Supplementary Material to

Protein Kinase Cβ Modulates Ligand-Induced Cell Surface Death Receptor Accumulation: A Mechanistic Basis for Enzastaurin-Death Ligand Synergy

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SUPPLEMENTAL METHODS

Plasmid construction and site-directed mutagenesis – cDNA encoding full-length Fas (GenBank accession # M67454) was amplified by RT-PCR and was cloned into pcDNA3.1 using EcoRI and XbaI sites or cloned into pCMS5A, which contains cDNA encoding EGFP-histone H2B driven by a separate SV40 promoter (47), using EcoRV and NotI sites. Mutations were introduced by site-directed mutagenesis using a Quikchange™ mutagenesis kit (Stratagene) according to the instructions of the supplier.

Plasmids that encode the C-terminal fragment of AP-180 tagged with c-Myc (c-AP180) and dnEps-15 were kindly provided by Ed Leof (Mayo Clinic, Rochester, MN). Full-length Cdc42 and Arf6 were amplified by RT-PCR and cloned into pcDNA3 using HindIII and NotI sites. dnCdc42 and dnArf6 were created by mutating Thr^{17} and Thr^{27}, respectively, to Asn using site-directed mutagenesis kits. pCMS5A-Fas Tyr^{291} was mutated to Phe using the same method as described above.

Metabolic labeling and recovery – 2 x 10^7 cells were washed once and resuspended in phosphate-free RPMI 1640 (Biosource) at a density of 5 x 10^6/ml. After ^{32}P-orthophosphate (GE Healthcare) was added to a final concentration of 150 μCi/ml, cells were incubated for an additional 3 h.

Fas-S tag was recovered from radiolabeled cells by pull-down with S protein-agarose as previously described (42). All steps were performed at 4 °C. In brief, cells were washed twice in ice cold PBS, incubated in lysis buffer with protease and phosphatase inhibitors [1% (w/v) Triton X-100, 150 mM NaCl, 30 mM Tris-HCl (pH 7.5), 10% (w/v) glycerol, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, 100 U/ml Trasylol, 1% (w/v) thiodiglycol, 20 nM microcystin] for 30 min and

sedimented at 12,000 x g for 15 min. S protein-agarose was added to the resulting supernatant, which was rotated end-over-end at least for 2 h at 4 °C. Beads were spun down at 10,000 x g for 1 min and washed four times with RIPA buffer containing phosphatase inhibitors [1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 150 mM NaCl, 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 1 mM sodium orthovanadate, 100 U/ml Trasylol, 50 mM NaF]. To elute protein for SDS-PAGE, beads were resuspended in sample buffer [4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 5% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue] and heated to 65 °C for 20 min.

Mass spectrometry –Fas-S peptide was isolated from 1 x 10^9 stably transfected K562 cells, subjected to SDS-PAGE and stained with Coomassie Blue. The tagged Fas band was excised, digested with trypsin, and analyzed by Qtof-MS/MS at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

Cell surface staining of Fas – For cell lines stably expressing mutant Fas constructs, cells were incubated with Apo-1-1 anti-Fas monoclonal antibody (Alexis) for 1 h on ice in the presence of 2% FBS. After two washes, cells were incubated with PE-conjugated anti-mouse IgG for 1 h. Cells were then fixed in 4% formaldehyde in PBS and analyzed by flow cytometry. Alternatively, 16 h after transfection with pCMS5A encoding Fas mutants, JM14A5 cells were stained with Apo-1-1 antibody followed by Alexa Fluor647-conjugated anti-mouse IgG. Fas expression was assessed in the GFP positive population.

Assessment of cell surface transferrin receptor – 16 h after transfection with plasmids that interfere with various endocytic pathways (indicated in Fig. S8) and pEGFP-N1 at an 8:1 ratio, Jurkat cells were treated with 20 nM PMA for 90 min at 37 °C, then quickly cooled by addition

of 4 volumes of ice-cold PBS. After two washes with ice-cold PBS, cells were stained with monoclonal anti-transferrin receptor antibody (BD Sciences) for 1 h on ice, washed, incubated with Alexa Fluor647-conjugated anti-mouse-IgG for 40 min, fixed with 4% formaldehyde in PBS and analyzed by flow cytometry. Staining with Alexa Fluor647 (reflecting cell surface transferrin receptor) was assessed by gating on the EGFP-positive (plasmid transfected) cells only.

Apoptosis assays – To assess the potential role of Fas phosphorylation in apoptosis, stable cell lines expressing wild type or mutant Fas were treated with CH-11 for 5 h, lysed in solution D and analyzed by flow cytometry. Alternatively, pCMS5A encoding wild type or mutant Fas was transfected into JM14A5 cells by electroporation as described above. After 16 h, cells were incubated with CH-11 for 5 h in the absence or presence of 20 nM PMA and stained with APC-conjugated annexin V. Annexin V binding (reflecting apoptosis) was assessed by gating on the EGFP-positive (plasmid-transfected) cells only.

Supplemental Figure Legends

Figure S1. PMA inhibits TRAIL-induced apoptosis in T98G cells. A, Log phase T98G cells were treated for 5 min with diluent or 20 nM PMA. TRAIL (2.5 ng/ml) was then added. At the completion of a 4-h incubation, combined adherent and floating cells were sedimented, fixed, stained with Hoechst 33258 and examined by fluorescence microscope for apoptotic morphological changes (32). Arrowheads in TRAIL treated sample, examples of apoptotic nuclei. **B,** summary of results from panel A and additional samples from the same experiment examined by an investigator who was blinded to the treatments. 300-600 cells were examined for each treatment. **C,** summary of results obtained when cells were treated with diluent or 25 ng/ml TRAIL in the absence or presence of 20 nM PMA. Error bars, mean ± S.D. of 3 independent experiments performed as illustrated in A and B.

Figure S2. PKCβ shRNA diminishes the effect of PMA on CH-11-induced apoptosis. 48 h after transfection of Jurkat cells with 40 μg empty vector or shRNA targeting the indicated PKC isoform (along with 5 μg plasmid encoding EGFP), cells purified on ficoll-Hypaque step gradients were treated for 5 h with 5 nM PMA along with 30 ng/ml CH-11. At the completion of the incubation, cells were stained with APC-conjugated annexin V and analyzed by 2-color flow cytometry (48). The percentage of $EGFP^+$ (shRNA-transfected) cells that bound annexin V is indicated to the right of each panel.

Figure S3. Enzastaurin inhibits the effect of PMA on CH-11-induced apoptosis. A, after a 5 min pretreatment with 20 nM PMA or diluent (0.04% ethanol) and 10 μM enzastaurin or diluent (0.02% DMSO), Jurkat cells were treated for 6 h with 12.5 ng/ml CH-11 agonistic anti-Fas antibody, fixed, stained with Hoechst 33258, and examined by fluorescence microscopy.

Arrowheads, examples of apoptotic cells. **B,** results from samples in panel A and additional samples from the same experiment.

Figure S4. Effect of a selective GSK3β inhibitor and an additional PKCβ inhibitor on PMA-induced protection from Fas-mediated apoptosis. A, Jurkat cells were preincubated with the GSK3β inhibitor LY2064827 (5 μM) for 90 min, then incubated for 5 h with 37.5 ng/ml CH-11 in the absence or presence of 20 nM PMA. To assess apoptosis, cells were stained with APC-conjugated annexin V and subjected to flow cytometry. **B,** Jurkat cells were pretreated for 5 min with the PKCβ inhibitor 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (PKCβi) at 10 μM, then treated for 6 h with the indicated concentration of CH-11 in the presence of diluent or 20 nM PMA. To assess apoptosis, cells were lysed in solution D and subjected to flow cytometry.

Figure S5. **PMA stimulates Fas phosphorylation. A,** Jurkat cells were radiolabeled for 3 h with 0.15 mCi/ml ^{32}P-orthophosphate without or with 20 nM PMA. FADD was immunoprecipitated using monoclonal anti-FADD antibody, subjected to SDS-PAGE, and analyzed by autoradiography (*right*) and blotted with FADD antibody (*left*). **B,** K562 cells stably expressing Fas-S peptide were radiolabeled under the conditions described for panel A. Tagged Fas was pulled down using S protein-agarose, subjected to SDS-PAGE, analyzed by autoradiography and blotted with anti-S-peptide antibody. **C,** example of mass spectrometric identification of a Fas phosphorylation site. Fas-S peptide was recovered on S protein-agarose and subjected to SDS-PAGE. After staining with Coomassie Brilliant, the band was excised, digested with trypsin and subjected to mass spectrometry. Numbers below mass spectrum indicated masses predicted from the indicated fragments if Ser^{225} (indicated by *) is

phosphorylated. Red and blue colors indicate fragments readily detectable in the chromatogram. **D,** diagram showing six phosphorylation sites identified in Fas by mass spectrometry. Among them, S225 and S333 were found only in PMA-treated samples. Domains of Fas are indicated as follows: ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain; DD, death domain; ID, inhibitory domain. **E,** sequence alignment showing conservation of S225 across species.

Figure S6. Functional analysis of cells stably expressing Fas with single phosphorylation site mutations. A, Parental Jurkat cells or JM14A5 cells stably transfected with the indicated construct were stained for cell surface Fas using APO-1-1 antibody followed by PE-conjugated anti-mouse IgG. *Gray*: isotype control; *Black line*: APO-1-1. **B,** clones expressing the indicated Fas mutant were treated with 50 ng/ml CH-11 for 5 h in the absence or presence of 20 nM PMA, lysed in solution D and analyzed by flow cytometry. Note that expression levels of the reconstituted Fas in JM14A5 cells were ~5-fold lower than endogenous Fas (panel A), providing a potential explanation for the lower CH-11-induced apoptosis in the reconstituted clones. **C,** after cells were treated with diluent or 50 ng/ml CH-11 in the absence or presence of 20 nM PMA, cell lysates (50 μg protein) were subjected to SDS-PAGE and blotted with anti-PARP antibody to assess caspase-mediated cleavage.

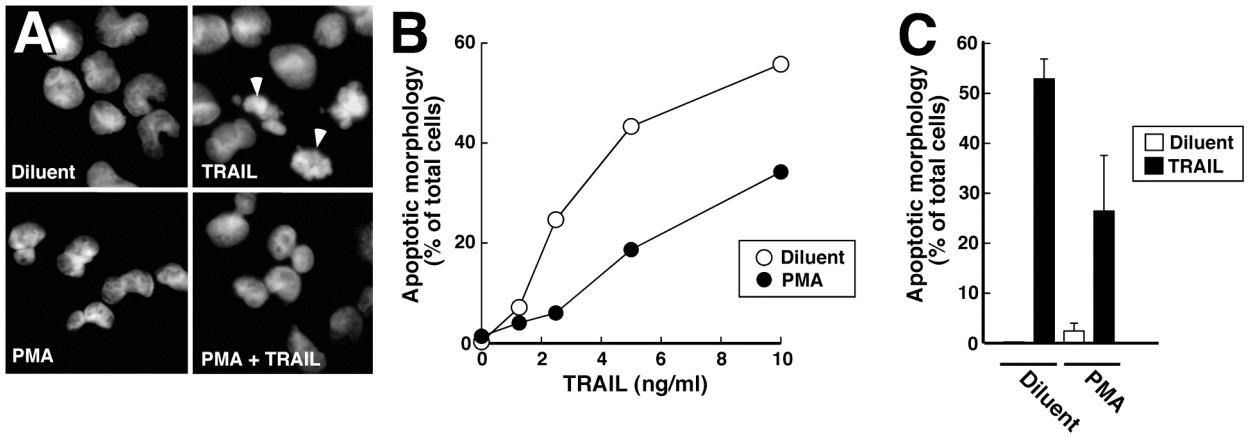
Figure S7. Functional analysis of cells transfected with Fas containing multiple phosphorylation site mutations. A, JM14A5 cells were transiently transfected with pCMS5A without insert (empty vector, e.v.) or pCMS5A encoding wild type (wt) Fas or Fas with 4 (Ser^{209}, Ser^{212}, $Thre^{214}$, Ser^{225}), 5 (4A plus Thr^{305}) or 6 (5A plus Ser^{333}) putative phosphorylation sites mutated to alanine. After 16 h, cells with stained by APO-1-1 followed by Alexa Fluor647-conjugated anti-mouse IgG. Dot plot shows EGFP positive cells (R2) and histograms show Fas

expression (*black*) *vs* isotype control (*gray*) in EGFP-positive populations. **B,** 16 h after transfection with e.v. or pCMS5A encoding the indicated Fas construct, JM14A5 cells were treated with diluent or CH-11 for 5 h and stained with Annexin-V-APC. Only EGFP positive cells were shown, and the percentage of Annexin-V positive cells in this population was given in the top-right quarter. **C,** Bar graph summarizing annexin V binding analyzed as illustrated in panel B. **D,** aliquots containing 1 x 10^8 JM14A5 cells were transfected with pCMS5A encoding the indicated Fas construct. After 16 h cells were treated with 500 ng/ml CH-11 at a cell density of 1 x 10^7/ml for 90 minutes in the absence or presence of 20 nM PMA. The Fas DISC was immunoprecipitated with rabbit anti-mouse IgM. Immunoprecipitates were subjected to SDS-PAGE and blotted with FADD.

Figure S8. Blockade of endocytic pathways does not inhibit Fas internalization. A, 16 h after Jurkat cells were transfected with pEGFP-N1 alone or in a 1:5 ratio with plasmids that inhibit clathrin-dependent endocytic pathways (c-AP180, dnEps-15) or clathrin-independent pathways (dnCdc42 and dnArf6, respectively), binding of anti-transferrin receptor (*upper panel*) or CH-11 (*lower panel*) was assessed by flow cytometry. **B,** JM14A5 cells were transfected with pCMS5A encoding wild type Fas (wt) or Fas Y291F. After 16 h, cells were treated with diluent or 125 ng/ml CH-11 in the absence or presence of 20 nM PMA for 90 minutes. CH-11 binding was assessed as described in the legend to Fig. 6C with gating on the EGFP positive population.

Figure S9. Enzastaurin enhances TRAIL-induced antiproliferative effects in HeLa and HCT116 cells. HeLa (A) or HCT116 cells (B) were incubated for 4 h with the indicated concentrations of TRAIL in the absence or presence of 10 μM enzastaurin. At the completion of the incubation, cells were washed, then incubated in drug-free medium until colonies formed. All values are normalized to cells treated with diluent alone (no enzastaurin or TRAIL). Because

enzastaurin has no effect by itself under the conditions of these assays, its ability to enhance the effects of TRAIL meets the definition of synergy (81). Error bars, ± 1 S.D. of triplicate aliquots.


A
Diluent
TRAIL
PMA
PMA + TRAIL
B
Apoptotic morphology
(% of total cells)
60
40
20
0
0
2
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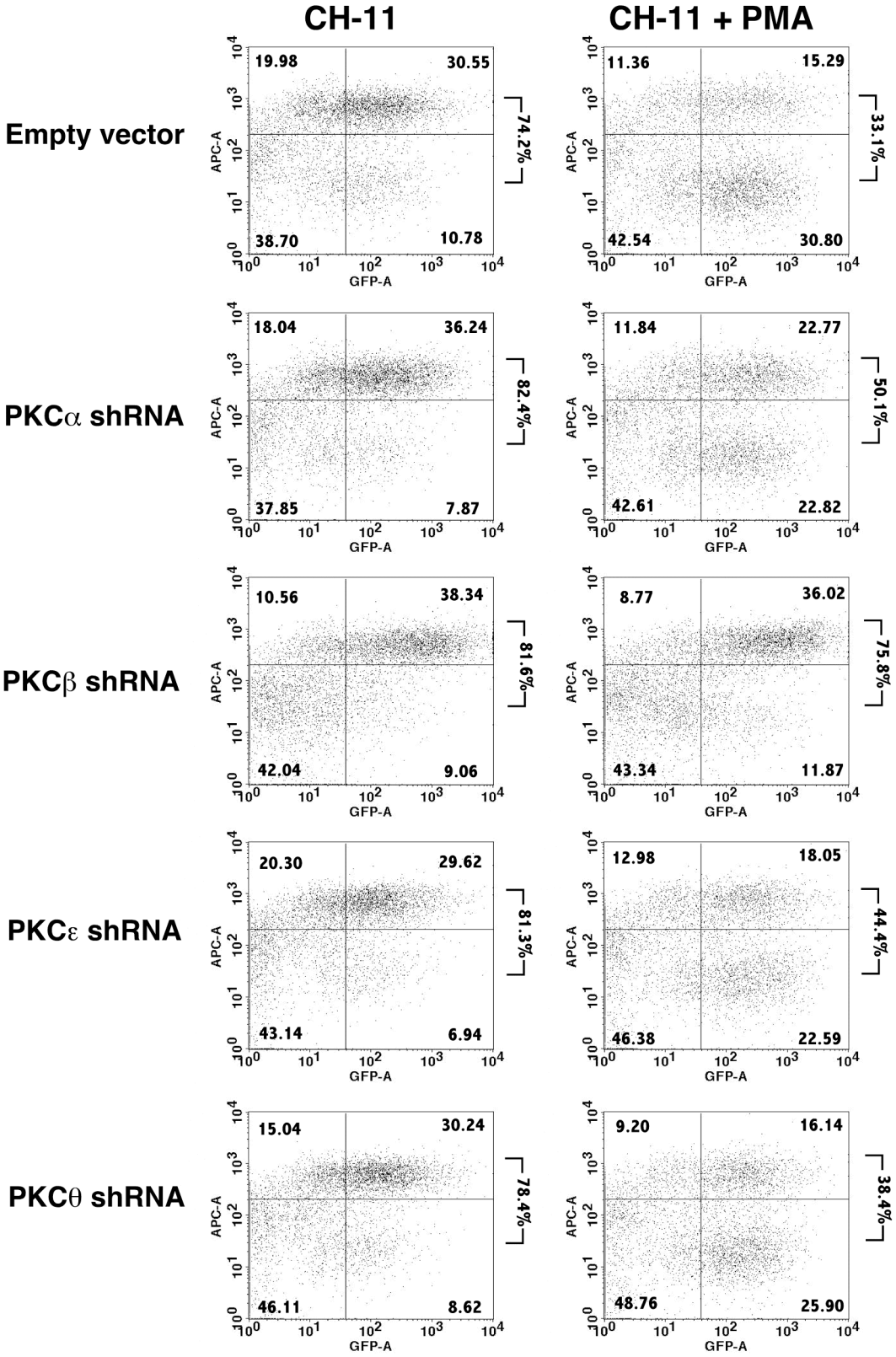
6
8
10
TRAIL (ng/ml)
Diluent
PMA
C
Apoptotic morphology
(% of total cells)
60
50
40
30
20
10
0
Diluent
TRAIL
Diluent
PMA

Figure S1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CH-11 + PMA
Empty vector
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PKCβ shRNA
PKCε shRNA
PKCθ shRNA
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GFP-A
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30.55
38.70
10.78
74.2%
11.36
15.29
42.54
30.80
33.1%
18.04
36.24
37.85
7.87
82.4%
11.84
22.77
42.61
22.82
50.1%
10.56
38.34
42.04
9.06
81.6%
8.77
36.02
43.34
11.87
75.8%
20.30
29.62
43.14
6.94
81.3%
12.98
18.05
46.38
22.59
44.4%
15.04
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46.11
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9.20
16.14
48.76
25.90
38.4%

Figure S2

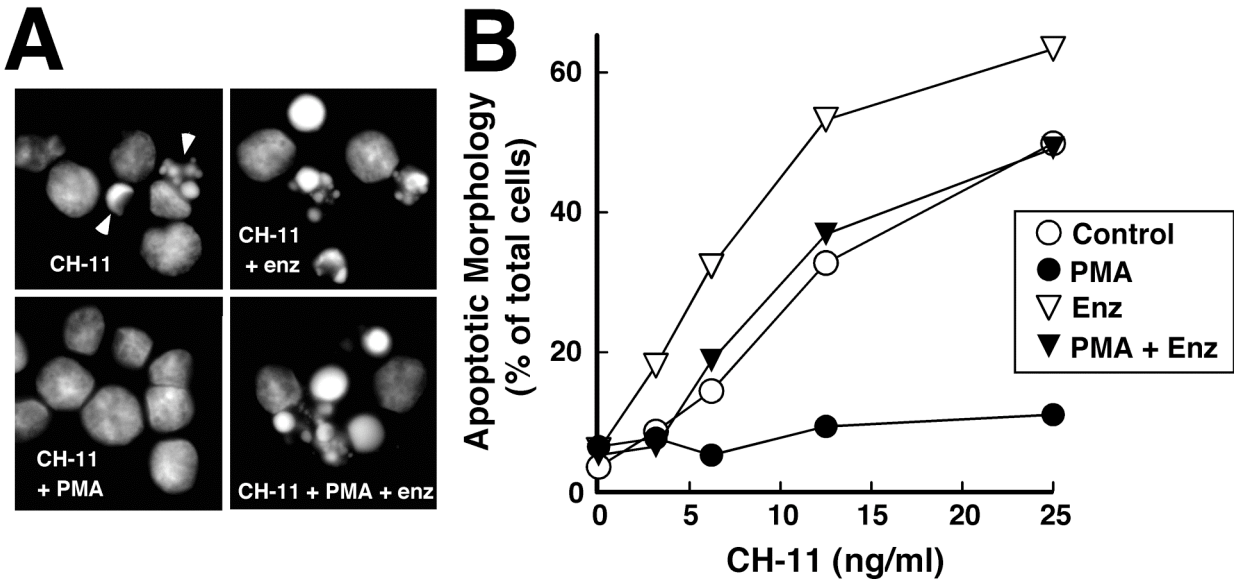

A
CH-11
CH-11
+ enz
CH-11
+ PMA
CH-11 + PMA + enz
B
Apoptotic Morphology
(% of total cells)
60
40
20
0
0
5
10
15
20
25
CH-11 (ng/ml)
Control
PMA
Enz
PMA + Enz

Figure S3

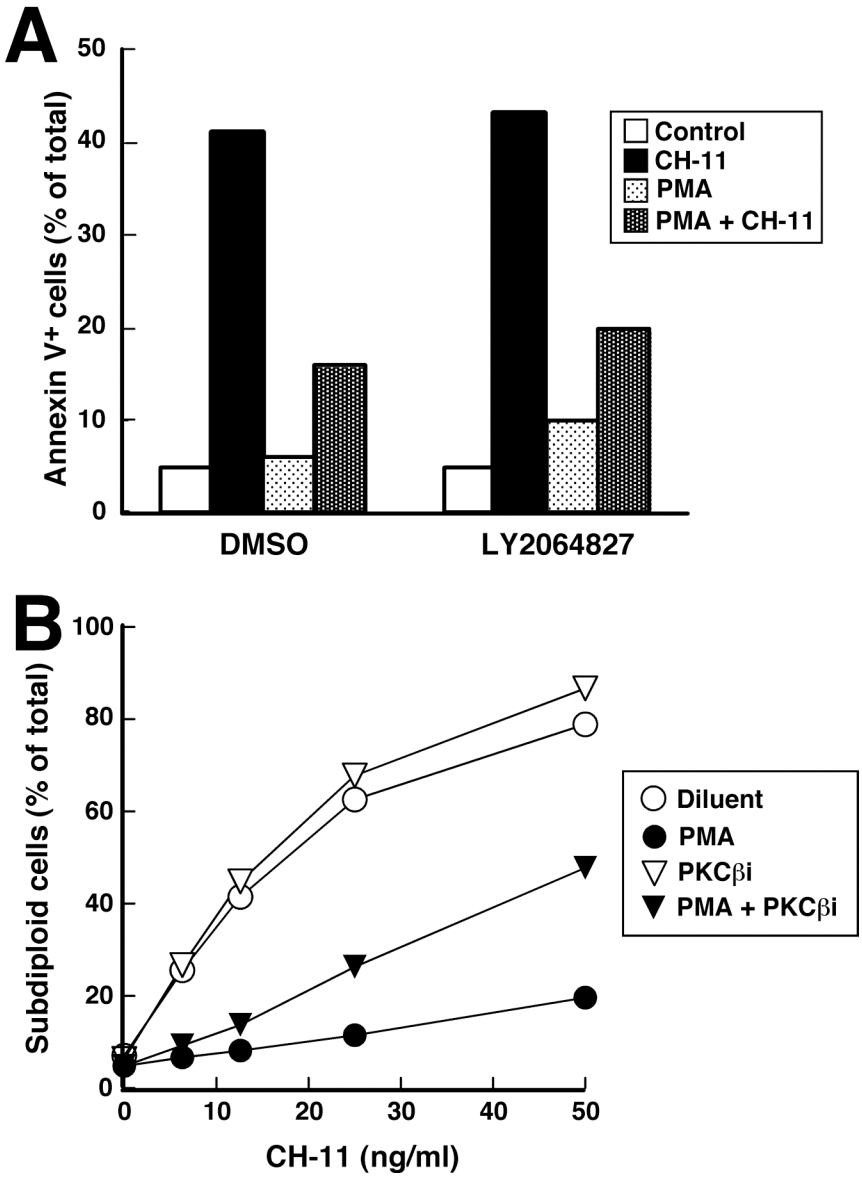
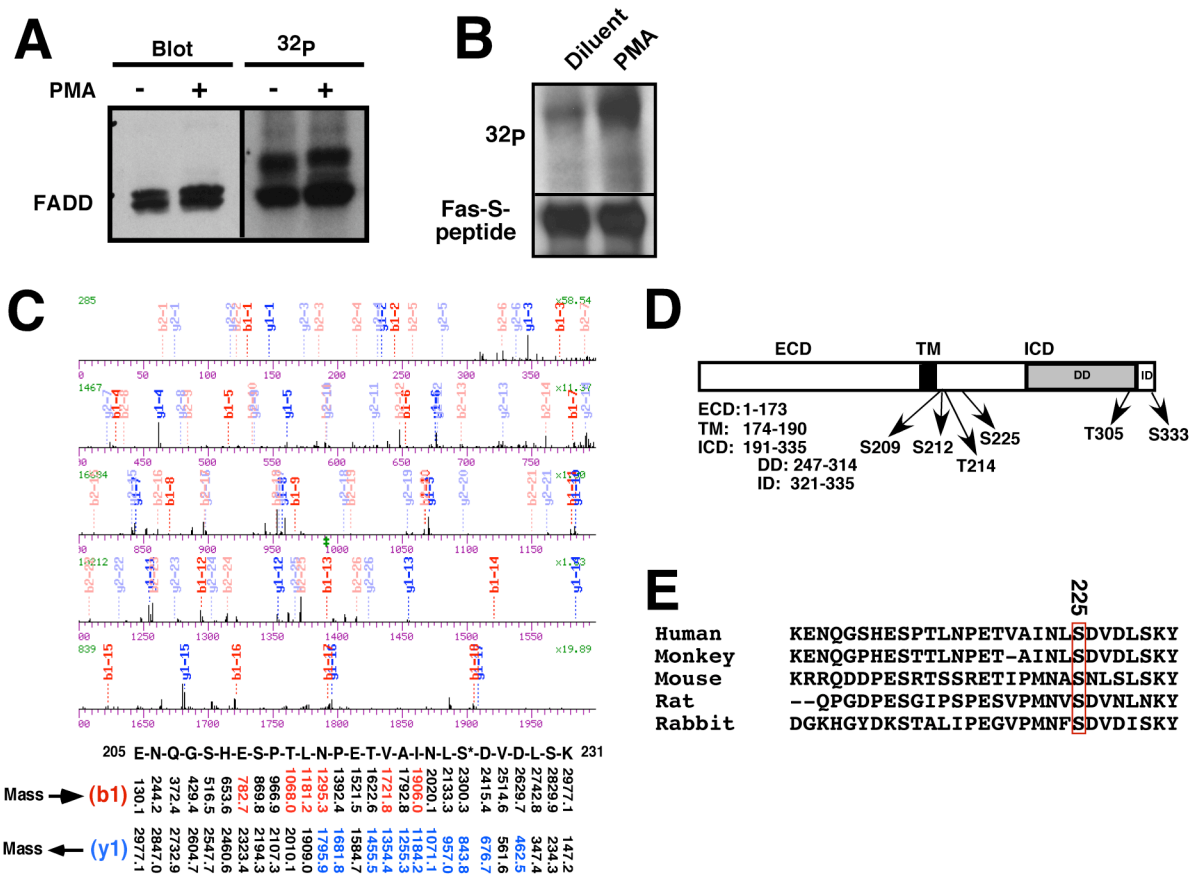

A
Annexin V+ cells (% of total)
50
40
30
20
10
0
Control
CH-11
PMA
PMA + CH-11
DMSO
LY2064827
B
Subdiploid cells (% of total)
100
80
60
40
20
0
0
10
20
30
40
50
CH-11 (ng/ml)
Diluent
PMA
PKCβi
PMA + PKCβi

Figure S4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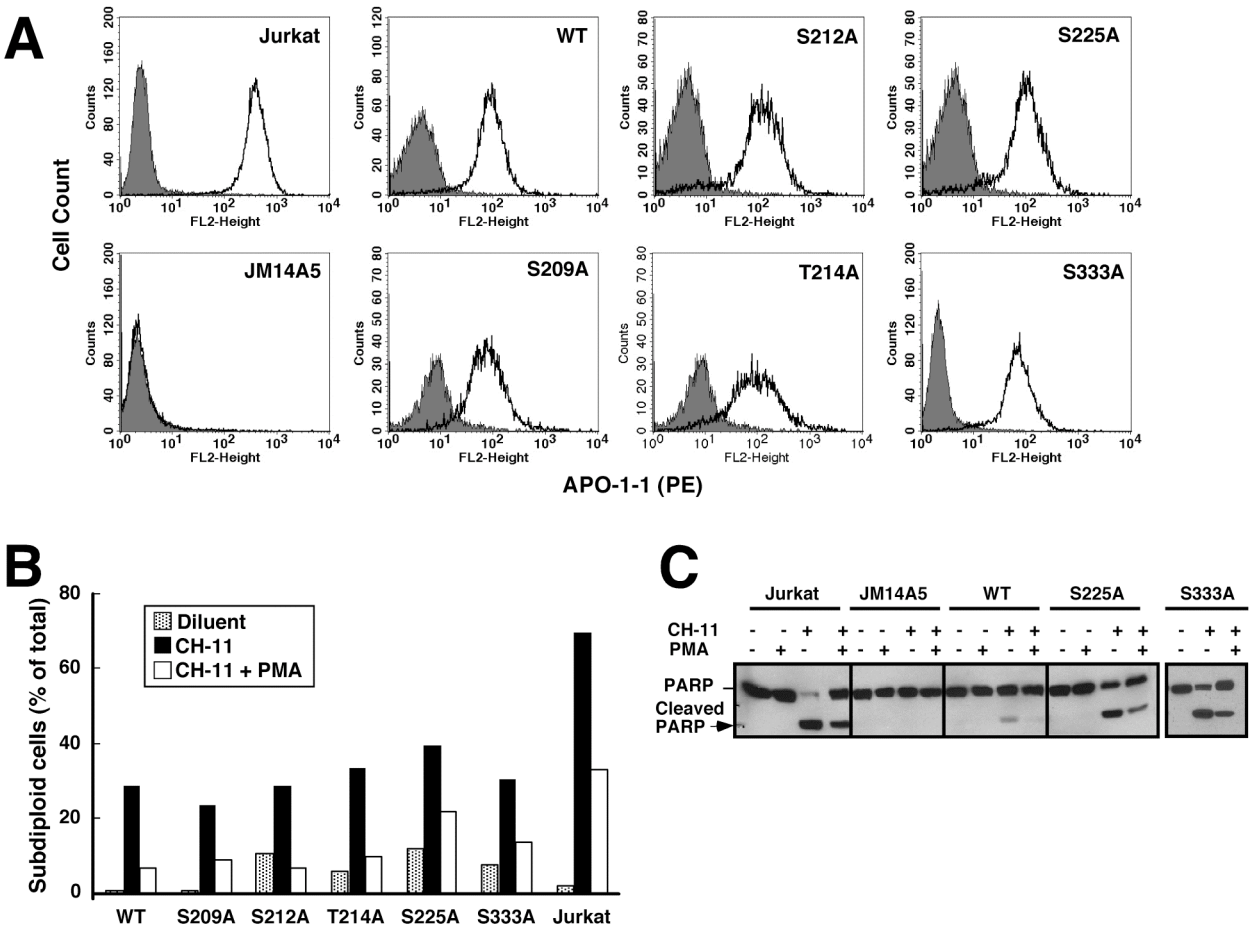
Blot
32P
PMA
- + - +
FADD
B
Diluent
PMA
32p
Fas-S-peptide
C
205 E-N-Q-G-S-H-E-S-P-T-L-N-P-E-T-V-A-I-N-L-S*-D-V-D-L-S-K 231
Mass (b1)
Mass (y1)
D
ECD
TM
ICD
DD
ID
ECD:1-173
TM: 174-190
ICD: 191-335
DD: 247-314
ID: 321-335
S209
S212
S225
T214
T305
S333
E
225
Human KENQGSHESPTLNPETVAINLSDVDLSKY
Monkey KENQGPHESTTLNPET-AINLSDVDLSKY
Mouse KRRQDDPESRTSSRETIPMNASNLSLSKY
Rat --QPGDPESGIPSPESVPMNVSDVNLNKY
Rabbit DGKHGYDKSTALIPEGVPMNFSDVDISKY

Figure S5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Jurkat
WT
S212A
S225A
JM14A5
S209A
T214A
S333A
Cell Count
Counts
FL2-Height
APO-1-1 (PE)
B
Diluent
CH-11
CH-11 + PMA
Subdiploid cells (% of total)
WT
S209A
S212A
T214A
S225A
S333A
Jurkat
C
Jurkat
JM14A5
WT
S225A
S333A
CH-11
PMA
PARP
Cleaved PARP

Figure S6

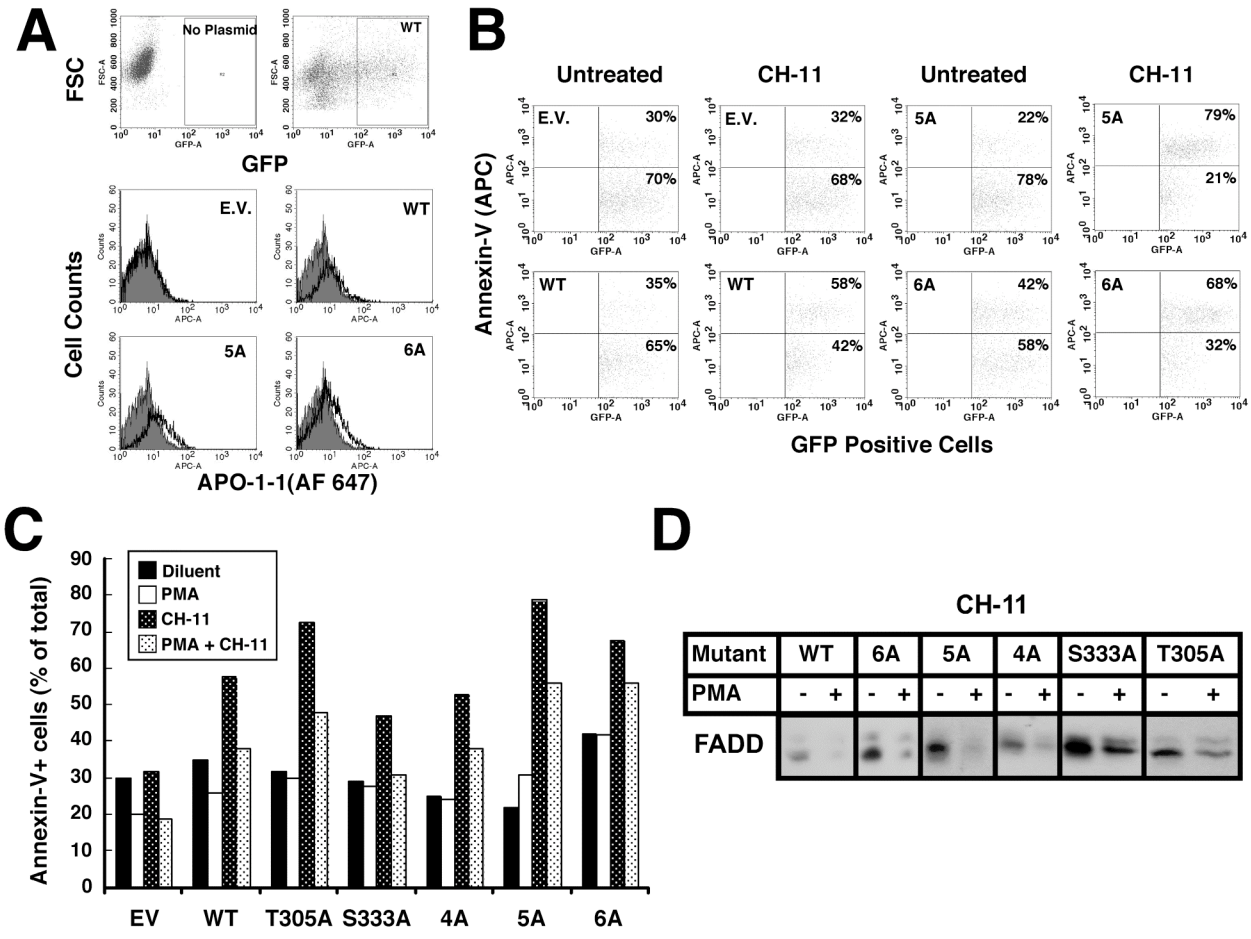
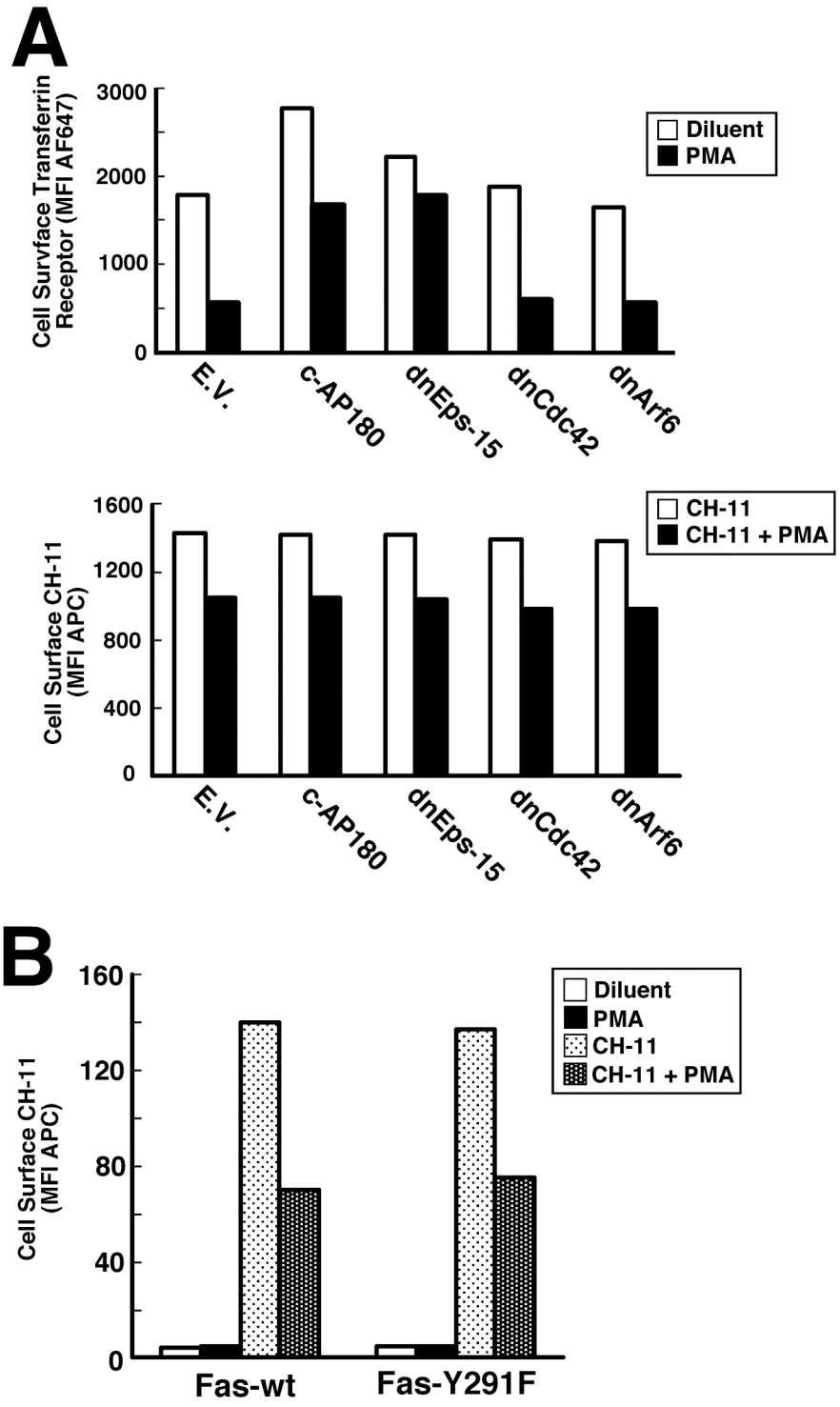

A
No Plasmid
WT
FSC
GFP
E.V.
WT
5A
6A
Cell Counts
APO-1-1(AF 647)
B
Untreated
CH-11
Untreated
CH-11
E.V.
30%
70%
E.V.
32%
68%
5A
22%
78%
5A
79%
21%
WT
35%
65%
WT
58%
42%
6A
42%
58%
6A
68%
32%
Annexin-V (APC)
GFP Positive Cells
C
Diluent
PMA
CH-11
PMA + CH-11
Annexin-V+ cells (% of total)
EV
WT
T305A
S333A
4A
5A
6A
D
CH-11
Mutant
WT
6A
5A
4A
S333A
T305A
PMA
FADD

Figure S7


A
Cell Survface Transferrin Receptor (MFI AF647)
3000
2000
1000
0
Diluent
PMA
E.V.
c-AP180
dnEps-15
dnCdc42
dnArf6
Cell Surface CH-11 (MFI APC)
1600
1200
800
400
0
CH-11
CH-11 + PMA
E.V.
c-AP180
dnEps-15
dnCdc42
dnArf6
B
Cell Surface CH-11 (MFI APC)
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120
80
40
0
Diluent
PMA
CH-11
CH-11 + PMA
Fas-wt
Fas-Y291F

Figure S8

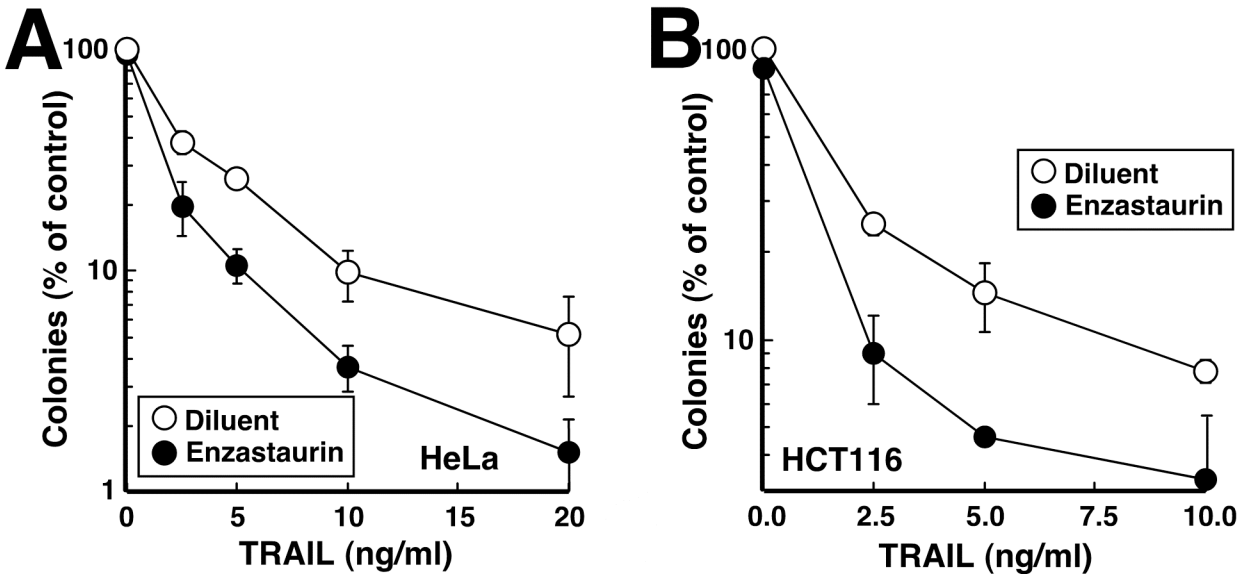
A
100
10
1
Colonies (% of control)
Diluent
Enzastaurin
HeLa
0
5
10
15
20
TRAIL (ng/ml)
B
100
10
Colonies (% of control)
Diluent
Enzastaurin
HCT116
0.0
2.5
5.0
7.5
10.0
TRAIL (ng/ml)

Figure S9