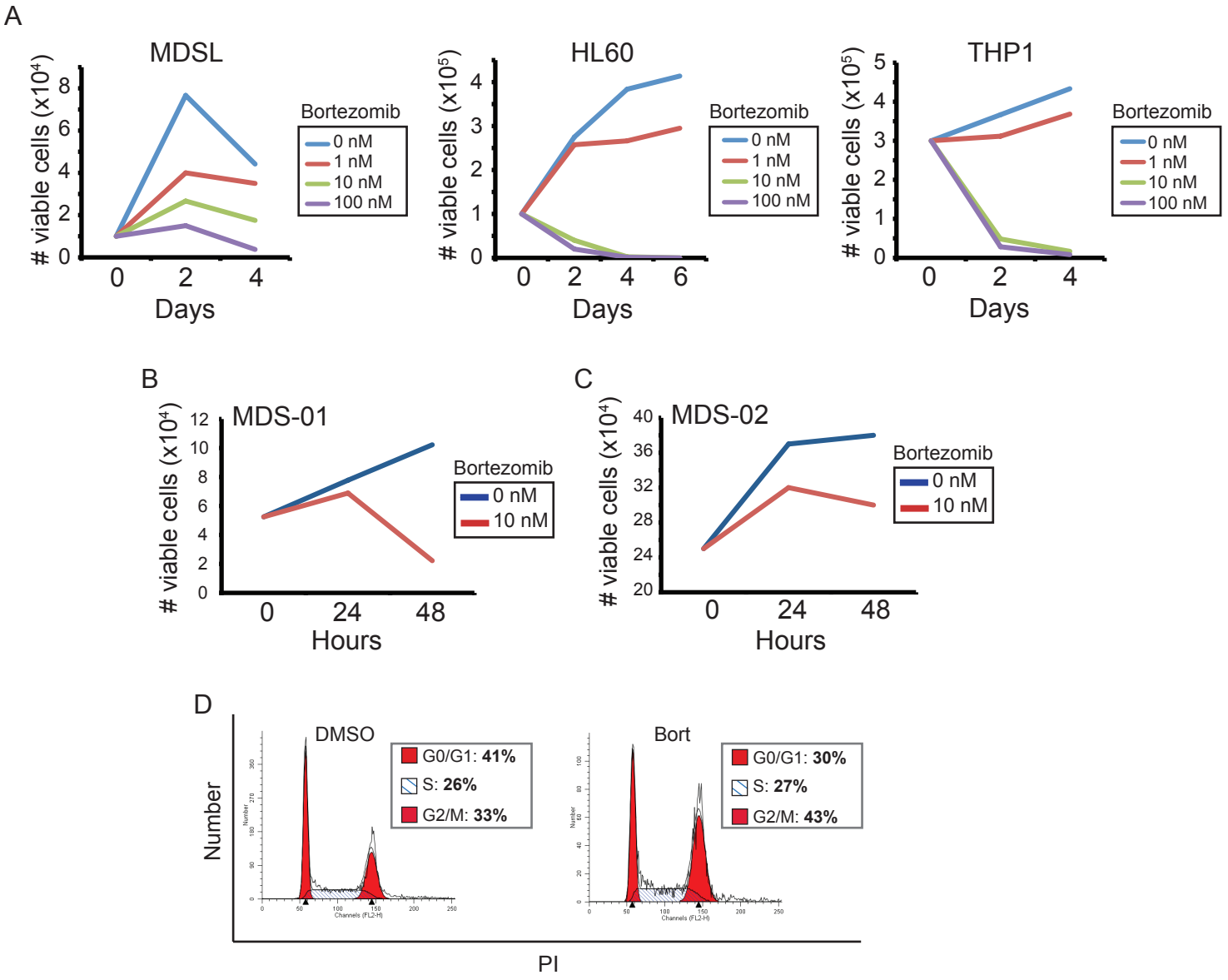
Supplemental Table 1. Proteasome subunit gene expression in TF-1 cells expressing shTRAF6.

Fold Change (shT6 vs vec)	up/down (shT6 vs vec)	Gene	Gene Description
1.8734876	down	PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
1.3505317	down	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
1.3500321	down	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
1.346279	down	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
1.1870465	down	PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3
1.1174587	down	PSME3	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)
1.0988634	down	PSMD10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10
1.0855404	down	PSMG3	proteasome (prosome, macropain) assembly chaperone 3
1.0690852	down	PSMG1	proteasome (prosome, macropain) assembly chaperone 1
1.0629337	down	PSMG4	proteasome (prosome, macropain) assembly chaperone 4
1.0629029	down	PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
1.0617175	down	PSMD9	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
1.051004	down	PSMD7	proteasome (prosome, macropain) 26S subunit, non-ATPase, 7
1.0454882	down	PSMB10	proteasome (prosome, macropain) subunit, beta type, 10 | chymotrypsin-like
1.03699	down	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
1.0353343	down	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
1.0338808	down	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
1.033211	down	PSMD6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6
1.0238222	down	PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6 | KIAA0391
1.0161107	down	PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4
1.0122166	down	PSMF1	proteasome (prosome, macropain) inhibitor subunit 1 (PI31)
1.0026861	down	PSMB4	proteasome (prosome, macropain) subunit, beta type, 4
1.2984062	up	PSMB5	proteasome (prosome, macropain) subunit, beta type, 5
1.1996164	up	PSMC3	proteasome (prosome, macropain) 26S subunit, ATPase, 3
1.193569	up	PSME4	proteasome (prosome, macropain) activator subunit 4
1.1910173	up	PSMD12	proteasome (prosome, macropain) 26S subunit, non-ATPase, 12
1.1774843	up	PSMD3	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
1.1671897	up	PSMD1	proteasome (prosome, macropain) 26S subunit, non-ATPase, 1
1.1637437	up	PSMC5	proteasome (prosome, macropain) 26S subunit, ATPase, 5
1.1555178	up	PSMB7	proteasome (prosome, macropain) subunit, beta type, 7
1.1280228	up	PSMB1	proteasome (prosome, macropain) subunit, beta type, 1
1.1222794	up	PSMD5	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5
1.1183304	up	PSMC6	proteasome (prosome, macropain) 26S subunit, ATPase, 6
1.1099048	up	PSMD2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2
1.1052538	up	PSMB3	proteasome (prosome, macropain) subunit, beta type, 3
1.1052232	up	PSMB2	proteasome (prosome, macropain) subunit, beta type, 2
1.0936346	up	PSMC2	proteasome (prosome, macropain) 26S subunit, ATPase, 2
1.0866498	up	PSMD4	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4
1.0845578	up	PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14
1.079188	up	PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4
1.0766393	up	PSMD13	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13
1.072969	up	PSMC6	proteasome (prosome, macropain) 26S subunit, ATPase, 6
1.0683745	up	PSMD11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11
1.0517234	up	PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
1.0504658	up	PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)
1.0481962	up	PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2
1.0440398	up	PSMB6	proteasome (prosome, macropain) subunit, beta type, 6
1.0342671	up	PSMC1	proteasome (prosome, macropain) 26S subunit, ATPase, 1
1.0275017	up	PSMA7	proteasome (prosome, macropain) subunit, alpha type, 7
1.0243065	up	PSMC1	proteasome (prosome, macropain) 26S subunit, ATPase, 1
1.012733	up	PSMA5	proteasome (prosome, macropain) subunit, alpha type, 5
1.0127015	up	PSMG2	proteasome (prosome, macropain) assembly chaperone 2

Supplemental Table 2. TRAF6 and drug gene signature overlap.

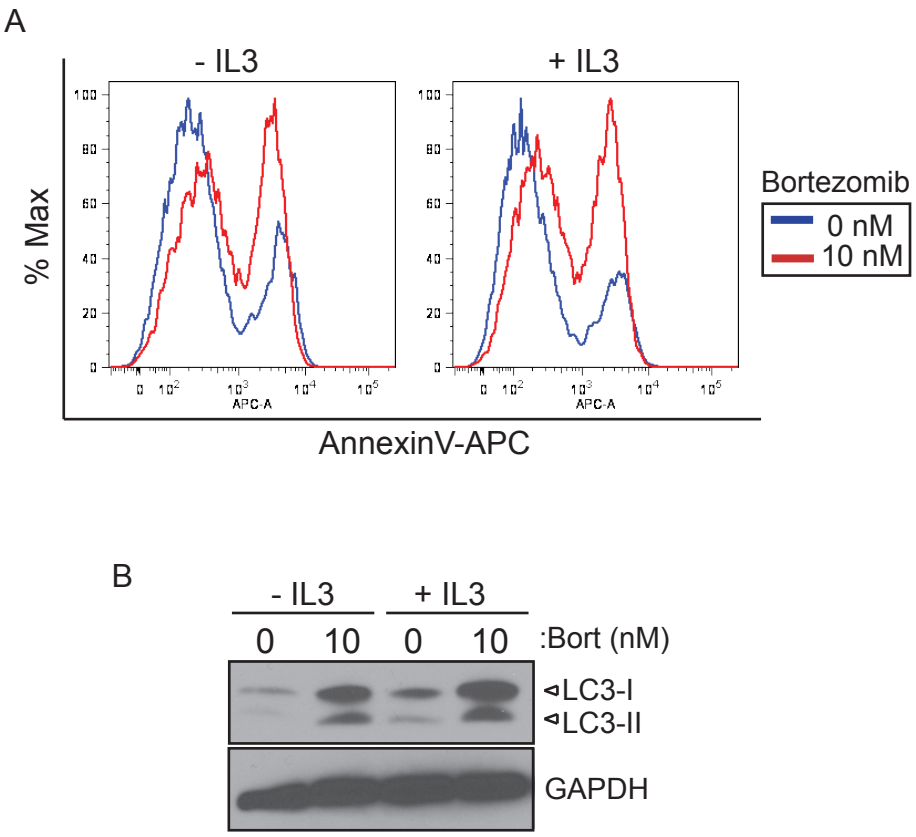
Name	P-value	Hit Count in Query List	Hit Count in Genome
Sirolimus	0.000014	10	97
Rapamycin	0.000185	30	784
Epigallocatechin gallate	0.001515	10	171
Disulfiram	0.01439	4	50
ALLN	0.002585	13	280
Bortezomib	0.042916	4	70

Supplemental Figure 1.



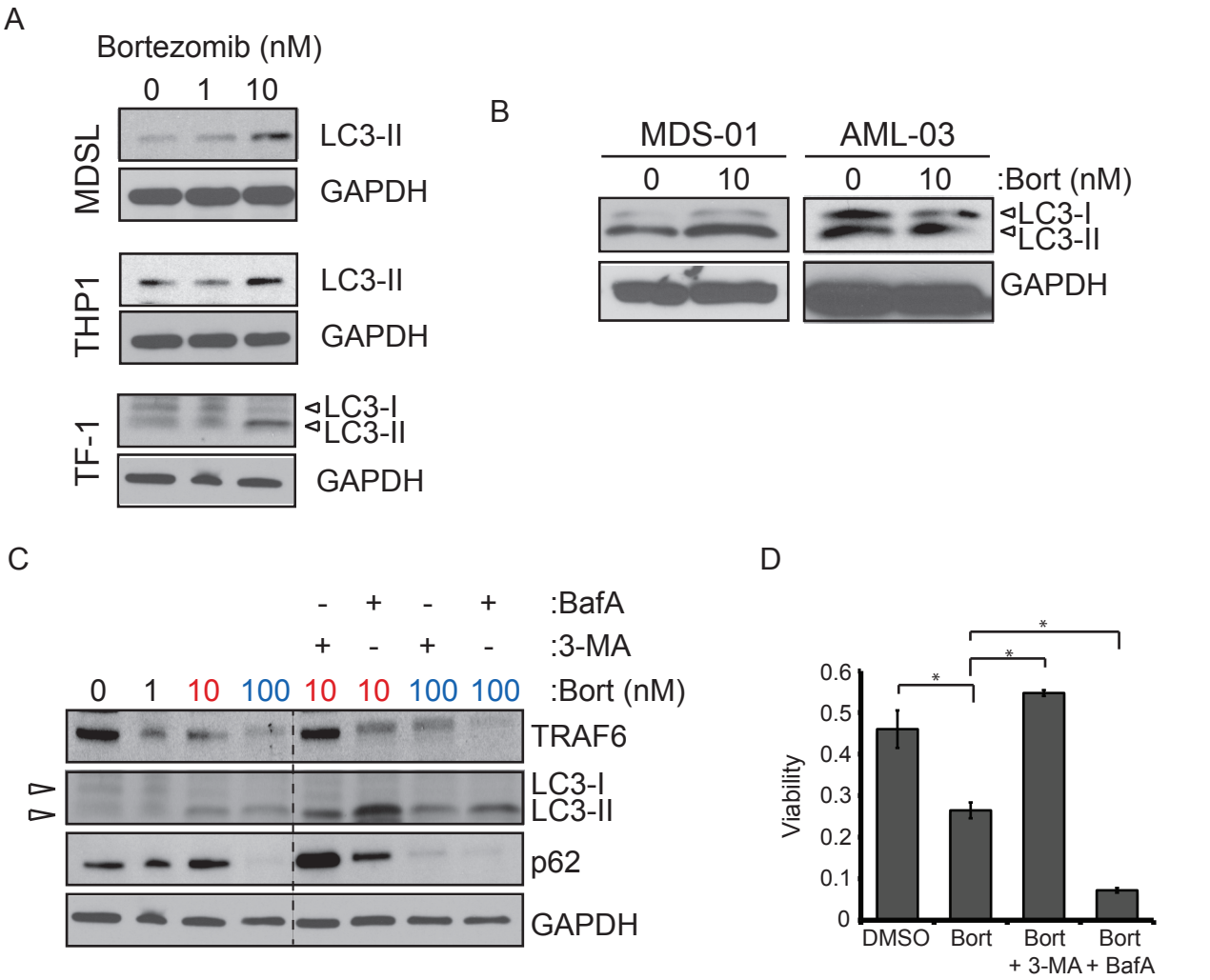
Inhibitory effect of Bortezomib on MDS and AML cell line viability and cell cycle. (A) The indicated cell lines were treated with increasing concentrations of Bortezomib for upto 6 days. Cell viability was measured by trypan blue exclusion. (B-C) Marrow cells from MDS patients (MDS-01 and MDS-02) were treated with DMSO or 10 nM Bortezomib and viable cells (trypan blue exclusion) were measured at the indicated time points. (D)) TF-1 cells were treated with Bortezomib for 24 hours and analyzed for cell cycle status by staining with propidium iodide (PI).

Supplemental Figure 2.



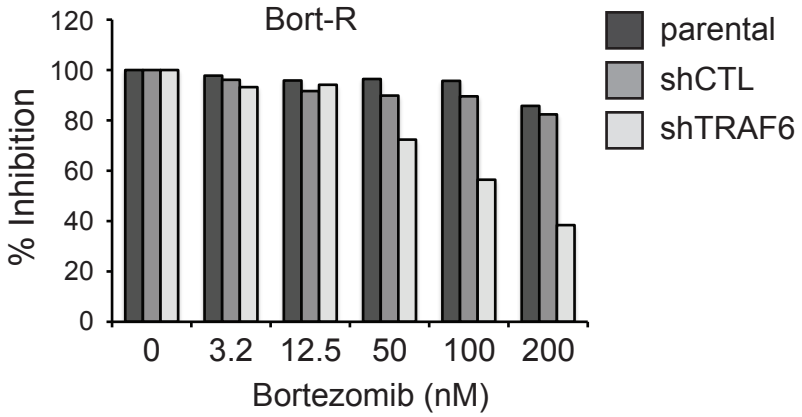
Effects of Bortezomib on viability and conversion of LC3B is not dependent on cytokines. TF-1 cells cultured with or without IL-3 were treated with Bortezomib for 24 hours and analyzed for cell viability by staining for AnnexinV-positive cells (A) and expression of LC3B by immunoblotting (B).

Supplemental Figure 3



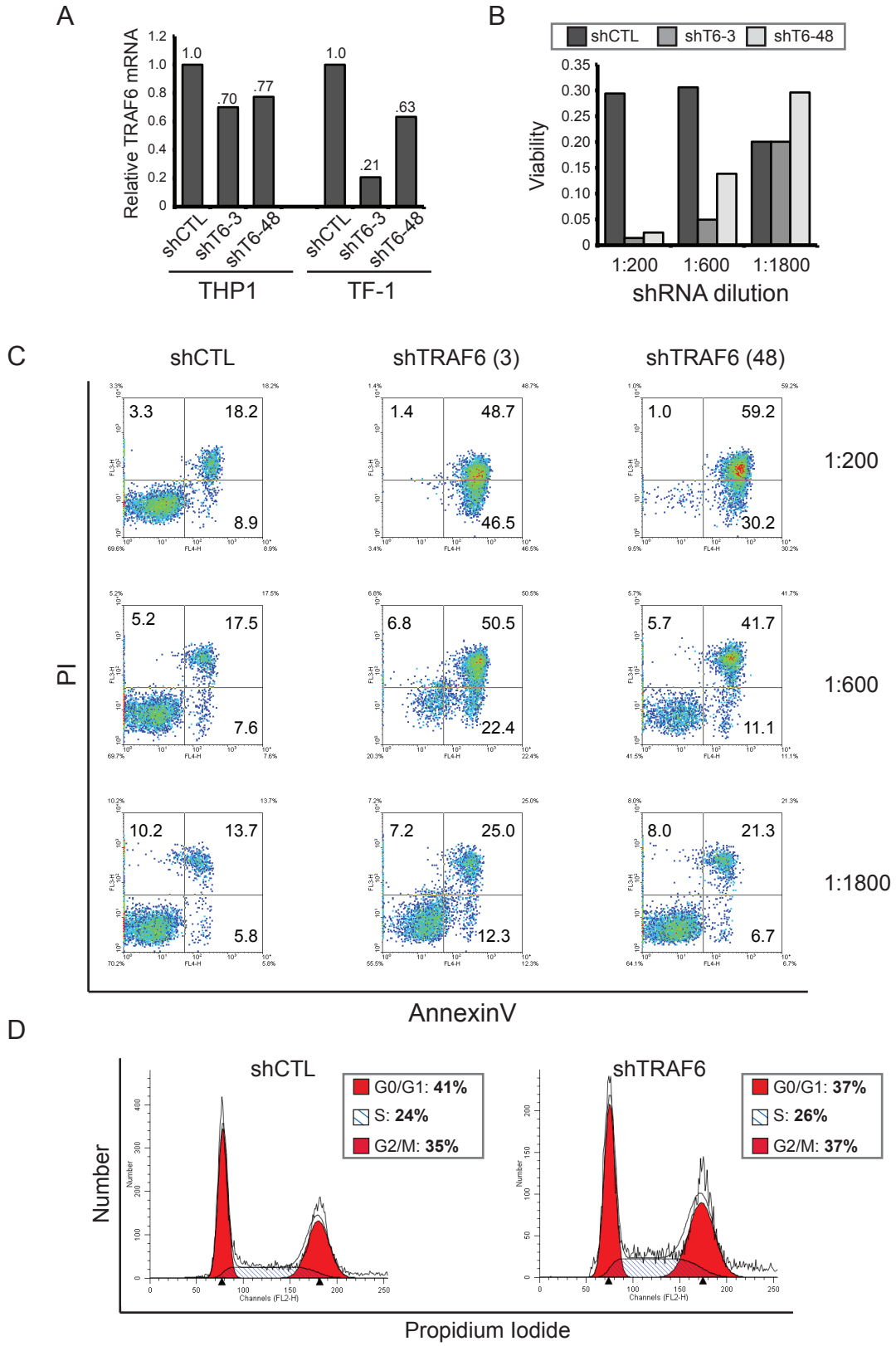
Bortezomib induces markers of autophagy in MDS/AML cells. (A-B) Cell lines and primary cells were cultured with the indicated amounts of Bortezomib for 24 hours. LC3B and GAPDH protein was determined by immunoblot analysis. (C) TF-1 cells were cultured with the indicated concentrations of Bortezomib and co-treated with BaflomycinA (100 nM; BafA) or 3-methyladenine (5 mM; 3-MA). Protein lysates were evaluated for TRAF6, LC3-I/II, p62, and GAPDH. (D) TF-1 cells were treated with Bortezomib (10 nM), 3-MA (5 mM), and/or BafA (100 nM) for 24 hours. Cell viability was measured by MTT assay.

Supplemental Figure 4.



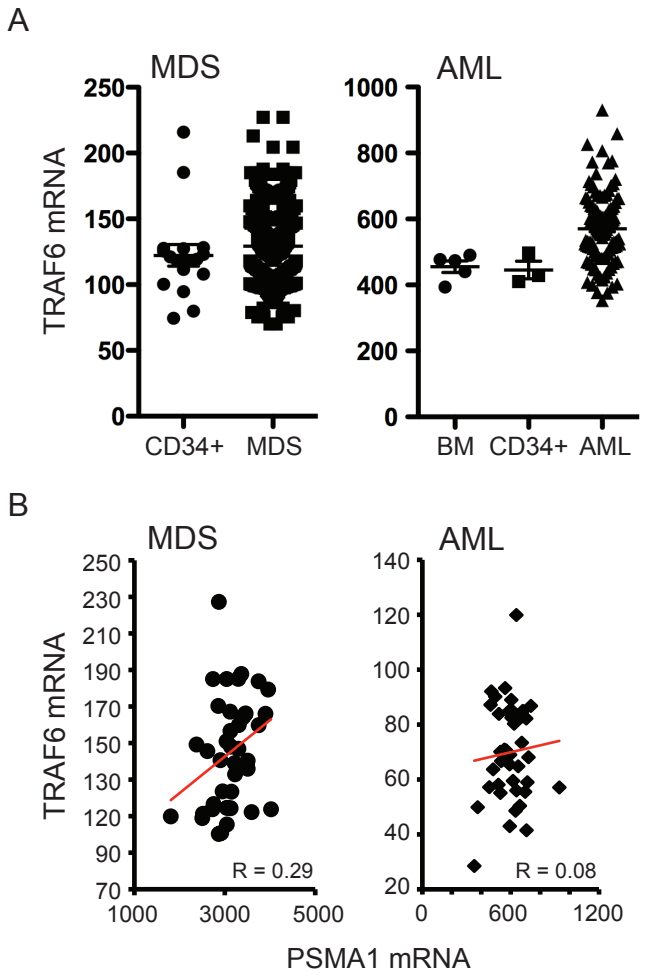
Evaluation of Bortezomib-resistant THP1 cells expressing shTRAF6. Bortezomib-resistant (Bort-R) THP1 cells were transduced with control shRNA (shCTL), shTRAF6, or mock transduced. Cell viability was determined at the indicated Bortezomib concentrations after 24 hour treatment. Parental and shCTL-transduced cells are resistant to Bortezomib. In contrast, knockdown of TRAF6 results in reduced viability when treated with Bortezomib.

Supplemental Figure 5.



Depletion of TRAF6 with independent shRNA-targeting constructs (A) HL-60 and THP-1 cells were transduced with two shTRAF6 constructs or vector control. Knockdown of TRAF6 mRNA was confirmed by qPCR . (B) TF-1 cells transduced with increasing concentrations of virus encoding shCTL or two independnet shTRAF6 constructs were evaluated for cell viability using the MTT assay. Cell viability is shown at the indicated viral dilutions. (C) Cell survival of transduced TF-1 cells was confirmed by AnnexinV/PI staining 5.5 days post-transduction with the indicated viral dilutions. (D) Cell cycle of TF-1 cells transduced with shCTL or shTRAF6 was determined by flow cytometry for PI incorporation. Flow plots show G0/G1, S, and G2/M phases of the cell cycle for shCTL and shTRAF6 expressing cells 4 days post transduction.

Supplemental Figure 6.



Expression of TRAF6 and PSMA1 in MDS and AML patients. (A) Expression of TRAF6 from previously published micrarray analyses is shown for MDS (Pellagatti, et al., 2006) and AML (Valk et al., 2004) CD34+ cells. (B) Co-expression of TRAF6 and PSMA1 in MDS (del(5q)) and AML (M2) is shown.