Farnesyltransferase inhibitor tipifarnib inhibits Rheb prenylation and stabilizes Bax in acute myelogenous leukemia cells

Husheng Ding,[1]* Jennifer S. McDonald,[1]* Seongseok Yun,[1,2] Paula A. Schneider,[1] Kevin L. Peterson,[1] Karen S. Flatten,[1] David A. Loegering,[1] Ann L. Oberg,[3] Shaun M. Riska,[3] Shengbing Huang,[4] Frank A. Sinicrope,[4] Alex A. Adjei,[5] Judith E. Karp,[6] X. Wei Meng,[1] and Scott H. Kaufmann[1,2]

[1]Division of Oncology Research, Department of Oncology; [2]Department of Molecular Pharmacology and Experimental Therapeutics; [3]Division of Biomedical Statistics, Department of Health Sciences Research; and [4]Division of Gastroenterology, Department of Medicine, Mayo Clinic College of Medicine, Rochester, MN; [5]Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY; and [6]Sidney Kimmel Cancer Center at Johns Hopkins, Baltimore, MD, USA

*HD and JSM contributed equally to this work.

©2013 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2013.087734
Manuscript received on March 11, 2013. Manuscript accepted on August 29, 2013.
Correspondence: Kaufmann.scott@mayo.edu

Supplementary Material To:

The Farnesyltransferase Inhibitor Tipifarnib Inhibits Rheb Prenylation and Stabilizes Bax in Acute Myelogenous Leukemia Cells

Husheng Ding, Jennifer S. McDonald, Seongseok Yun, Paula A. Schneider,
Kevin L. Peterson, Karen S. Flatten, David A. Loegering, Ann L. Oberg, Shaun M. Riska,
Shengbing Huang, Frank A. Sinicrope, Alex A. Adjei, Judith E. Karp, X. Wei Meng,
and Scott H. Kaufmann

SUPPLEMENTARY MATERIALS AND METHODS

Materials. Murine monoclonal anti-RasGRP1 was generated as described.[16] Antibodies that recognize the indicated antigens were obtained as follows: phospho-Ser^{473}-Akt, phospho-Thr^{308}-Akt, Akt, phospho-Ser^{2448}-mTOR, mTOR, phospho-Thr^{389}-S6-kinase, S6 kinase, phospho-Ser^{235,236}- and phosphor-Ser^{240,244}-S6 and S6 from Cell Signaling Technology (Beverly, MA); HDJ-2 from Neomarkers/Thermo (Fremont, CA); Rheb from Invitrogen (Carlsbad, CA); heat shock protein 90β (Hsp90β) from David Toft (Mayo Clinic, Rochester, MN); 6A7 anti-active Bax antibody from EMD Millipore (Billerica, MA); and Bcl-2 family members as previously described.[51] Reagents were purchased from the following suppliers: DNA oligonucleotides from Integrated DNA Technologies (Coralville, IA); 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega (Madison, WI); and phenazine methosulfate and rapamycin from Sigma-Aldrich (St. Louis, MO). Lentiviral constructs encoding short hairpin RNAs (shRNAs) that target Bax (nucleotides 113-131, GenBank™ accession number NM_138761) and Puma (nucleotides 784-802, GenBank™ accession number NM_014417) were obtained from Open Biosystems or constructed as reported previously [25]. All other reagents were obtained as previously described.[51]

Cell culture. ML-1 and HL-60 cells from M. Kastan (Duke University, Durham, N.C.) and R.T. Abraham (Pfizer, Pearl River, NY), respectively, were propagated at densities of <1 x 10^6 cells/ml in RPMI 1640 medium containing 40 units/ml penicillin G, 40 μg/ml streptomycin, 1 mM glutamine and 10% heat-inactivated fetal bovine serum (medium A). U937 cells were from American Type Culture Collection (Manassas, VA) were grown in medium A containing 4.5 mg/ml glucose, 10 mM HEPES (pH 7.4), 0.15% (w/v) sodium bicarbonate and 1 mM pyruvate (medium B). All lines were authenticated by short tandem repeat profiling in the Mayo Advanced Genomics Technology Center (most recently in July, 2012) and passaged less than three months before use. To generate resistant U937 cells, parental cells were diluted to a density of 1 x 10^5/ml and exposed to increasing concentrations of tipifarnib starting with 25 nM and increasing in 2-fold increments over a 6-month period as cells repeatedly grew back to a density of 4 - 8 x 10^5/ml. Thereafter cells were cultured at twice-weekly intervals or cloned by limiting dilution (1 cell/10 wells) in medium A containing 800 nM tipifarnib. U937 cells stably expressing Bax shRNA or Puma shRNA were generated by lentiviral transduction,[26] selection for stable integrants in 2 μg/ml puromycin after 48 h, and cloning of the resulting puromycin-resistant cells by limiting dilution. U937 cells stably expressing Rheb M184L in pBabePuro were similarly generated by retroviral transduction[52] and selection.

Clinical samples. After informed consent was obtained under the aegis of Institutional Review Board-approved protocols, sequential bone marrow aspirates were harvested from patients prior to therapy and prior to drug ingestion on day 8 of treatment with twice daily tipifarnib, which was administrated as part of a phase II trial of single-agent tipifarnib in AML[27] or a recently described tipifarnib/etoposide phase II trial.[28] Immediately after isolation, mononuclear cells were prepared on ficoll-Hypaque gradients, washed with serum-free RPMI 1640 medium, and prepared for electrophoresis.[29]

Immunoprecipitation and immunoblotting. Immunoprecipitation of active Bax was performed using methods previously described for the detection of active Bak,[16] but substituting the 6A7 anti-active Bax antibody. Blotting of immunoprecipitates was performed as described.

Whole cell lysates were prepared from cell lines as described previously.[29] In brief, cells were washed in serum-free RPMI-HEPES and solubilized in 6 M guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.5 at 21° C), 10 mM EDTA, 1% (v/v) ß-mercaptoethanol, and 1 mM α-phenylmethylsulfonyl fluoride (freshly added from a 100 mM stock in anhydrous isopropanol). Following reaction with iodoacetamide, cell lysates were dialyzed sequentially into 4 M urea followed by 0.1% (v/v) SDS and lyophilized.[29] Aliquots were resuspended in SDS sample buffer at 5 mg protein/ml (assayed by the bicinchoninic acid method),[53] separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with various antibodies.[30]

MTS assay. Parental or tipifarnib-resistant cells were sedimented at 100 x g for 5 min, washed once in serum-free medium and resuspended in medium A. Aliquots containing ~2 x 10^4 cells in 120 μl medium B were incubated at 37° C with varying concentrations of tipifarnib for 6 d. After reaction with MTS and phenazine methosulfate as instructed by the supplier, plates were incubated for 2-6 h to obtain an absorbance of 0.5-1.0 at 490 nm in control samples.

RNA isolation and microarray. RNA was isolated in on three separate days from 20 x 10^6 cells treated for 48 h with 0.1% DMSO or tipifarnib (800 nM, U937 and ML-1; 3200 nM, HL-60) or resistant cells growing in the same tipifarnib concentration using the Qiagen RNeasy kit. After analysis using an Agilent BioAnalyzer 2100 to confirm RNA quality, RNA was reverse transcribed, labeled and hybridized to Affymetrix U133A 2.0 microarrays using standard techniques.

Microarray analyses and Connectivity Map assessment. Analyses were performed on the log base 2 scale. The probes were then corrected for GC content[33] followed by nonlinear normalization[34] applied to the perfect match data from all chips together, since the majority of genes were expected to be similarly expressed across all samples. Probes were then summarized to the probeset level via RMA.[35] Empirical Bayes linear models[36] were then used to test the hypothesis of differential expression between samples treated with diluent vs. tipifarnib. Probes with a fold change in the tipifarnib treated cells of at least 2.0 and p value <0.05 were used in further analysis at the Broad Institute's Connectivity Map website (www.broadinstitute.org/cmap/newQuery). The version used (build02) contained 7000 genome-wide expression profiles derived from treating various human cell lines with 1309 bioactive small molecules. Enrichment of the induced and repressed genes of a signature within each Connectivity Map treatment profile was estimated with a metric based on the Kolmogorov-Smimov statistic and combined to produce a "connectivity score" as previously described.[37]

Quantitative RT-PCR. After total RNA was isolated from control or tipifarnib-treated U937 cells, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed in triplicate using 100 ng RNA and TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Carlsbad, CA) per the supplier's instructions. Using Bax (Hs00180269_m1) and Puma (Hs00248075_m1) probe sets, PCR was performed on a ABI Prism 7900HT Real_Time System using a program consisting of 48 °C for 30 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data analysis was performed using the following equations: $\Delta C_t = C_t$(sample)-C_t(endogenous control); $\Delta\Delta C_t = \Delta C_t$(sample)-$\Delta C_t$(untreated); and Fold Change = 2-$\Delta\Delta C_t$.

SUPPLEMENTARY REFERENCES

51. Dai H, Meng XW, Lee S-H, Schneider PA, Kaufmann SH. Context-dependent Bcl-2/Bak Interactions Regulate Lymphoid Cell Apoptosis. Journal of Biological Chemistry. 2009;284:18311-22.
52. Mow BMF, Chandra J, Svingen PA, Hallgren CG, Weisberg E, Kottke TJ, et al. Effects of the Bcr/Abl Kinase Inhibitors STI571 and Adaphostin (NSC 680410) on Chronic Myelogenous Leukemia Cells *in Vitro*. Blood. 2002;99(2):664-71.
53. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of Protein Using Bicinchoninic Acid. Analytical Biochemistry. 1985;150:76-85.

SUPPMENTARY FIGURE LEGENDS

Figure S1. Tipifarnib inhibits proliferation in Jurkat T-cell ALL cells. Aliquots containing 1 x 10^5 Jurkat cells/ml were treated with the indicated concentrations of tipifarnib or diluent continuously, with cells being counted and diluted into fresh medium with drug every 2 days. Graph at left indicates cumulative cell number/ml after taking into account dilution. Graph on right shows cell number on day 6 relative to diluent-treated controls.

Figure S2. Induction of apoptosis in U937 cells. A, U937 cells were fed with fresh medium containing 400 nM tipifarnib every two days to yield the indicated exposure time, then stained with propidium iodide and subjected to flow microfluorimetry. **B,** results of assays shown in panel A and additional time points. **C-F,** after treatment with diluent or 800 nM tipifarnib for 6 days, U937 cells were stained with APC-annexin V and examined by flow microfluorimetry (C,D) or fixed with 3:1 methanol:acetic acid, stained with Hoechst 33258 and examined by fluorescence microscopy (E, F). Circle in E, typical group of apoptotic fragments counted as one apoptotic cell. Error bars in D and F, mean ± SD of 3 independent experiments.

Figure S3. Lack of detectable effect of tipifarnib on signaling upstream and immediately downstream of Akt. After U937 cells were treated for 24 h with the indicated tipifarnib concentration, whole cell lysates (the same samples shown in Figure 3) were subjected to immunoblotting with antibodies that recognize the indicated antigens. GAPDH served as a loading control. Grey arrow indicates farnesylated HDJ-2 and black arrow indicates unfarnesylated antigen.

Figure S4. Tipifarnib-induced mRNA changes in AML cell lines are similar to other PI3 kinase/mTOR inhibitors. A, after U937, HL-60 or ML-1 were treated for 48 h with diluent or tipifarnib at 800, 1600, or 800 nM, respectively, cDNA was prepared and hybridized to Affymetrix 133 2.0 microarrays as described in the Methods. Numbers in each circle indicate the number of mRNAs for which changes were statistically significance ($p < 0.05$) and more than 2-fold in amount between control and tipifarnib-treated cells. **B,** correlation coefficient between mRNA changes induced by tipifarnib (panel A) in the present study and mRNA changes described at the Broad Institute Connectivity website in multiple experiments with each drug.

Figure S5. Further characterization of tipifarnib-resistant U937 cells. A, immunoblot of whole cell lysates from parental U937, bulk culture of U937 selected for growth in 800 nM tipifarnib, and three separate tipifarnib-resistant clones isolated by limiting dilution. **B,** tipifarnib-resistant U937 cells growing in 800 nM tipifarnib (lanes 1-4) or parental cells without drug (lanes 5-8) were treated with 30 μM cycloheximide for 0-48 h in the continued presence (lanes 1-4) or absence (lanes 5-8) of tipifarnib, then subjected to SDS-PAGE followed by immunoblotting with antibodies that recognize the indicated antigens. GAPDH served as loading a loading control.

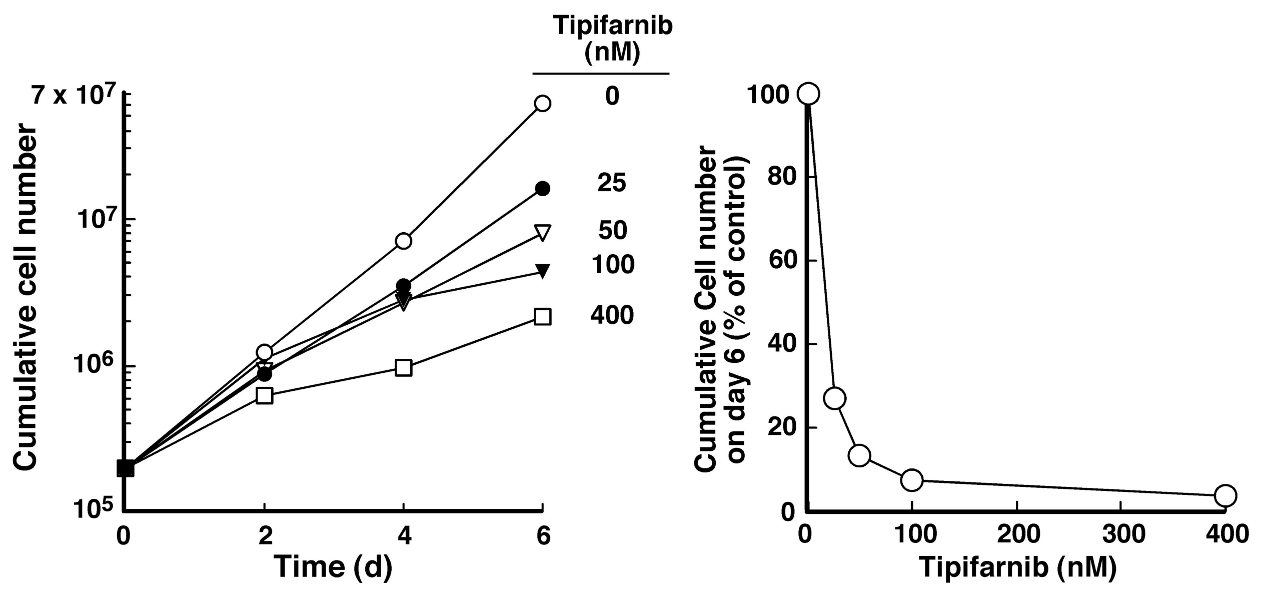

Figure S1

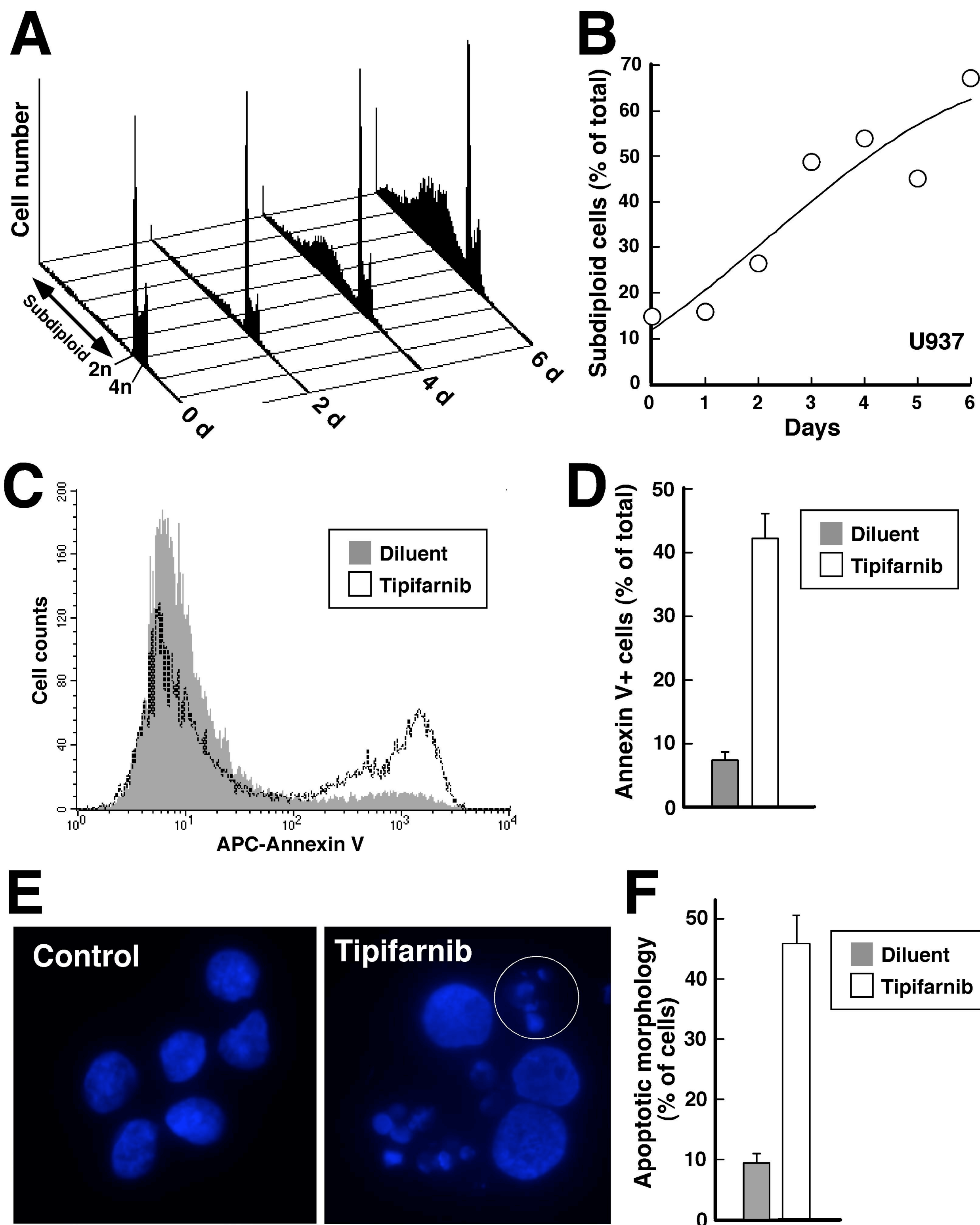

Figure S2

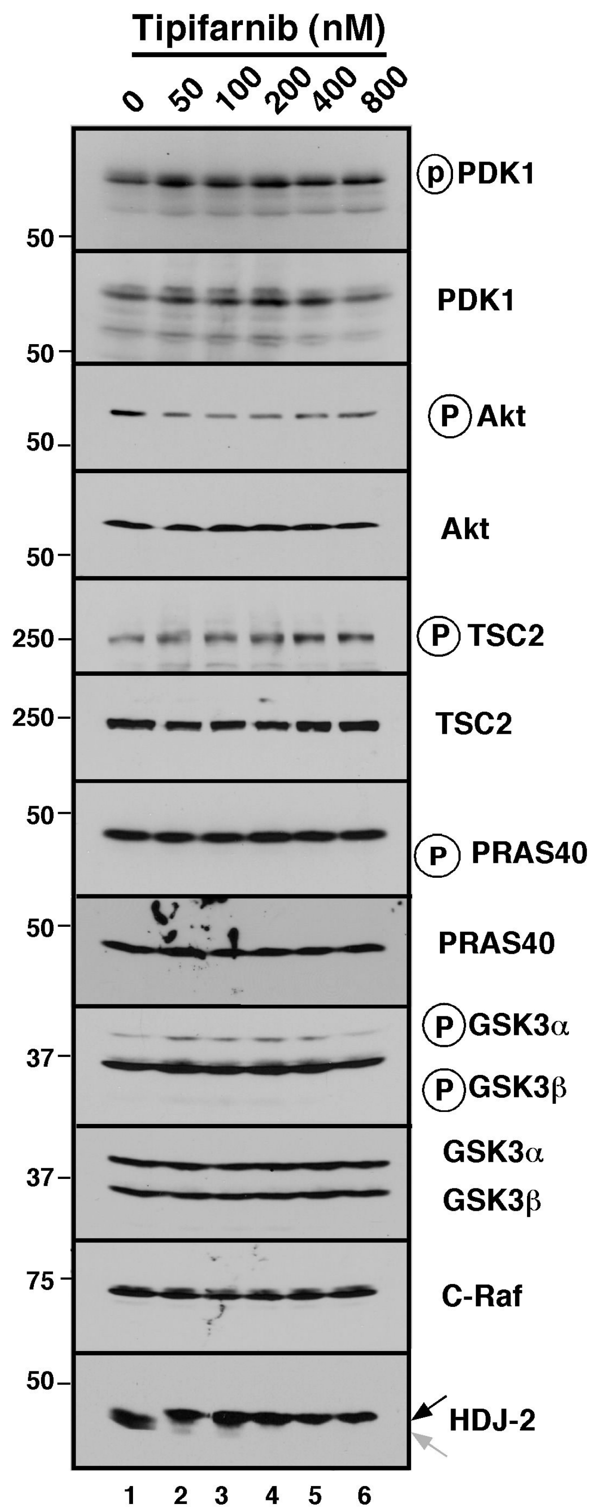

Figure S3

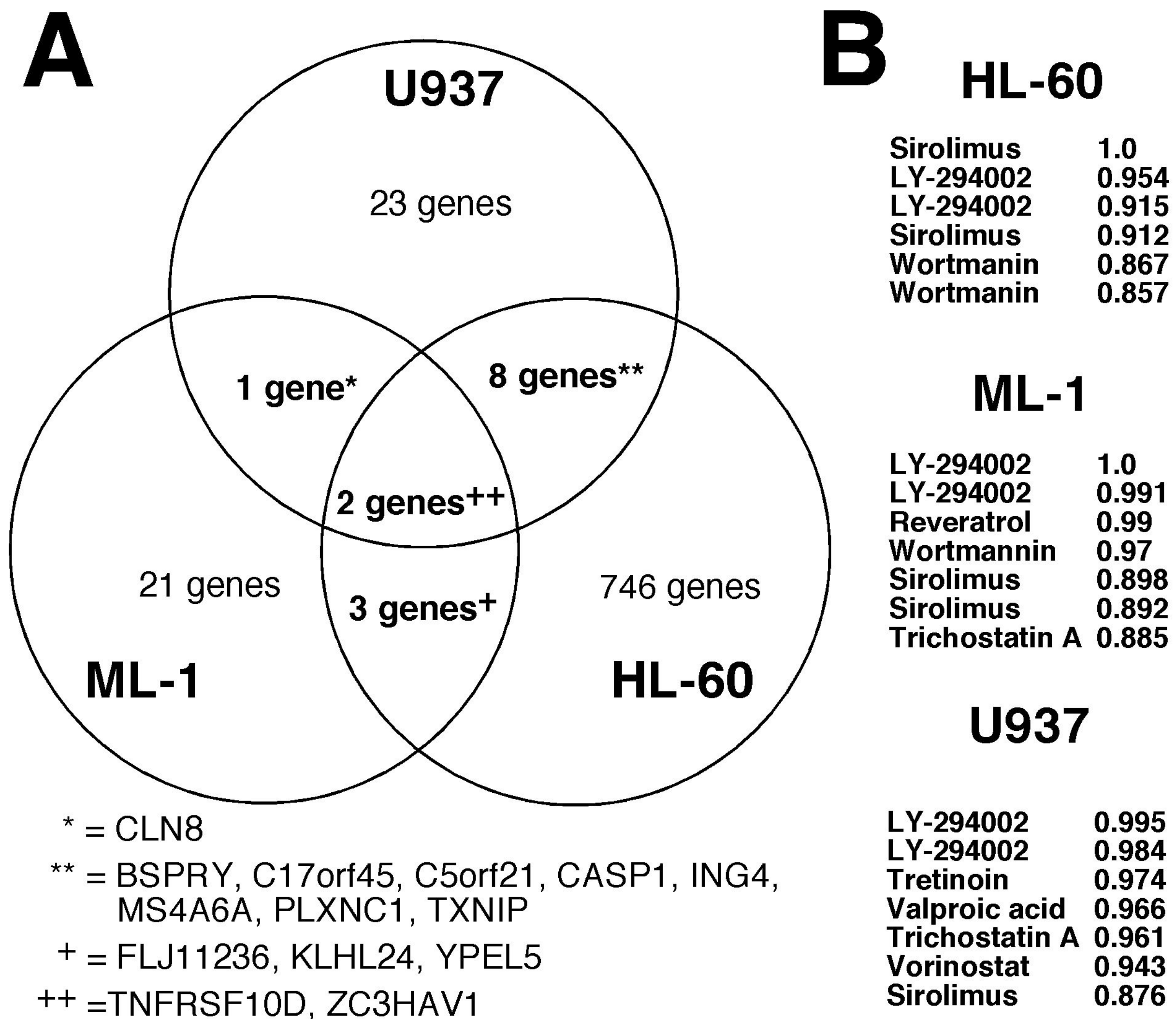

Figure S4

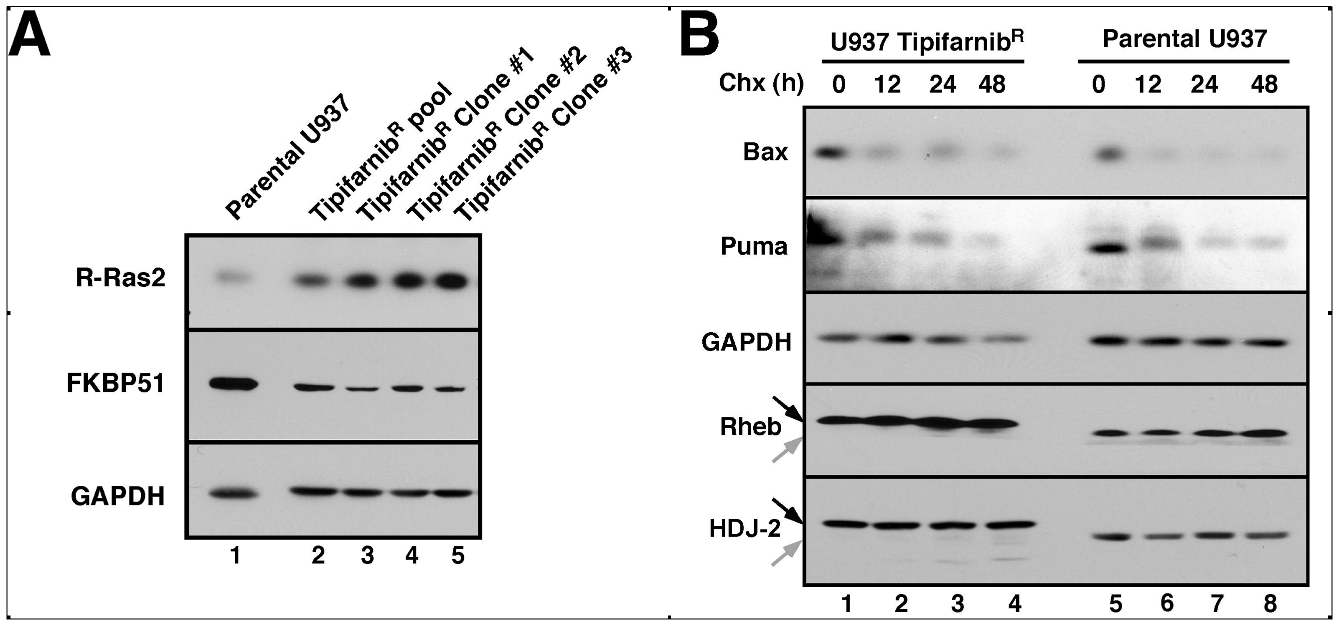

Figure S5