Supporting Information

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SI Materials and Methods

Reagents and Cell Lines. H89 [1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)], forskolin, purified calcineurin, and calmodulin were from Sigma. Tacrolimus (FK506) was from LC Laboratories. Fura-2-AM(Acetoxymethyl 2-[5-[bis[(acetoxymethoxy-oxo-methyl)methyl]amino]-4-[2-[2-[bis[(acetoxymethoxy-oxo- methyl)methyl]amino]-5-methyl-phenoxy]ethoxy]benzofuran-2-yl]oxazole-5-carboxylate) and BAPTA-AM [1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)] were from Life Technologies. Recombinant protein phosphatase 1 (PP1) and purified PP2A were from New England Biolabs and Millipore, respectively. Okadaic acid was from Calbiochem. WEHI7.2 cells and Jurkat cells were cultured as described (1). S49 cells (wild-type; 24.3.2), protein kinase A (PKA)-negative cells (24.6.1), and cells lacking $G\alpha_s$ (94.15.1) were purchased from the University of San Francisco Cell Culture Facility and cultured following the facility's directions. Peripheral blood samples from untreated patients with chronic lymphocytic leukemia (CLL) were obtained with informed consent, and CLL cells were isolated and cultured as described (1).

Immunoprecipitation and Immunoblotting. The procedures for immunoprecipitation and immunoblotting were described previously (2). The following antibodies were used: anti-Bcl-2 (Santa Cruz, sc-7382), anti-beta-actin (Sigma, A2228), anti-inositol 1,4,5-trisphosphate receptor 1 ($InsP_3R$-1; Santa Cruz, sc-6093, or Novus, NB120-5908), anti-phospho-$InsP_3R$-1-serine (Ser) 1755 (Cell Signaling, 3760), anti-NFATc2 (Santa Cruz, sc-7296), anti-dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32; Santa Cruz, sc-271111 or Cell Signaling, 2306), anti-phospho-DARPP-32-threonine (Thr) 34 (Cell Signaling, 5393), anti-calcineurin (BD, 610260), anti-PKA (Cell Signaling, 4782), and anti-PP1α (Santa Cruz, sc-271762). The intensity of bands on immunoblots was quantified using ImageJ.

Anti-CD3 Stimulation Associated with Immunoblotting and Ca^{2+} Imaging. Five million cells were suspended in 1 mL extracellular buffer (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Hepes at pH7.5, and 5 mM glucose). Anti-CD3 antibodies (Becton Dickenson) were added to 20 μg/mL and rotated at room temperature for the indicated times. Cells were centrifuged at 300 × *g* for 2 min at 4 °C and lysed in 50 μL RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3PO_4) for immunoblot analysis. In some experiments, WEHI7.2 cells were incubated with H89 or FK506 (or DMSO vehicle) at 37 °C for the indicated times before anti-CD3 stimulation. In other experiments, WEHI7.2 cells were preincubated with DMSO vehicle or okadaic acid at concentrations ranging from 1 nM to 2 μM before adding anti-CD3 antibody. CLL cells were treated with 10 μM TAT-$IDP_{DD/AA}$ dissolved in water or a corresponding scrambled control peptide, as described previously (1). Techniques for single-cell digital imaging of Fura-2-AM-loaded cells, including the use of EGTA and BAPTA-AM to decrease Ca^{2+} levels, were described previously (1). Cells plated on microscope coverslips are covered with extracellular buffer, and anti-CD3 antibodies are added after baseline Ca^{2+} levels have been recorded for several minutes.

RNA Interference. To knock down the expression levels of Bcl-2 and DARPP-32, 10 million and 5 million Jurkat cells, respectively, were transfected with ON-TARGET plus Smartpool siRNA (Dharmacon). Cells were washed with PBS and suspended in 200 μL Opti-MEM media (Life Technologies) in a 2-mm electroporator cuvette (BioExpress). Two micromoles nontargeting or specific siRNA for human Bcl2 or DARPP-32 genes were mixed with cells and subjected to electroporation with a single square pulse at 140 V for 10 ms with a BioRad GenePulser Xcell. Cells were allowed to recover overnight in RPMI-1640 medium with 10% (vol/vol) FBS and supplements.

In Vitro Phosphatase Assays. WEHI7.2 cells were treated with 10 μM forskolin for 5 min, washed with cold PBS, and lysed in CHAPS lysis buffer (1% CHAPS, 50 mM Tris·HCl at pH 7.5, 100 mM NaCl, 2 mM EDTA, protease inhibitor mixture; Roche). Cell lysate was centrifuged at 20,000 × *g* for 15 min at 4 °C. The resulting supernatant was incubated with 100 μL protein G-agarose beads (Millipore) for 2 h to remove nonspecific binding. The supernatant was incubated with anti-$InsP_3R$-1 or anti-NFATc2 antibodies and incubated with rotation overnight at 4 °C, followed by rotation with 10 μL protein G agarose for 2 h at 4 °C. The beads were washed three times each with lysis buffer, followed by phosphatase buffers. Buffers and reaction conditions for PP1/PP2A phosphatase was described previously (3). For the calcineurin assay, beads were washed and suspended in 100 μL phosphatase buffer (20 mM Tris·HCl at pH 8.0, 100 mM NaCl, 10 nM calyculin A) in the presence/absence of 100 nM purified calcineurin, 100 nM calmodulin, 5 mM EGTA, or 100 nM $CaCl_2$ and incubated at 30 °C for the indicated times. The beads were washed four times with phosphatase buffer and boiled for 5 min in 20 μL SDS-sample buffer. The eluted proteins were resolved by SDS/PAGE and analyzed by immunoblotting, using ImageJ to quantify intensity of bands.

Apoptosis Assays. Apoptotic nuclei were quantified in Hoechst 33342–stained cells by epifluorescence microscopy (Zeiss Axiovert S100), using a 40× fluorescent oil objective (Carl Zeiss) and a Hamumatsu Orca camera at room temperature. From 200 to 400 cells were visualized and counted in duplicate in each treatment group.

Statistical Analysis. The Student *t* test was used to assess statistical differences between groups. A two-tailed *P* value of 0.05 was used as the threshold for significance.

1. Zhong F, et al. (2011) Induction of Ca^{2+}-driven apoptosis in chronic lymphocytic leukemia cells by peptide-mediated disruption of Bcl-2-IP3 receptor interaction. *Blood* 117(10):2924–2934.
2. Rong YP, et al. (2008) Targeting Bcl-2-IP_3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals. *Mol Cell* 31(2):255–265.
3. Ammosova T, et al. (2011) Protein phosphatase-1 activates CDK9 by dephosphorylating Ser175. *PLoS ONE* 6(4):e18985.

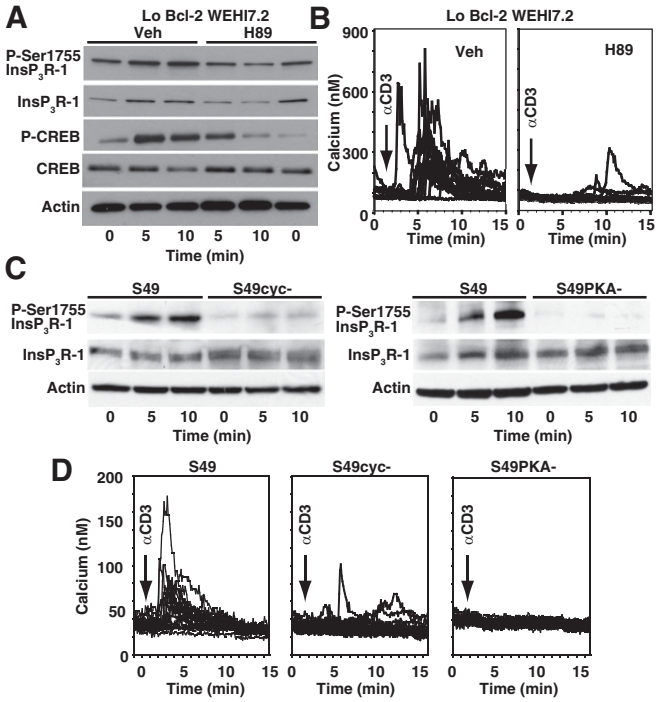

Fig. S1. PKA phosphorylates Ser1755 $InsP_3R$-1 after anti-CD3-antibody-mediated T cell receptor (TCR) activation. (*A*) Immunoblots representative of three experiments in Lo Bcl-2 WEHI7.2 cells indicating that pretreatment with the PKA inhibitory H89 (20 μM for 30 min) inhibits both cAMP response element-binding protein (CREB) phosphorylation and $InsP_3R$-1 Ser1755 phosphorylation induced by 20 μg/mL anti-CD3 antibody. Veh, DMSO vehicle for H89. (*B*) individual single cell Ca^{2+} traces (representative 20 cells) representative of two experiments (each examining 85 cells per treatment) in which pretreatment with 20 μM H89 for 30 min inhibited Ca^{2+} elevation induced by 20 μg/mL anti-CD3 antibody. (*C*) Immunoblots representative of three experiments demonstrating that increased phosphorylation of Ser1755 $InsP_3R$-1 after anti-CD3 antibody-mediated TCR activation is abrogated by mutations that inhibit PKA activity. (*D*) Individual single-cell Ca^{2+} traces (20 cells) representative of 85 cells in each panel, indicating that PKA is required for optimal Ca^{2+} responses to anti-CD3 antibody.

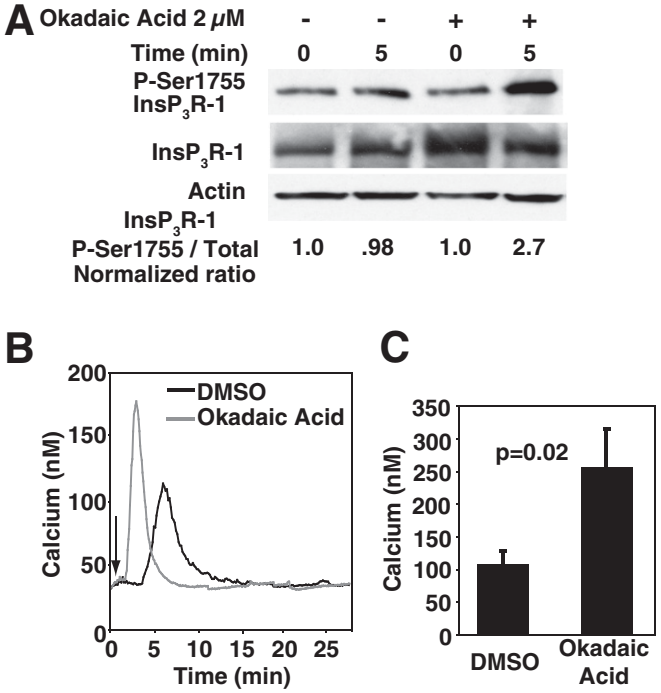

Fig. S2. Pharmacologic inhibition of PP1 reverses the inhibitory effect of Bcl-2 on anti-CD3-induced elevation of P-Ser1755 $InsP_3R$-1 and Ca^{2+}. (*A*) WEHI7,2 cells expressing high levels of Bcl-2 were pretreated for 60 min with either DMSO vehicle (–) or okadaic acid (+) (final concentration, 2 μM) before adding 20 μg/mL anti-CD3. Immunoblotting was performed on cells immediately before (0 min) and 5 min after adding anti-CD3. The results, confirmed in two similar experiments, indicate that okadaic acid pretreatment increases P-Ser1755 $InsP_3R$-1 levels. (*B*) WEHI7.2 cells were treated with okadaic acid as in *A*, and cytoplasmic Ca^{2+} concentration was continuously measured by digital imaging for 25 min after anti-CD3 addition (arrow). Shown are representative Ca^{2+} traces in single cells, indicating that okadaic acid pretreatment increases anti-CD3–induced Ca^{2+} elevation. (*C*) Summary of peak Ca^{2+} elevation induced by anti-CD3 in multiple experiments performed as described in *B*. Symbols represent mean ± SEM in three separate experiments. In each experiment, Ca^{2+} was measured in 85 cells for each treatment. The findings indicate that okadaic acid pretreatment at a concentration of 2 μM increases anti-CD3-induced Ca^{2+} elevation compared with control vehicle DMSO.

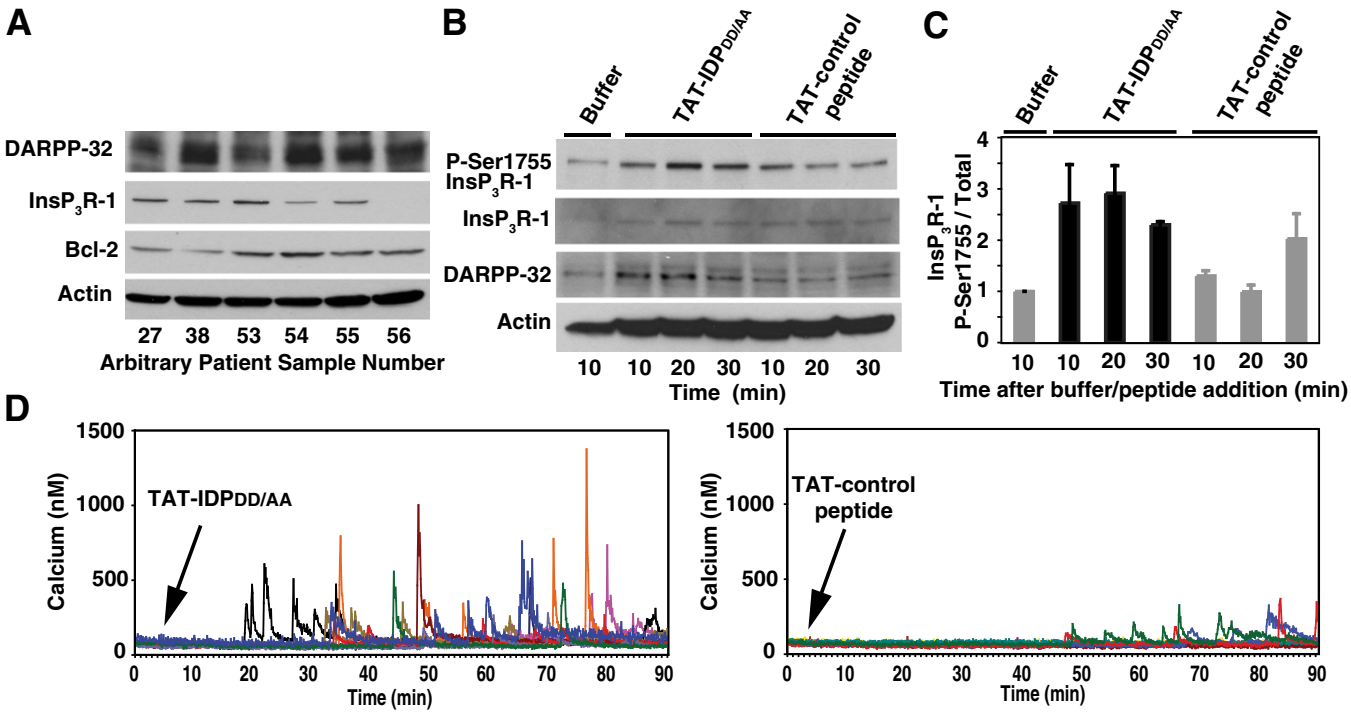

Fig. S3. DARPP-32 in CLL cells and Bcl-2 regulation of P-Ser1755 $InsP_3R$-1 levels. (*A*) DARPP-32 detection by immunoblotting in CLL cells from six different CLL patients. (*B*) Immunoblots indicating that the synthetic peptide TAT-$IDP_{DD/AA}$ (10 μM), but not control peptide, reverses Bcl-2-mediated repression of P-Ser1755 $InsP_3R$-1 levels, representative of three experiments, each using a different patient sample. (*C*) P-Ser1755 $InsP_3R$-1 levels relative to total $InsP_3R$-1 levels in CLL cells treated with buffer, 10 μM TAT-$IDP_{DD/AA}$, or 10 μM TAT-control peptide (mean ± SE, $n = 3$). (*D*) Single-cell traces recording Ca^{2+} elevation induced by TAT-$IDP_{DD/AA}$ or control peptide, each at 10 μM final concentration, representative of four experiments using primary CLL samples from different patients.