SUPPLEMENTARY APPENDIX

Inhibition of 4EBP phosphorylation mediates the cytotoxic effect of mechanistic target of rapamycin kinase inhibitors in aggressive B-cell lymphomas

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Supplementary Materials and Methods:

Cell lines and drugs. Jeko-1, Mino, Z138, Rec-1, Jvm2, Maver-1, Ramos, Raji, Daudi, Su-dhl-16,Su-dhl-6, Tmd8, U2932, Dohh2 and ROS-50 cells were maintained in RPMI 1640; Granta and HEK-293T cells were maintained in DMEM; Val cell was maintained in IMDM with 55 μM 2-mercaptoethanol. All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Torin1, ABT-737 and ABT-199 were from Chemscene (Monmouth Junction, NJ); AZD-8055 was from ChemieTek (Indianapolis, IN); WEHI-539 was from ApexBio (Boston, MA); Temsirolimus was from Sigma-Aldrich (St. Louis, MO).

Cell proliferation and apoptosis assays. Viable cell metabolism was determined by using the CellTiter 96 AQueous kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, cells were placed at a density of 2-5×10^5 cells/ml and treated with drug or vector for 72 hours. The cells were then incubated with MTS reagent for 2h, and 490 nm absorbance was quantified with a plate reader. Coefficient of drug interaction (CDI) were calculated by the following equation: CDI = AB/ (A × B), where AB is the inhibitory ratio of the combined group to control group; A or B is the ratio of single agent group to control group, respectively. CDI less than 0.7 indicates that two drugs have significant synergy.

Apoptosis were determined by using Annexin-V staining kit (BD Biosciences, San Jose, CA). Cells were treated with drug or vector for 24 or 48 h and then pelleted and washed once with cold phosphate-buffered saline (PBS). The cell pellets were then resuspended in 1X binding buffer at a concentration of 10^6 cells/ml. 100 μl of the suspension was transferred to a culture tube and incubated with Annexin V-FITC and propidium iodide or Annexin V-PE and 7-AAD, 5 μl of each, for 15 min at 25°C, protecting from light. The stained sample were then diluted with 400 μl of 1X binding buffer and analyzed by FACSCalibur flow cytometry (BD Biosciences).

Immunoblotting and antibodies. Cell lysates were separated and immunoblotted by using SDS-PAGE (Bio-Rad, Hercules, CA) or Bolt® Bis-Tris system (Life technology, Carlsbad, CA). Blots were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), and the band intensity was quantified by using Odyssey software v2.0.

Antibodies for immunoblotting in this study were as follows: 4EBP1 (#9644), 4EBP2 (#2845), p-4EBP1$^{Thr37/46}$ (#2855), RPS6 (#2217), p-RPS6$^{S235/236}$ (#4858), EIF4E (#9742), BCL-2 (#2870), MCL1 (#5453), p-AKT^{S473}(#4060), p-AKT^{T308} (#13038) and p- GSK3-β (#9336) were from Cell Signaling Biotechnology; BCLX (#556361) and GSK3-β (#610202) were from BD Biosciences (San Jose, CA); AKT (#1080-1) was from Epitomics (Burlingame, CA); Actin (#1616) was from Santa Cruz Biotechnology (Dallas, TX); Rictor (#70374) was from Abcam (Cambridge, MA).

Supplementary Table S1: The sgRNA sequences of CRISPR-CAS9 experiments

4EBP1-sg1	GAGCACCACCCGGCGAGTGG	4EBP2-sg1	GCTGATGGCCACGGTGCGGG
4EBP1-sg2	GGGCTCATCACTGGAAGGGC	4EBP2-sg2	GCGCTCACCTCCCGGTGTGG
4EBP1-sg3	TAGCCCAGAAGATAAGCGGG	4EBP2-sg3	GCAGGGTGGGGTCTGAGCCA
Rictor-sg1	TTACCTCGGGTCAGATCCAG	Rictor-sg3	AGCACTTCAGAATCCAGAGG
Rictor-sg2	GTTTGTTTTCATACAGTTTG		

Supplementary Figure Legends:

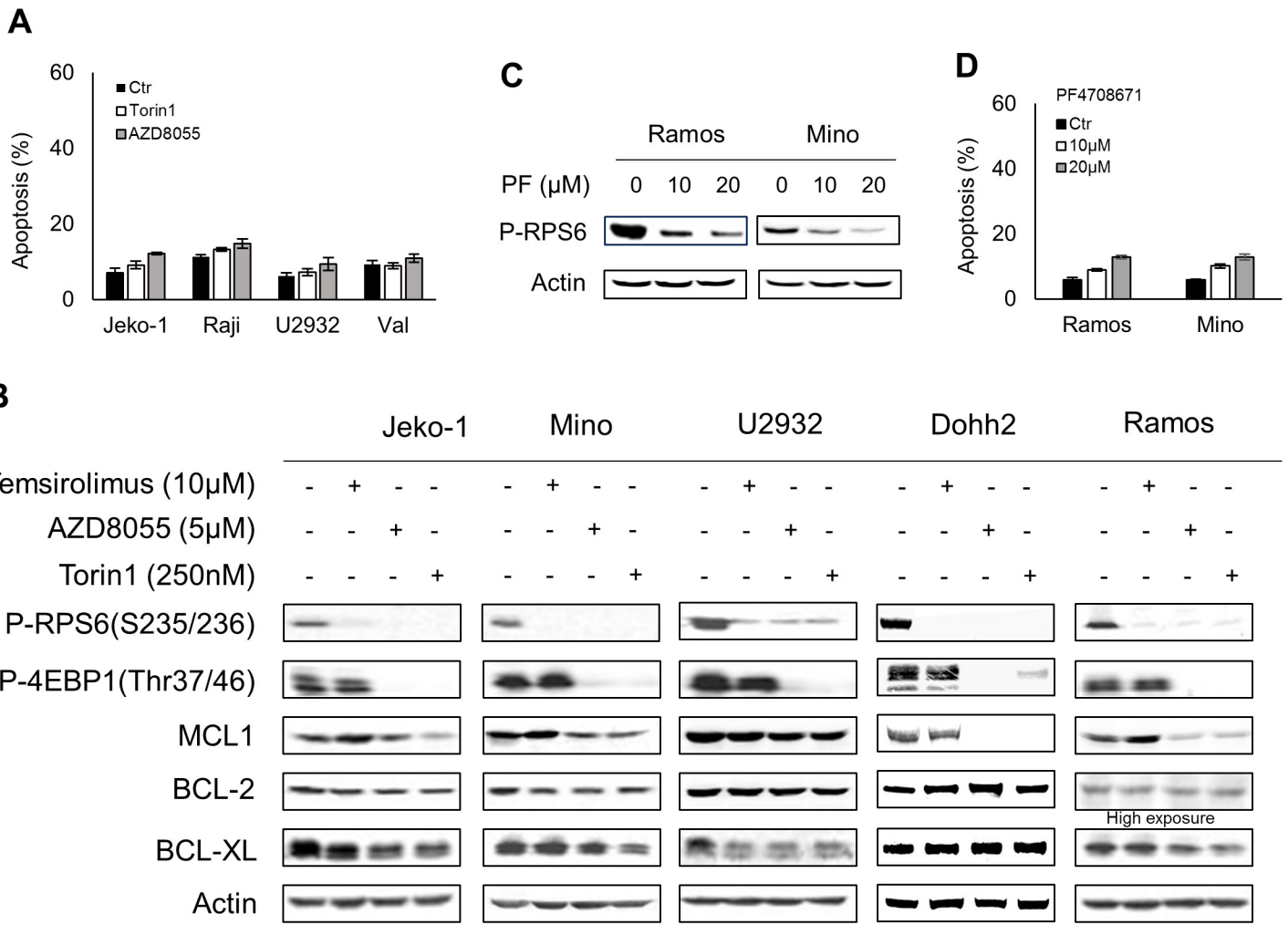
Supplementary Figure S1. Inhibition of p70S6K has little effect on TORKI-induced apoptosis in aggressive B lymphoma cells. (A) Resistant cells were treated with TORKi for 96h, and the apoptosis was measured. (B) Lymphoma cells were treated with Temsirolimus, AZD8055, or Torin-1 for 24 h, and immunoblotting was performed using the indicated antibodies. (C and D) Ramos and Mino cells were treated with PF4708671, a p70S6K selective inhibitor, and measured with p-RPS6 (S235/236) and apoptosis (48h). All apoptosis was evaluated using flow cytometry with Annexin V and PI double staining. Data shown are average of two experiments and are presented as mean ± SEM.

Supplementary Figure S2. BH3 profiling of TORKI-resistant aggressive lymphoma cells. The assays were performed using 80 μM BH3-only peptides as illustrated in Figure 6.

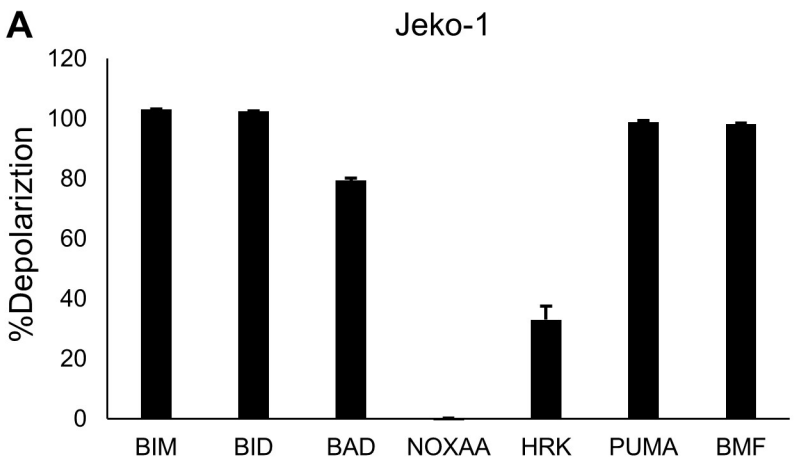
Supplementary Figure S3. Combination of TORKI with other pro-apoptotic drugs. (A) Mino cells were treated with AZD8055 and ABT-199 (targeting BCL-2) or WEHI-539 (targeting BCL-

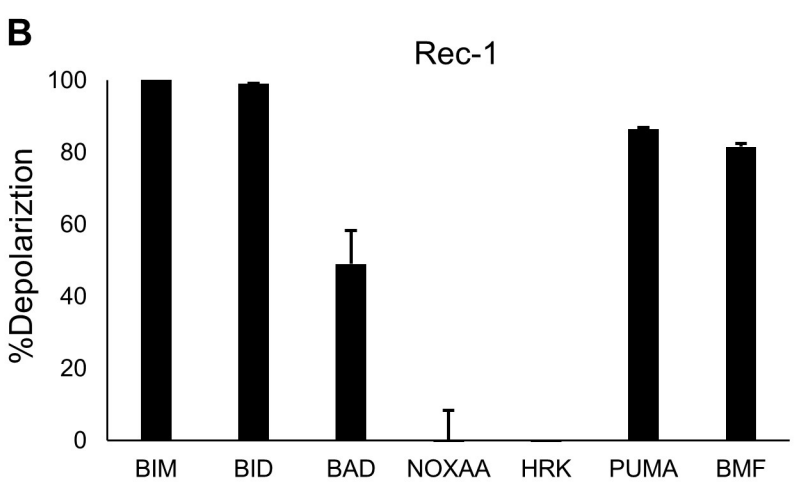
XL), individually or in combination. The apoptosis was evaluated using flow cytometry with Annexin V and PI double staining 48h after the treatment. (B) Mino cell were treated with AZD8055 and ABT-737, individually or in combination, and cell viability was measured by MTS assay 72 h after the treatments. (C and D) DHL cells Ros-50 and Dohh2 were treated with Torin1 and ABT-199 for 48 h, individually or in combination. Live cells were evaluated by measuring apoptosis with Annexin V and PI double staining. **: $P<0.01$; ***: $P<0.001$. Results are shown as the mean ± SEM of two experiments for apoptosis assay and three experiments for MTS assay.

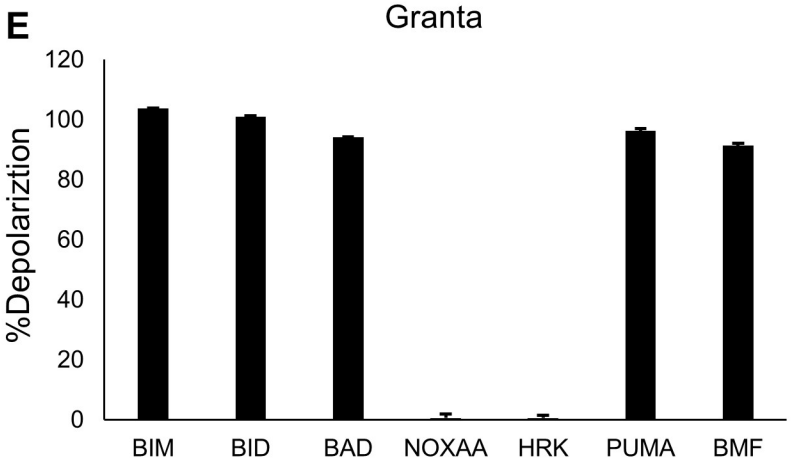
Supplementary Figure S1

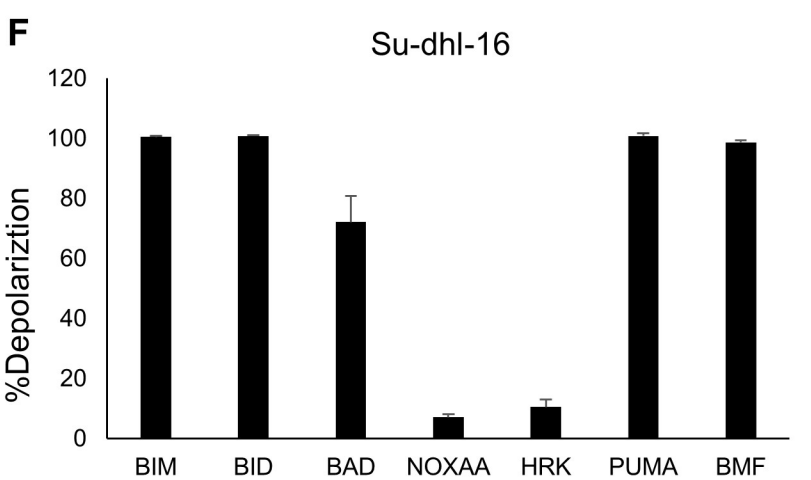


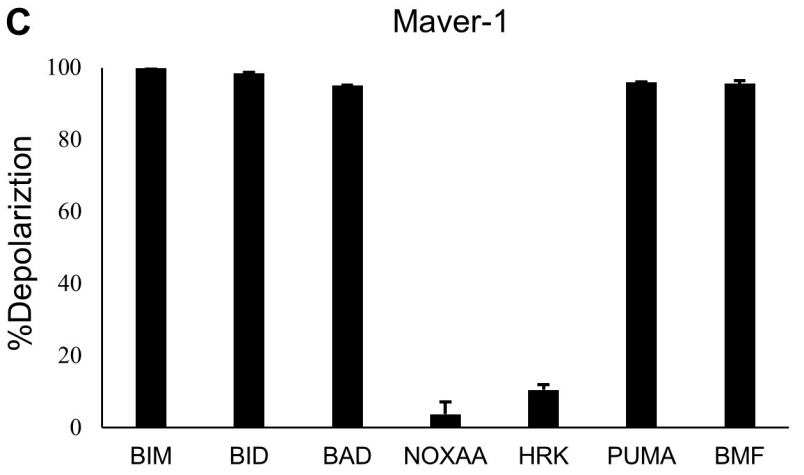
Supplementary Figure S2

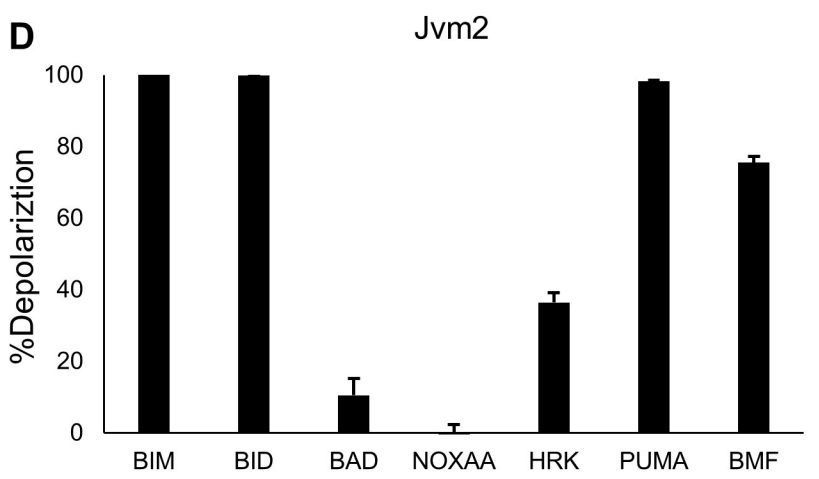


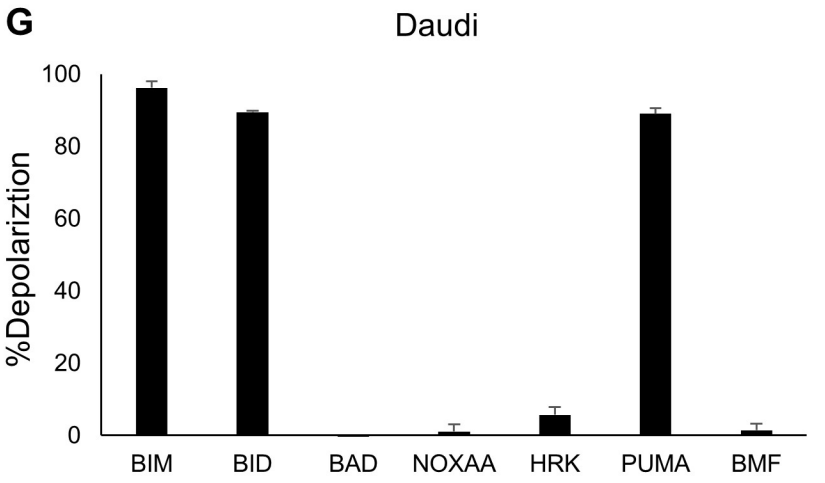


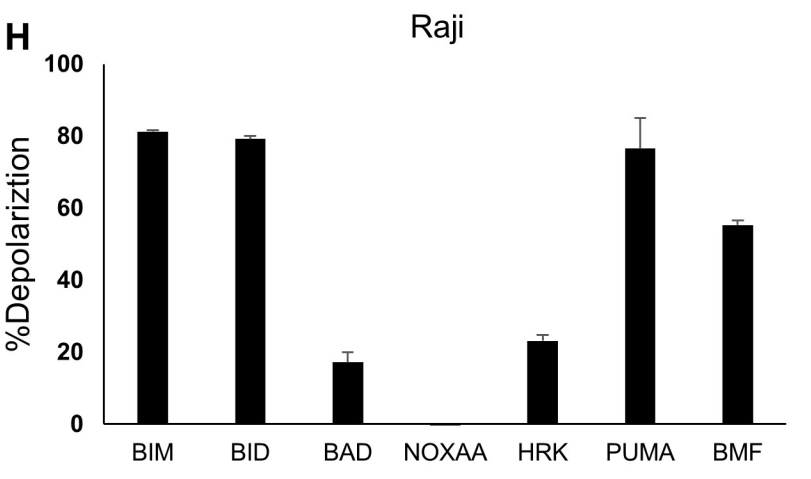












Supplementary Figure S3