

Co-administration of the mTORC1/TORC2 inhibitor INK128 and the Bcl-2/Bcl-xL antagonist ABT-737 kills human myeloid leukemia cells through Mcl-1 down-regulation and AKT inactivation

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Supplementary Methods

Immunoprecipitation and Immunoblotting: The antibodies used in these studies were: Cleaved caspase-3, ERK1/2, p-ser⁴⁷³-AKT, p-thr³⁰⁸-AKT, p-ser⁷⁰-4EBP1, Bcl-xL (Cell Signaling Technology; Beverly, MA). Bax, Bcl-2, and Mcl-1 (PharMingen; San Diego, CA). Poly(ADP-ribose) Polymerase (PARP) (Biomol Research Laboratories, Plymouth Meeting, PA). AIF, cytochrome c, Bak, AKT (Santa Cruz Biotechnology, Santa Cruz, CA). Bim, and α -tubulin (Calbiochem).

Mutation analysis: Primary AML samples were analyzed for mutations in 50 cancer associated genes using Next Generation Sequencing (NGS). Briefly, libraries for each sample were generated from 10 ng of dsDNA using Ion AmpliSeqTM Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA). This panel consists of 207 amplicons covering near 2,800 COSMIC mutations in 50 cancer-associated genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL. Sequencing was conducted on Ion 316TM chips and run on the Ion Torrent PGM instrument using the Ion PGMTM Sequencing 200 kit v2 (Life Technologies) following the manufacturer's recommendations.

Generation of Ba/F3 mutants: Ba/F3 cells were purchased from the DSMZ and were maintained in RPMI-1640 medium with 10% FBS and 2 ng/ml of murine IL-3. Ba/F3 cells carrying FLT3 mutations were generated as follow: Total RNA was extracted from the MV4-11 cells using the RNeasy kit (Qiagen) and cDNA was synthesized using AccuScript High-Fidelity Reverse Transcriptase (Agilent Technologies). FLT3-ITD was amplified and cloned into pLVX-IRES-ZsGreen1 vector (clontech) using PCR In-Fusion HD cloning kit (Clontech). FLT3-F691L point mutation was introduced into FLT3-ITD cDNA using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All inserts were fully sequenced using primer walking DNA sequencing (Eurofins MWG Operon Huntsville, AL). Lentiviruses carrying FLT3-ITD or

FLT3-ITD-F691L were generated and used to infect Ba/F3 wild type cells as previously described ¹. Following 3 days of culture in the presence of IL-3, IL-3 was removed and cells were cultured for an additional 3 weeks. The cell population selected was confirmed for GFP expression by flow cytometry in association with FLT3 mutant expression and increased STAT5 phosphorylation by Western blot analysis.

Statistical analysis: The significance of differences between experimental conditions was determined using the Student's t test for unpaired observations. Survival rates were analyzed by Kaplan–Meyer and comparisons of survival curves and median survival were analyzed by logrank test. Animals that deceased due to causes unrelated to disease or treatment toxicity (e.g., sacrifice) were considered censored for survival analysis.

Reference

1. Rahmani M, Aust MM, Attkisson E, Williams DC, Jr., Ferreira-Gonzalez A, Grant S. Inhibition of Bcl-2 antiapoptotic members by obatoclax potently enhances sorafenib-induced apoptosis in human myeloid leukemia cells through a Bim-dependent process. *Blood*. 2012;119(25):6089-6098.

Legends to Supplementary Figures

Supplementary Figure 1. Inhibition of Bcl-2 or Bcl-2/Bcl-xL markedly enhances INK128

lethality in AML cells. A) U937 cells displaying tet-inducible dual knock-down of Bcl-2 and Bcl-xL were left untreated or pre-treated with 1 µg/ml doxycycline (Dox) for 48 hr, then exposed to the designated concentration of INK128 or Rapamycin for 7 hr, after which cell growth and viability was assessed using the Cell-Titer-Glo luminescent assay. **B)** NOD/SCID γ mice were injected via tail vein with U937 cells exhibiting tet-inducible Bcl-2/Bcl-xL dual knock-down and expressing luciferase. Mice were fed either with regular or doxycycline-supplemented pellets (200 mg/kg, Bio-Serv, Frenchtown, NJ). At the designated intervals, mice were imaged using the IVIS 200 system. U937 **(C)**, and MV4-11 **(D)** cells were treated with INK128 and ABT-737 alone or in combination for 24 hr after which cell growth and viability was assessed using Cell-Titer-Glo luminescent assay and median dose effect analysis was performed using CalcuSyn software. The agent concentrations used were: For U937 cells, ABT-737: 250, 500, 750, 1000 nM; INK128: 100, 200, 300, and 400 nM. For MV4-11, ABT-737: 5, 10, 15, and 20 nM; INK128: 50, 100, 150, and 200 nM. **E)** MV4-11, MOLM-13, and KG-1 cells were treated with INK128 (100 nM for MV4-11 and MOLM-13 cells; 200 nM for KG-1 cells) \pm ABT-199 (10 nM for MV4-11 and MOLM-13 cells; 500 nM for KG-1 cells) for 24 hr, after which the extent of cell death was assessed by Annexin V/PI staining assay. Error Bars: S.D of 3 independent experiments; *, $p < 0.05$ for combined treatment compared to either agent alone.

Supplementary Figure 2. Activity of FLT3 inhibitors sorafenib or quizartinib in Ba/F3 cells

expressing FLT3-ITD or FLT3-ITD F691L mutations. Wild type Ba/F3 cells or Ba/F3 cells expressing FLT3-ITD or FLT3-ITD F691L were treated with the designated concentrations of sorafenib or quizartinib for 24 hr after which cell growth and viability were assessed using the CellTiter-Glo luminescent assay. Values represent the means for 3 independent experiments \pm S.D.

Supplementary Figure 3. Mcl-1, Bcl-2, or Bcl-xL oppose INK128/ABT-737-mediated cell

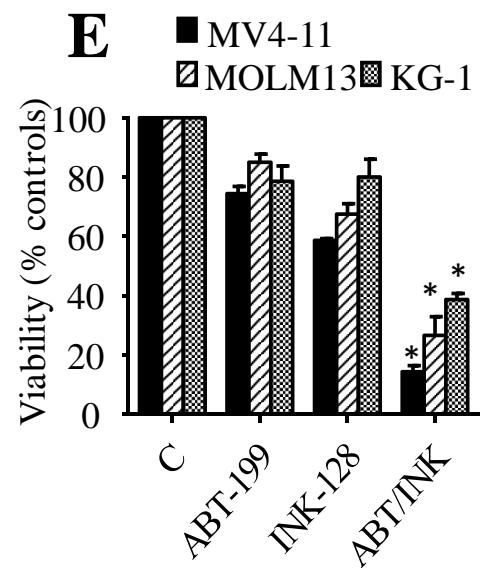
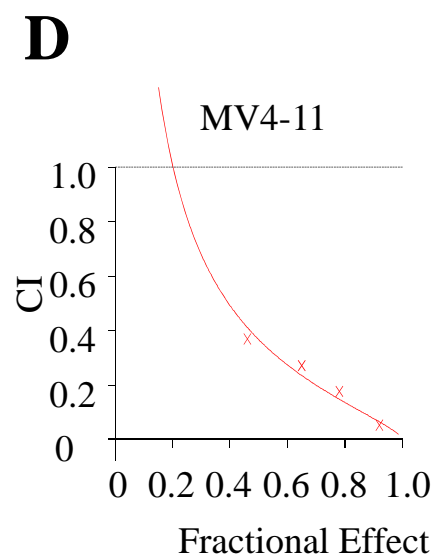
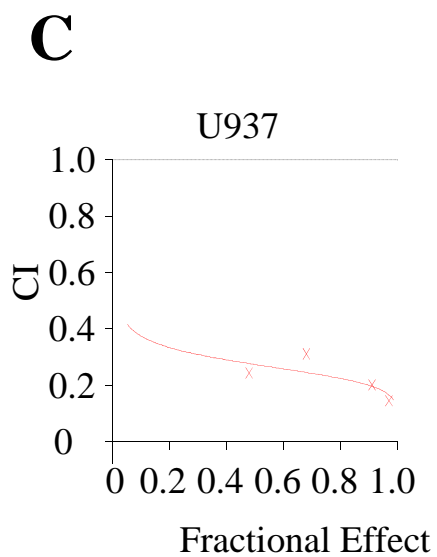
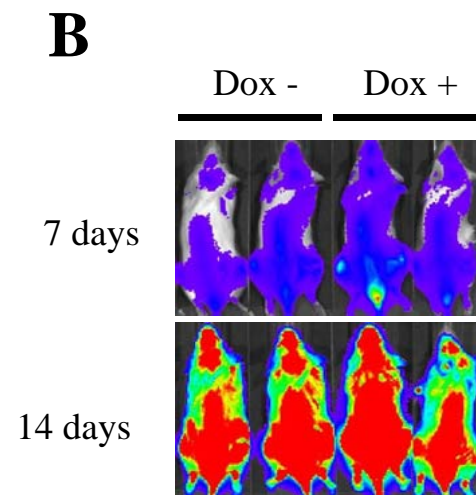
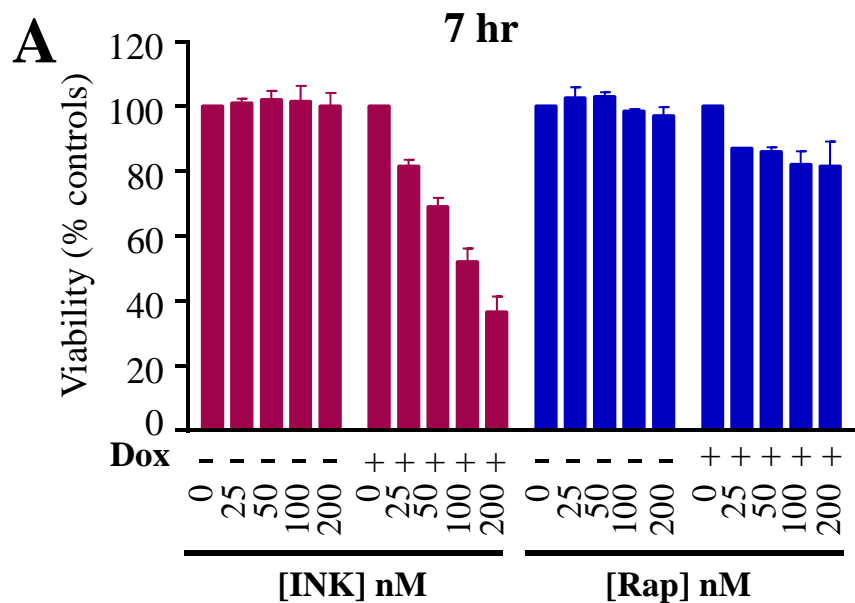
death in AML cells. A) MV4-11 cells were exposed to 10 nM ABT-737 and 100 nM INK128 individually or together for 24 hr, after which cells were lysed and protein lysates subjected to Western blot analysis. **B)** U937 cells ectopically expressing Mcl-1 or their empty vector control counterparts (pCEP) were exposed to 200 nM INK128 \pm 500 nM ABT-737 for 24 hr, after which

cell growth and viability were assessed using the CellTiter-Glo luminescent assay. Values represent the means for 3 independent experiments \pm S.D. **C)** Western blot analysis in untreated U937 cells ectopically expressing Bcl-2, Bcl-xL or the empty vector pREP. **D)** Assessment of cell growth and viability using the CellTiter-Glo Luminescent assay following cell exposure to 200 nM INK128 \pm 500 nM ABT-737 for 24 hr. Error Bars: S.D of 3 independent experiments; *, $p < 0.01$ in each case.

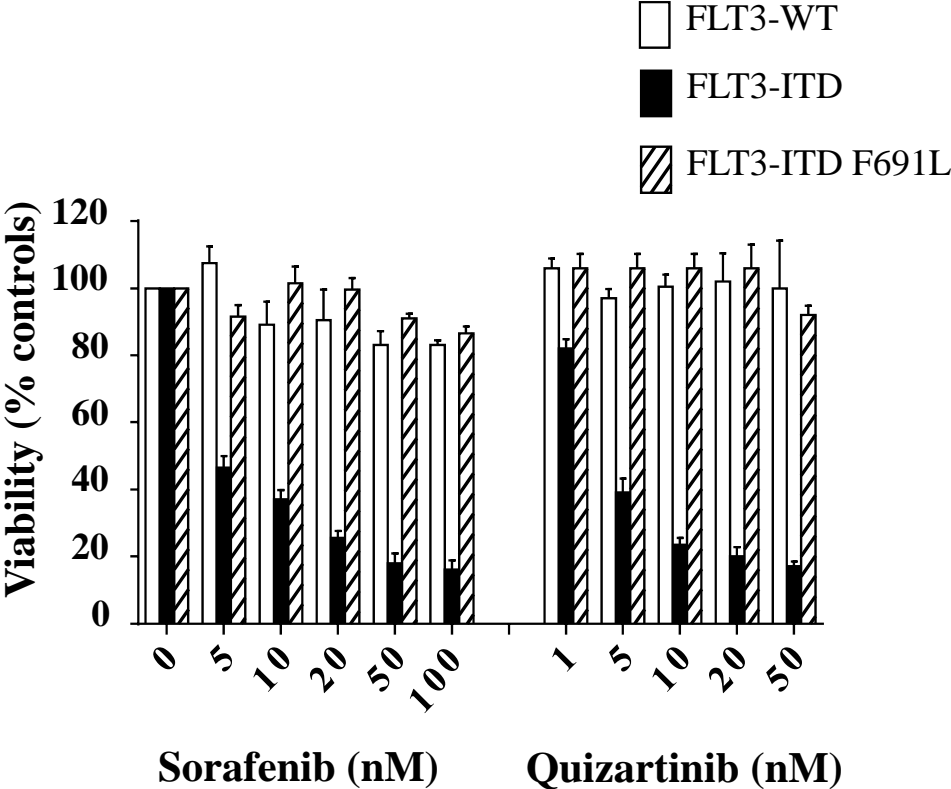
Supplementary Figure 4. Modulation of mTOR inhibitor/ABT-737 anti-leukemia activity by AKT. A-B) U937 or MV4-11 cells were exposed to the designated concentrations of rapamycin or INK128 for 4 hr after which cell were lysed and protein lysates were subjected to Western blot analysis. **C)** Assessment of cell growth and viability using the CellTiter-Glo Luminescent assay in MV4-11 cells following 24 hr exposure to the indicated agents. The doses used in this study were: 10 nM ABT-737; 100 nM INK128; 100 nM rapamycin; and 2 μ M AKTi (AKT inhibitor VIII). Values represent the means for at least 3 independent experiments \pm S.D. *, $p < 0.05$. **D)** Blots (from Figure 8C) were quantified using Image Studio Software (Li-Cor Biosciences), and results were normalized for ERK1/2 loading controls. The data shown represent the means for 2 sets of animals.

Supplementary Figure 5. Effect of combined treatment with INK128 and ABT-737 on animal weight. NOD/SCID gamma mice bearing MV4-11-derived systemic xenograft were treated with INK128 (0.5 mg/kg) and ABT-737 (80 mg/kg) alone or in combination for the designated intervals, after which animal body weights were measured. Data involve at least 5 mice/condition.

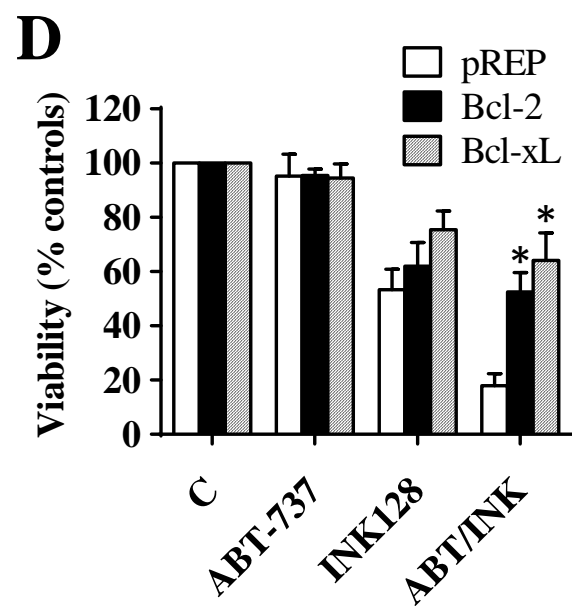
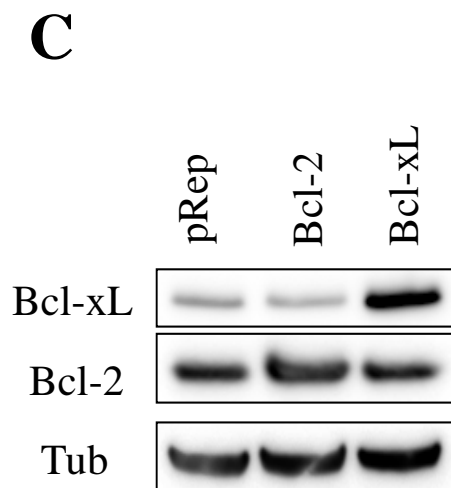
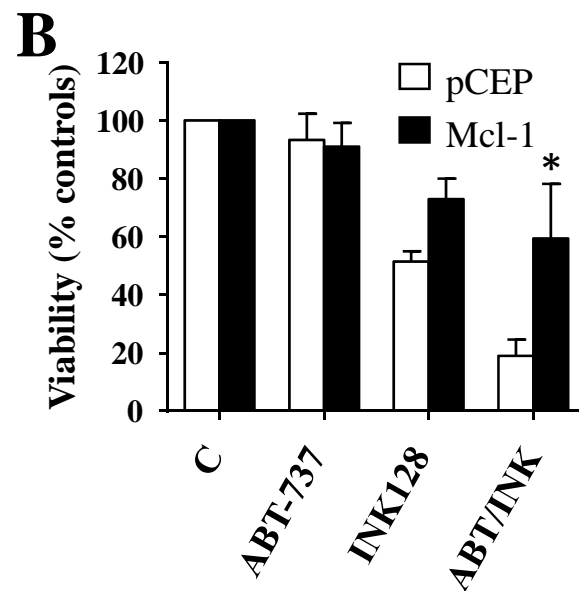
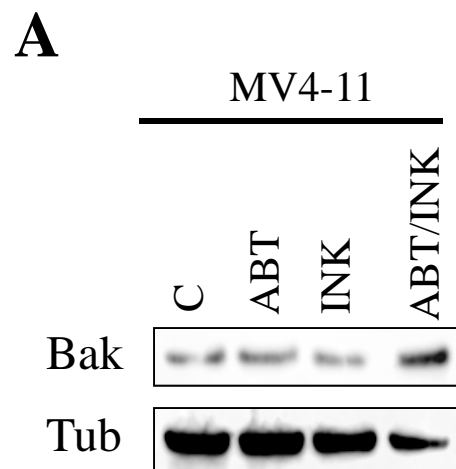
Supplementary Figure 1



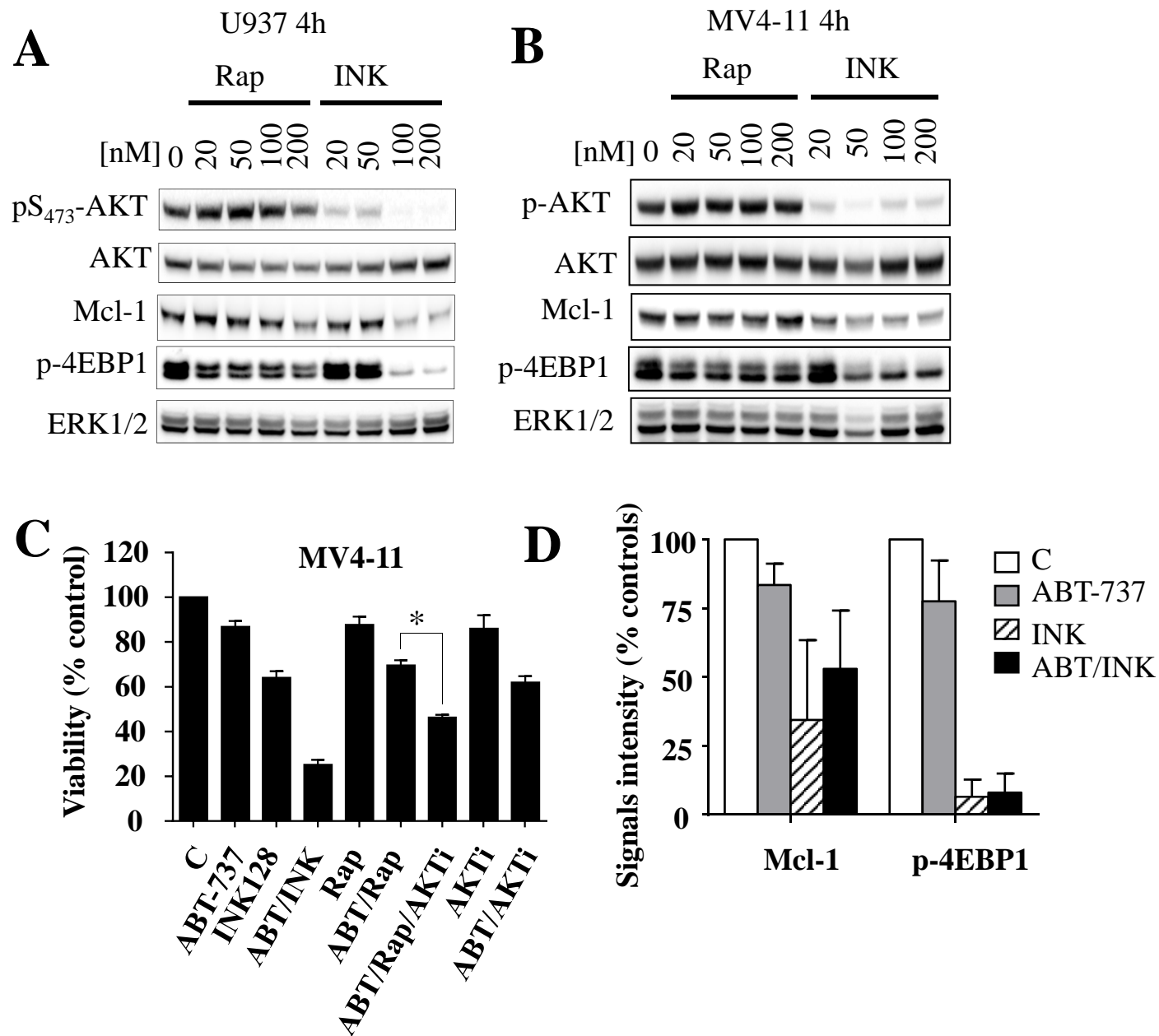
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

