Supporting Information

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SI Materials and Methods

Antibodies and Reagents. BAPTA-AM [1,2-bis-(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl) ester] and 2-APB (2-aminoethoxydiphenyl borate) were purchased from Calbiochem. Indo-1 AM, Hoechst 33248, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), and tetramethyl rhodamine methyl ester (TMRM) were purchased from Sigma-Aldrich. Fura-2PE3-AM was obtained from Euromedex, and enzastaurin (LY317615) was obtained from SynVec. Soluble CD95L (gp190-CD95L) was generated in the laboratory (1). The anti-leukemia inhibitory factor isotype-matched negative control 1F10 (IgG) mAb and the anti-CD95L 10F2 were generated in our laboratory (2, 3). Anti–caspase-8 (C15) and anti-CD95 mAbs (APO1-3) were purchased from Axxora. Anti-human CD95 (DX2) and anti-human Fas-associated death domain protein (FADD) mAbs (clone 1) were purchased from BD Biosciences. Anti-protein kinase C (PKC) β1 and anti-PKCβ2 Abs were purchased from Santa Cruz Biotechnology. Anti-human Orai1 and stromal interaction molecule 1 (STIM1) (extracellular epitope) rabbit polyclonal Abs were obtained from Abcam and Alomone Labs, respectively. Anti–caspase-8 mAb (C15) was purchased from Axxora.

Plasmids, Transfections, and Selection of Cell Lines. Jurkat cells (5×10^6 cells in 0.3 mL) were electroporated with 10 μg of DNA at 300V with a single pulse using the BTM 830 electroporation generator (BTX Instrument Division, Harvard Apparatus). For selection of stable clones, Jurkat cells were transfected as described earlier and then placed in a medium supplemented with 1.8 mg/mL of neomycin. GFP-, GFP-fused Orai1–, and Orai1E106A-expressing cells were cloned by limiting dilution and then selected by flow cytometery based on GFP expression. Of note, all selected clones expressing GFP fused to either WT or mutated Orai1 exhibited a relatively weak fluorescence intensity compared with cells transfected with GFP alone, suggesting that only clones expressing weak amounts of Orai1 survived, and thus that calcium homeostasis may be altered without lethality only in a limited range of values in these cells (Fig. S5*A*). Silencing experiments were performed by lentiviral transduction of H9 and Jurkat T cells using validated shRNAmir-pGIPZ vectors for Orai1 (RHS4430-98715881 and -101067842), PKCβ (RHS4430-101063880 and -101068999), or a nontargeting shRNAmir-pGIPZ vector as a negative control (Open Biosystems). To improve the percentage of transduced T cells, living cells were harvested at 72 h after transduction, and green cells (pGIPZ encoding GFP) were sorted using a FACSAria flow cytometer/cell sorter (BD Bioscience).

Western Blot and Death-Inducing Signaling Complex (DISC) Analysis. After stimulation, cells were lysed for 30 min at 4 °C in lysis buffer [25 mM Hepes (pH 7.4), 1% vol/vol Triton X-100, 150 mM NaCl, and 2 mM EGTA, supplemented with a mix of protease and phosphatase inhibitors; Sigma-Aldrich]. Protein concentration was determined by the bicinchoninic acid method (Pierce) in accordance with the manufacturer's protocol. Proteins were separated in a 10% SDS/PAGE and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 15 min with TBST [50 mM Tris, 160 mM NaCl, and 0.05% vol/vol Tween 20 (pH 7.8)] containing 5% wt/vol dried skimmed milk (TBSTM). The primary antibody was incubated overnight at 4 °C in TBSTM under agitation. The membrane was washed intensively in TBST, after which peroxidase-labeled anti-rabbit (Invitrogen) or anti-mouse (GE Healthcare) was added for 45 min. After intensive washing, the proteins were visualized with the ECL Substrate Kit (GE Healthcare).

For DISC analysis, $10–20 \times 10^6$ cells were preincubated with the indicated drugs for 30 min before stimulation with 1 μg/mL of APO1-3 for 15 min at 4 °C (0 min) or 37 °C (15 min). Cells were harvested and lysed, and then CD95 was immunoprecipitated using protein A-Sepharose beads (Sigma-Aldrich) for 2 h. After extensive washing, the immune complex was resolved using SDS/PAGE and evaluated by Western blot analysis.

Cell Death Assays. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay (4). In brief, 4×10^4 cells were cultured for 24 h in flat-bottomed 96-well plates with the indicated concentrations of CD95L in a final volume of 100 μL. Then 15 μL of MTT (5 mg/mL in PBS) solution was added, and after 4 h of incubation at 37 °C, absorbance was measured at a 570-nm wavelength.

Cell death also was assessed by measuring the loss of mitochondrial potential ($\Delta\Psi m$). For this, cells were preincubated with 10 nM $DiOC_6$ or 200 nM TMRM for 15 min and then stimulated with CD95L. The loss of cell fluorescence was measured by flow cytometry.

For assessment of the plasma membrane integrity of CD95L-treated cells, GFP-fused Orai1- or Orai1E106A-expressing leukemic T-cell clones were incubated for the indicated times with CD95L, then mixed with 20 μg/mL of propidium iodide (Sigma-Aldrich) for 1 h and immediately analyzed by flow cytometry. Propidium iodide-positive cells were considered dead cells (i.e., loss of plasma membrane integrity).

Confocal Microscopy. Cells were untreated or treated with 100 ng/mL of CD95L for 15 min, then fixed in PBS containing 4% wt/vol paraformaldehyde for 10 min at 4 °C. The aldehyde groups were quenched for 10 min using a solution of PBS supplemented with 5% FCS. For PKCβ staining, cells were permeabilized in PBS supplemented with 5% FCS/ 0.1% saponin for 5 min. Then cells were incubated with 1 μg/mL of the anti-CD95 (APO1-3), anti-Orai1, anti-STIM1, anti-PKCβ1, or anti-PKCβ2 mAb in PBS/1% wt/vol BSA for 60 min at room temperature. CD95 was measured using secondary Alexa Fluor 594-coupled goat anti-mouse Ab, and Orai1, STIM1, PKCβ1 and PKCβ2 were measured using Alexa Fluor 488-conjugated donkey anti-rabbit Ab (Invitrogen) for 60 min at room temperature. In GFP-fused Orai1- or Orai1E106A-expressing Jurkat cells, CD95 and PKCβ2 were revealed using secondary Alexa Fluor 594-coupled goat anti-mouse Ab and Alexa Fluor 633-conjugated donkey anti-rabbit Ab, respectively. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich). Images were acquired with a Zeiss LSM 510 META confocal microscope with an Apoplan 63× objective.

Statistical Analyses. The significance of differences was calculated using the *t* test or Mann–Whitney *U* test ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

1. Legembre P, Beneteau M, Daburon S, Moreau JF, Taupin JL (2003) Cutting edge: SDS-stable Fas microaggregates, an early event of Fas activation occurring with agonistic anti-Fas antibody but not with Fas ligand. *J Immunol* 171:5659–5662.

2. Legembre P, Moreau P, Daburon S, Moreau JF, Taupin JL (2002) Potentiation of Fas-mediated apoptosis by an engineered glycosylphosphatidylinositol-linked Fas. *Cell Death Differ* 9:329–339.

3. Taupin JL, et al. (1993) Immunogenicity of HILDA/LIF either in a soluble or in a membrane-anchored form expressed in vivo by recombinant vaccinia viruses. *Scand J Immunol* 38:293–301.

4. Weichert H, Blechschmidt I, Schröder S, Ambrosius H (1991) The MTT assay as a rapid test for cell proliferation and cell killing: Application to human peripheral blood lymphocytes (PBL). *Allerg Immunol (Leipz)* 37:139–144.

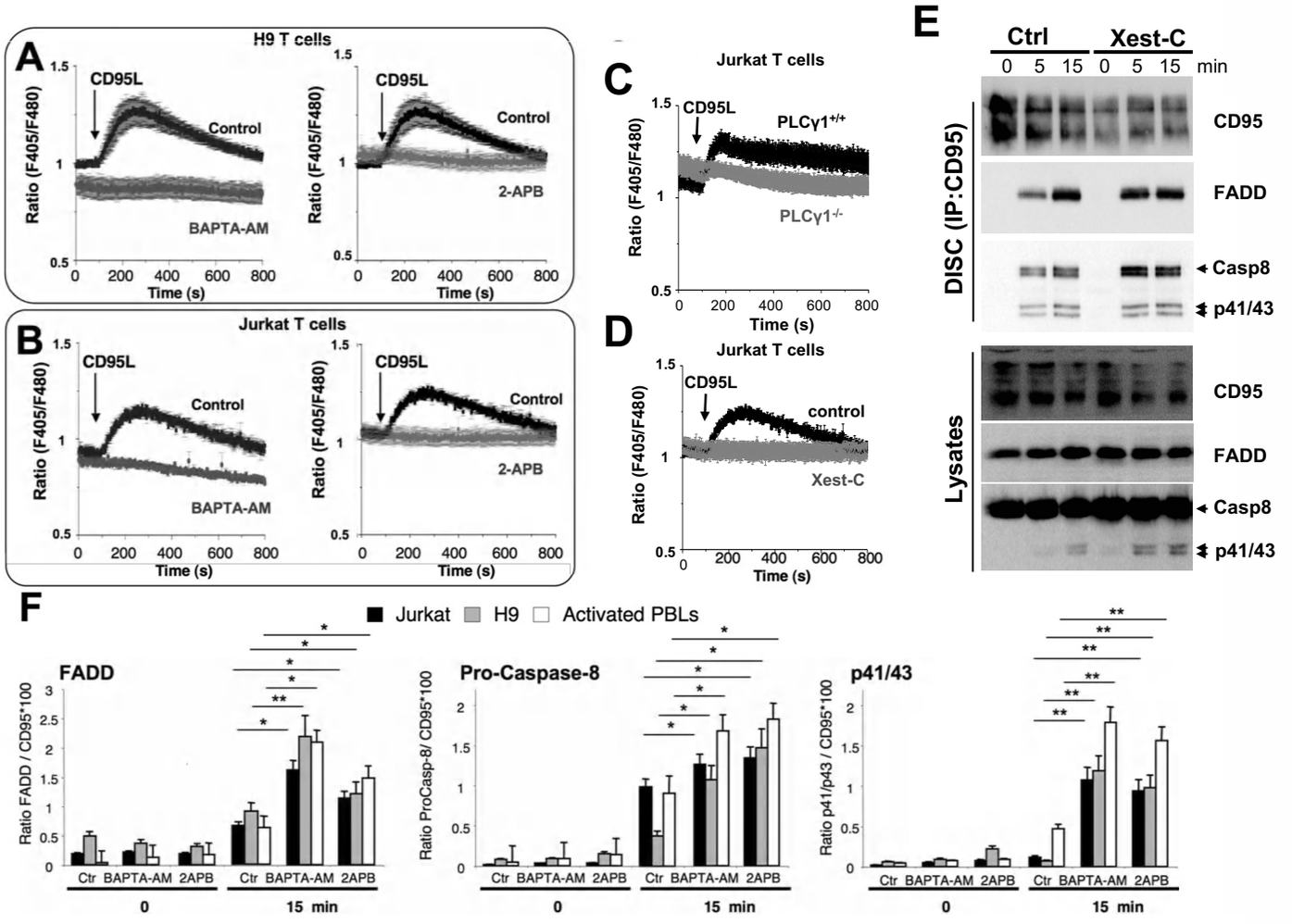

Fig. S1.CD95-mediated mobilization of endoplasmic reticulum-stored Ca2+ inhibits DISC formation. $[Ca^{2+}]_i$ as indicated by the ratio (F405 nm/F480 nm) was measured in the cell population using Indo-1 AM. Cells were bathed in a saline solution containing 0.8 mM Ca^{2+} (referred to as normal medium). Black arrows indicate the application of CD95L (100 ng/mL). Data represent the mean ± SD of four independent experiments. *(A)* The H9 T-cell line was pre-incubated for 30 min with 5 μM BAPTA-AM (left) or 44μM 2-APB (right). Then cells were stimulated with 100 ng/mL of CD95L, and $[Ca^{2+}]_i$ modifications were recorded. *(B)* The Jurkat T-cell line was treated as in A. *(C)* $[Ca^{2+}]_i$ modification was recorded in phospholipidase Cγ1 (PLCγ1)-deficient Jurkat cells ($PLC\gamma1^{-/-}$) and their PLCγ1-reconstituted counterparts ($PLC\gamma1^{+/+}$). *(D)* Pretreatment (for 30 min) of the Jurkat T cells with the inositol 1,4,5-triphosphate receptor antagonist xestospongin-C (2 μM) abrogated the CD95-mediated Ca^{2+} signal induced on addition of CD95L (note that control treatment is the same as in B). *(E)* H9 T cells were pretreated with xestospongin-C (Xest-C; 2 μM) and then stimulated with 1 μg/mL of the agonist anti-CD95 mAb APO1-3 at 4° C (0 min) or 37° C (5 and 15 min). Then cells were lysed, and CD95 was immunoprecipitated using coupled protein A-Sepharose beads. The immune complex was resolved in a 10% SDS/PAGE and analyzed using the indicated antibodies. The data are representative of three independent experiments. *(F)* Densitometric analysis of the DISC in Fig. 1B. Jurkat, H9, and activated peripheral blood lymphocytes (PBLs) were pretreated with BAPTA-AM, 2-APB, or DMSO (ctr), and then stimulated for 15 min with the agonist anti-CD95mAb APO1-3 (1 μg/mL) or left untreated (0). CD95 was immunoprecipitated, the immune complex was resolved by SDS/PAGE, and CD95, FADD, and caspase-8 immunoblotting was performed. p41/43 corresponds to the first step of pro–caspase-8 cleavage. For each immunoblot, the band intensity was quantified by densitometry using ImageJ software, and ratios of the intensity of the indicated proteins versus immunoprecipitated CD95 were calculated. The significance of differences was calculated using the nonparametric Mann–Whitney U test. *P < 0.05; **P < 0.01.

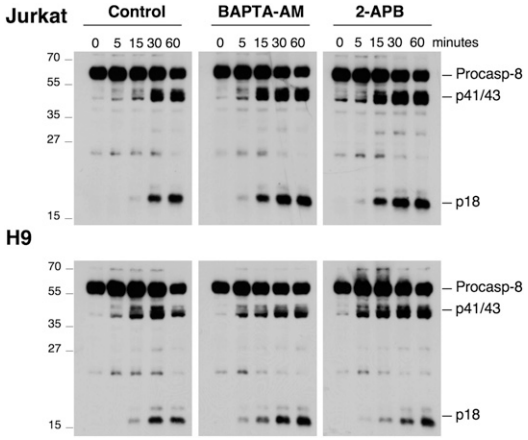

Fig. S2. Inhibition of the CD95-mediated Ca^{2+} signal accelerates caspase-8 cleavage. The indicated T cells were preincubated in the presence or absence of BAPTA-AM (5 μM) or 2-APB (44 μM) for 30 min and then incubated with 100 ng/mL of CD95L for the indicated times. Cells were lysed, and 100 μg of protein was loaded per lane and resolved in a 10% SDS/PAGE. Anti–caspase-8 immunoblotting was performed. Cleavage products of the pro–caspase-8 (p41/43 and p18) are a hallmark of activated caspase-8.

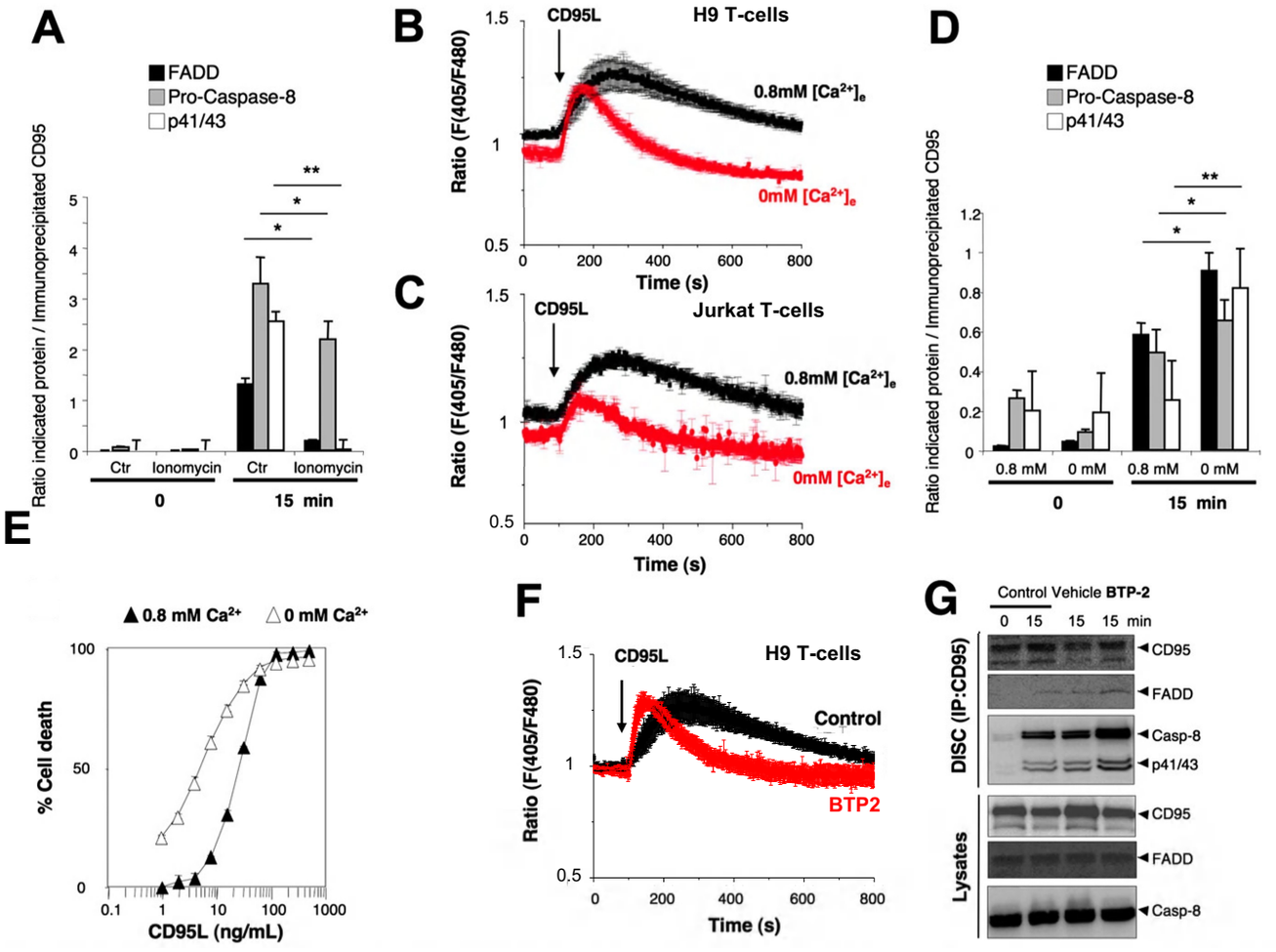

Fig. S3. CD95-mediated Ca^{2+} influx hampers the apoptotic signal. *(A)* Densitometric analysis of the DISC formed in cells pretreated with ionomycin. Jurkat T cells were exposed to ionomycin (1 μM) before stimulation with CD95L (100 ng/mL) at the indicated times, as described in Fig. 1C. For each immunoblot, the band intensity was quantified by densitometry using ImageJ software, and ratios of the intensity of the indicated proteins versus immunoprecipitated CD95 were calculated. The significance of differences was calculated using the nonparametric Mann–Whitney U test. *P < 0.05; **P < 0.01. *(B and C)* Cytosolic Ca^{2+} was assessed in cell populations using Indo-1 AM. An overlay of the mean ± SD of four independent experiments is shown. Black arrows indicate the application of CD95L (100 ng/mL). *(B)* The H9 T-cell line was bathed in a medium containing 0.8 mM Ca^{2+} or a medium complemented with 2 mM EGTA (0 mM Ca^{2+}), and cells were exposed to CD95L (100 ng/mL). *(C)* Jurkat T cells were treated as in B (control treatment is the same as in figure S1B and S1D). *(D)* Activated PBLs were stimulated with APO1-3 (1 μg/mL) in a control medium (0.8 mM) or in free Ca^{2+} medium (0 mM Ca^{2+}). Then the DISC was analyzed as described in Fig.1E, and the band intensity was quantified by densitometry using ImageJ software. Ratios of the intensity of the indicated proteins versus immunoprecipitated CD95 were calculated. The significance of differences was calculated using the nonparametric Mann–Whitney U test. *P < 0.05; **P < 0.01. *(E)* Jurkat T cells were incubated in medium containing 0.8 mM Ca^{2+} (control) or in the absence of extracellular Ca^{2+} (medium supplemented with 2mM BAPTA) and treated for 16 h with indicated concentrations of CD95L. Cell death was assessed by the MTT assay. Data represent the mean ± SD of three independent experiments. *(F)* Cytosolic Ca^{2+} was assessed in cell populations using Indo-1 AM. H9 T cells were preincubated for 30 min with or without the SOCE inhibitor BTP2 (500 nM), and then 100 ng/mL of CD95L was applied, as indicated by the black arrow (Control treatment of H9 cells is the same as in B). The mean ± SD of three independent experiments is shown under control (black trace) and treated (red trace) conditions. *(G)* H9 T cells were untreated or treated for 30 min with DMSO (vehicle) or BTP2 (500 nM) and then incubated with 1 μg/mL of APO1-3 for the indicated times. Cells were lysed, CD95 was immunoprecipitated, and the DISC was analyzed. Total lysates were added as a loading control.

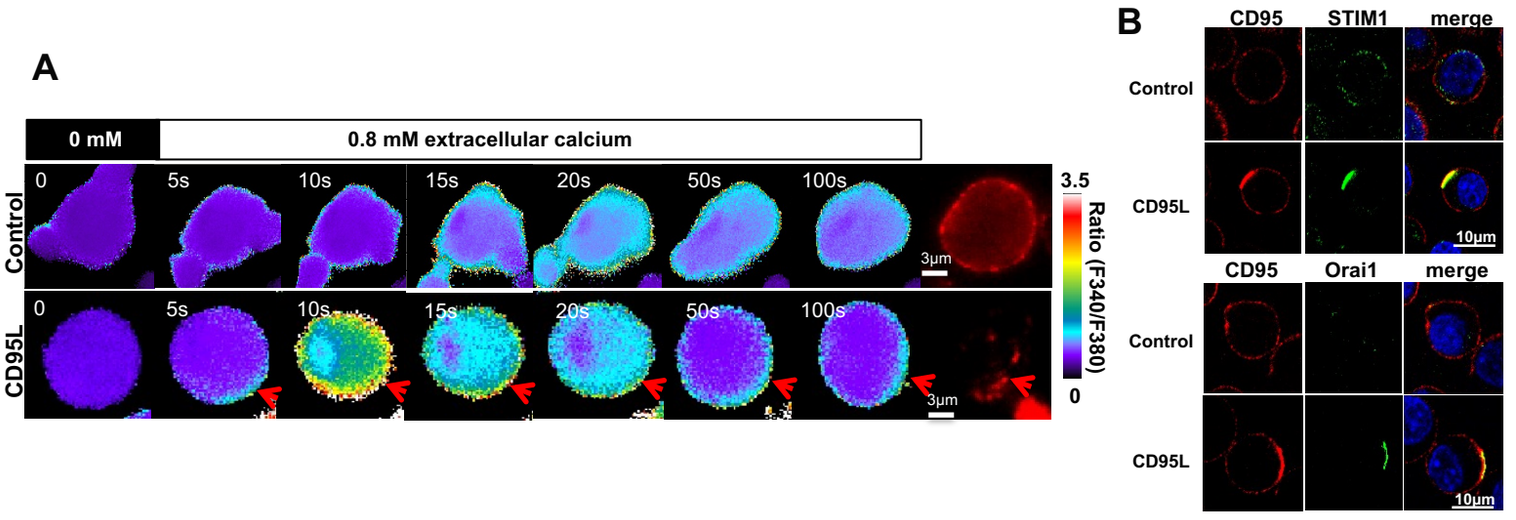

Fig. S4. CD95 engagement leads to the redistribution of Orai1/STIM1 in CD95-Cap and to localized entry of Ca^{2+}. *(A)* CD95 was stained as described in Materials and Methods; Jurkat cells were untreated (control) or treated with 100 ng/mL of CD95L. CD95 labeling was analyzed using a conventional videomicroscopy setup, and images of the fura-2PE3 fluorescence (F340nm/F380nm) were obtained every 5 s to monitor intracellular Ca^{2+} concentration. Cells were bathed in a Ca^{2+}-free extracellular medium (black bar), and the extracellular calcium concentration was increased to a final concentration of 0.8 mM (white bar). Red arrows indicate the position of the CD95-Cap. *(B)* The Jurkat T-cell line was incubated in the presence or absence of 100 ng/mL of CD95L for 15 min. Cells were fixed and stained for CD95, STIM1, or Orai1. Nuclei are depicted in blue.

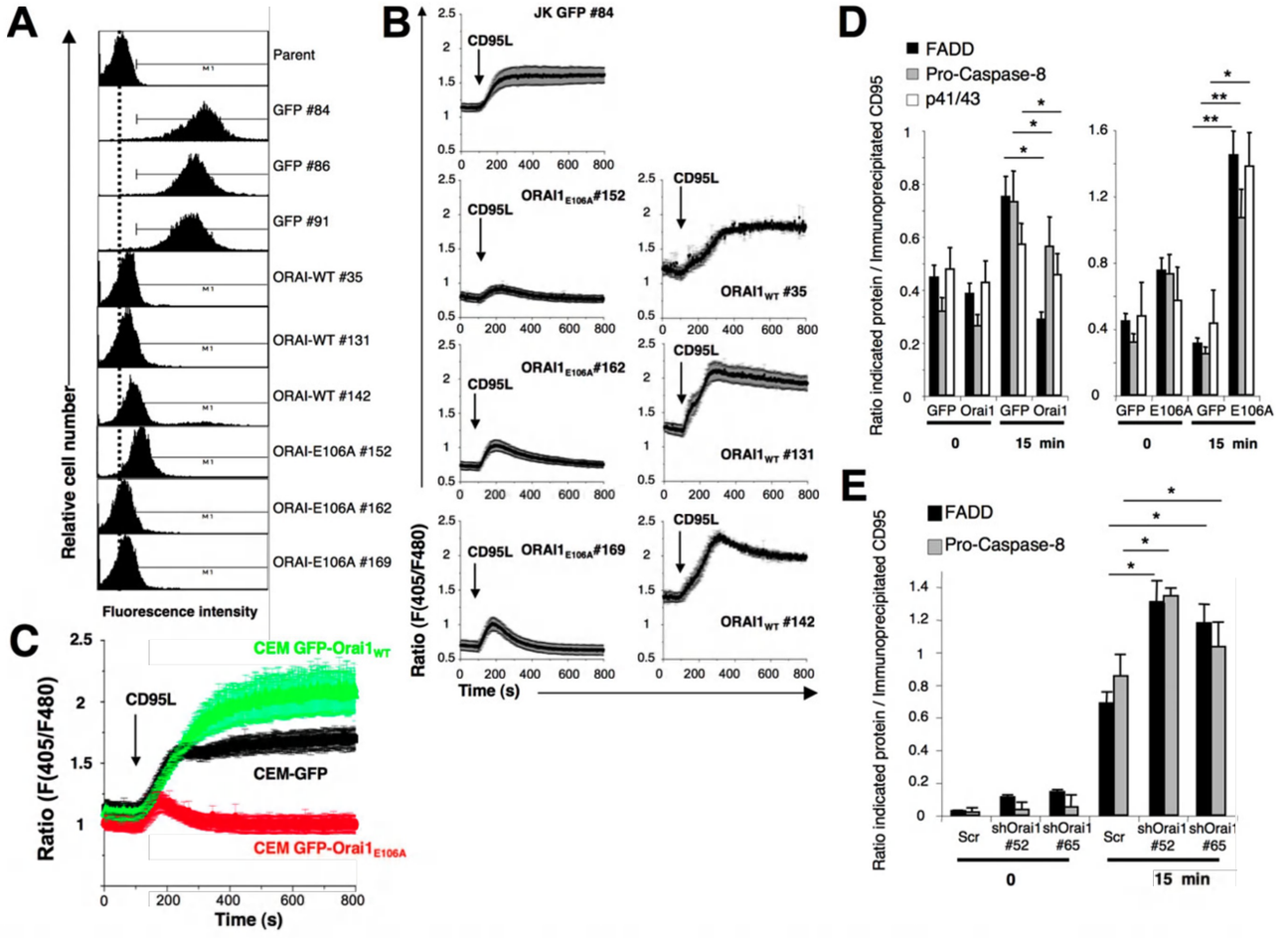

Fig. S5. Role of Orai1 in the CD95-mediated Ca^{2+} response and DISC formation. (*A*) The Jurkat cell line was transfected with GFP alone or GFP fused to the WT or dominant negative Orai1 (Orai1E106A) cDNA. Several clones were isolated, in which the level of GFP expression was assessed by flow cytometry. (*B*) $[Ca^{2+}]_i$ was assessed in the cell population. The indicated T-cell clones were loaded with Indo-1 AM and then stimulated with 5 ng/mL of CD95L. The black arrows correspond to the times of CD95L addition. Values represent mean ± SD of four independent experiments. (*C*) The CEM T-cell line was transfected with GFP-encoding (black), GFP-Orai1–encoding (green), or GFP-Orai1E106A–encoding (red) vectors. Clones were isolated, and the CD95-mediated Ca^{2+} response was analyzed. $[Ca^{2+}]_i$ was assessed in the cell population as described in *B*. The black arrow indicates CD95L addition. Data represent mean ± SD of three independent experiments. (*D*) Densitometric analysis of the DISCs formed in GFP-, Orai1-, and Orai1E106A-expressing Jurkat cells (Fig. 2*D*). Band intensity was quantified by densitometry using ImageJ software. Ratios of the intensity of the indicated proteins versus immunoprecipitated CD95 were calculated. The significance of differences was determined using the nonparametric Mann–Whitney *U* test. $*P < 0.05$; $**P < 0.01$. (*E*) Densitometric analysis was performed on the DISCs formed in Jurkat cells infected with lentivirus encoding scrambled (scr) or Orai1-targeting shRNAs (Fig. 2*F*). Band intensity was quantified by densitometry using ImageJ software. Ratios of the intensity of the indicated proteins versus immunoprecipitated CD95 were calculated. The significance of differences was determined using the nonparametric Mann–Whitney *U* test. $*P < 0.05$.

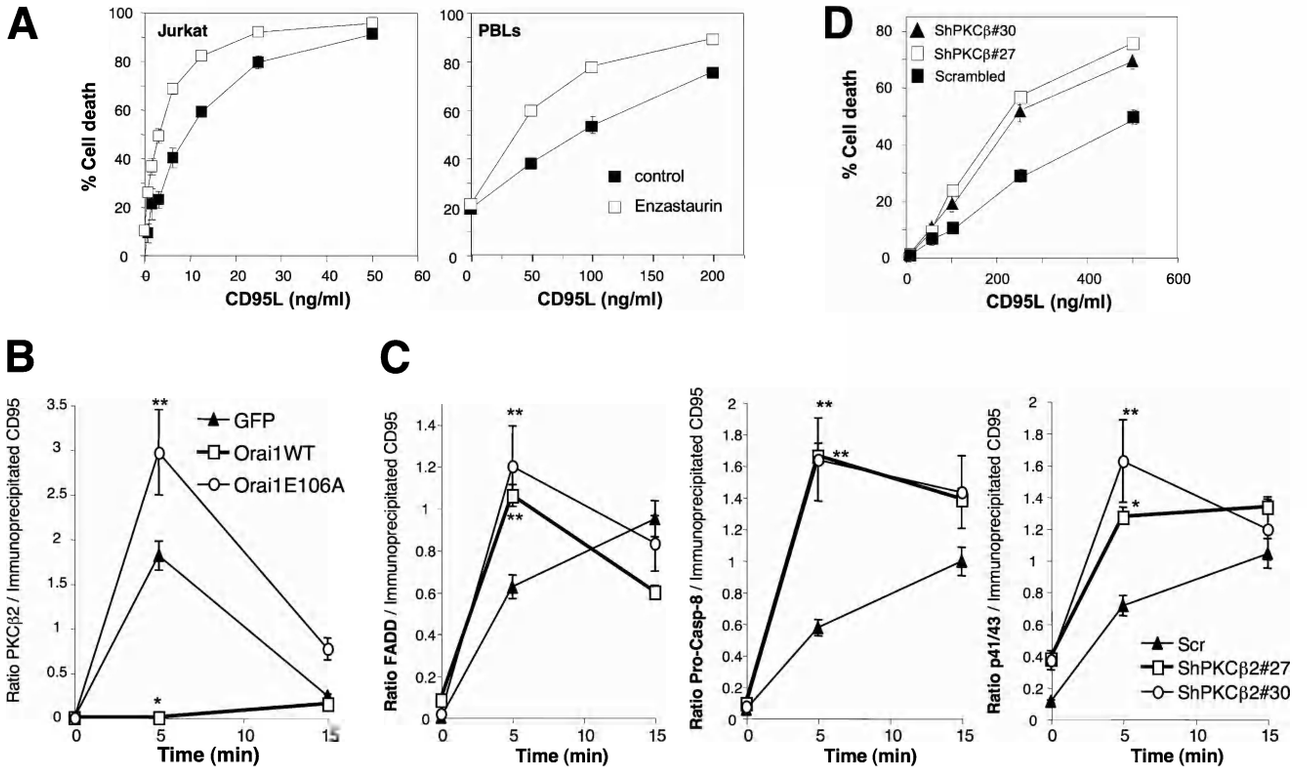

Fig. S6. PKCβ2 inhibits the CD95-mediated apoptotic signal. (*A*) Jurkat T cells and activated PBLs were preincubated for 15 min with 5 μM PKCβ inhibitor LY317615 (enzastaurin) and then incubated for 4 h with CD95L. Cell death was assessed by measuring the decrease in ΔΨm. Data represent mean ± SD of three independent experiments. (*B*) According to Fig. 3*D*, the recruitment of PKCβ2 in the DISCs of GFP-, Orai1-, or Orai1E106A-expressing Jurkat T cells was assessed by densitometry using ImageJ software. The ratios of intensity of PKCβ2 versus immunoprecipitated CD95 were calculated. The significance of differences was evaluated using the nonparametric Mann-Whitney *U* test. $*P < 0.05$; $**P < 0.01$. (*C*) Densitometric analysis was performed on the DISCs formed in H9 cells infected with lentivirus encoding scrambled (scr) or PKCβ2-targetting shRNAs (Fig. 3*E*). Band intensity was quantified by densitometry using ImageJ software. The ratios of intensity of indicated protein versus immunoprecipitated CD95 were calculated. The significance of differences was evaluated using the non-parametric Mann–Whitney *U* test. $*P < 0.05$; $**P < 0.01$. (*D*) H9 T cells infected with lentivirus encoding either scrambled or two different PKCβ2-targeting ShRNAs were incubated for 4 h with the indicated concentrations of CD95L, and cell death was assessed by measuring the decrease in ΔΨm. Data represent the mean ± SD of three independent experiments.

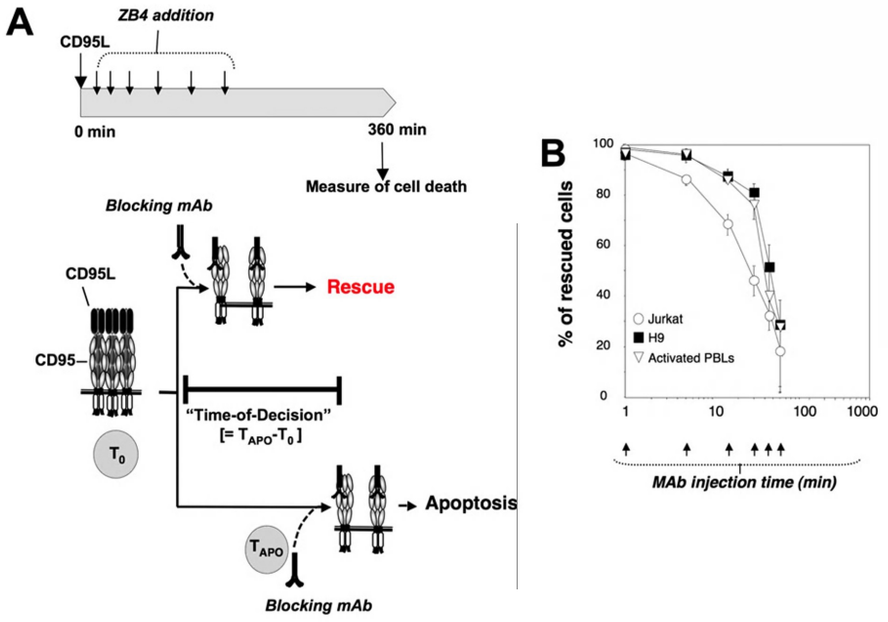

Fig. S7. Reversibility of the CD95-mediated apoptotic signal. (*A*) Schematic of the protocol used to reverse the CD95L–CD95 interaction using an antagonist anti-CD95 mAb ZB4 (black arrows). T_0 corresponds to the time at which CD95L was added to the cells, and T_{APO} represents the minimal incubation time required for the CD95–CD95L interaction to trigger an irreversible apoptotic signal. (*B*) The indicated cells were incubated for 6 h with a concentration of CD95L triggering 100% cell death (10 ng/mL for Jurkat cells, 100 ng/mL for H9 cells, and 500 ng/mL for PBLs). Blocking anti-CD95 mAb ZB4 (2.5 μg/mL) was subsequently added at the indicated times in the medium containing cells and CD95L, and cell death was assessed after 6 h by measuring the decrease in $\Delta\Psi m$. Data represent mean ± SD of three independent experiments.