Supplemental Data

Table S1. Combinations of Gαi and Gαq dependent GPCR ligands yield synergistic Ca^{2+} responses.

	C5a	UDP	UTP
UDP	Y	nd	N
UTP	Y	N	nd
LPA	N	Y	Y
S1P	Y	Y	nd
Spir	N	Y	Y

Apparent Gα Linkage
i
q
i/q

NIH-3T3 cells stably expressing C5aR or Ro2 were stimulated with combinations of C5a (10 nM), UTP (100 μM), UDP (100 μM), LPA (50 nM), S1P (50 nM), and Spiradoline (Spir, 100 nM). Ligands dependent on PTx-sensitive G-proteins are labeled in blue. Ligands showing partial sensitivity to PTx are labeled in green. Ligands insensitive to PTx and presumed to be dependent on Gαq are labeled in red. "Y" indicates the presence of synergy from dual-ligand stimulations, inferred from replicate assays, and "N" indicates the absence of synergy. Combinations for which synergy was not determined are marked 'nd'.

Figure S1. Expression of mPLCβ3 in PLCβ2/3$^{-/-}$ BMDM restores synergistic Ca^{2+} responses to levels observed in WT BMDM. Single cell Ca^{2+} assays were performed on WT or PLCβ2/3$^{-/-}$ BMDM transduced with retrovirus encoding YFP-FLAG or YFP-mPLCβ3. Cells were stimulated with C5a (0.75 nM), UDP (500 nM), or C5a+UDP, and the Ca^{2+} responses were measured by integration over 2.25 min. The predicted additive (Pred. Additive) response was calculated as the sum of the UDP and C5a responses. Synergy ratios calculated as the ratio of the observed UDP+C5a response to the predicted additive response are displayed in Fig. 2E. Responses by transduced cells were measured in multiple assays of each of 3 independent batches of infected BMDM. Values shown are mean +/- SEM from n=5-6 samples per condition. *$p<0.05$.

Figure S2. Ligand dose-responses. Intracellular Ca^{2+} levels were calculated for Fura-2 loaded NIH-3T3-C5aR cells. Cells were stimulated with varied concentrations of (A) UTP, UDP or C5a and (B) LPA, S1P or PAF. Values are mean +/- SEM from n=4-8 samples per point.

Figure S3. Robust synergy is observed for ligands that stimulate little to no Ca^{2+} response individually. Intracellular Ca^{2+} levels were calculated for Fura-2-loaded NIH-3T3-C5aR cells. (A) Cells were stimulated with 1 or 10 nM C5a alone or in combination with 100 μM UDP. (B) Cells were stimulated with 25 or 100 μM UDP alone or in combination with 10 nM C5a. Peak offsets for C5a alone were 0-15 nM (median = 8 nM) while UDP yielded no measurable responses. Values shown are mean +/- SEM of n=4 samples per condition per assay from a representative assay of n=4 with similar results.

Figure S4. C5a stimulated Ca^{2+} responses and synergistic responses with UTP are PTx-sensitive. NIH-3T3 cells stably expressing C5aR were treated for 18 h with buffer or 50 ng/ml PTx and Ca^{2+} assays were performed to assess single-ligand and synergy responses. (A) Responses of cells to 10 nM C5a with or without pretreatment with PTx. (B) Responses of cells pretreated with buffer to 10 nM C5a, 100 μM

UTP, or C5a+UTP. The predicted additive response is shown, and the calculated synergy ratio for this assay = 2.0. (C) Responses of cells pretreated with PTx to 10 nM C5a, 100 μM UTP, or C5a+UTP. The predicted additive response is shown, and the calculated synergy ratio for this assay = 1.3. Values are mean from n=4 samples from a representative assays of two with similar results.

Figure S5. Alternate Gαi-linked GPCRs provide synergy with UTP and UDP. (A) Responses to LPA and S1P are PTx-sensitive. NIH-3T3 cells pretreated with PTx (50 ng/ml for 18 h) were stimulated with 100 μM UTP, 2.5 μM LPA, or 1 μM S1P. (B) UDP synergizes with LPA or S1P. Cells were stimulated with 100 μM UDP, 50 nM LPA, and 50 nM S1P alone or in combination. Peak offset synergy ratios for dual ligand stimulations were 4.9 and 3.7 for UDP+LPA and UDP+S1P respectively. Results are from representative assays of n=3-4 each. (C) NIH-3T3 cells stably expressing the Ro2 RASSL form of the human kappa opioid receptor respond to spiradoline (Spir) in a PTx-sensitive manner. Cells treated with or without PTx (50 ng/ml for 18 h) were stimulated with 100 nM Spir. (D) Spiradoline synergizes with UTP. Cells were treated with 100 μM and 500 nM Spir alone or in combination. Quantitation of responses was by peak-offset for each condition. The synergy ratio for the dual-ligand stimulation was 3. Results are from a representative assay of n=4 with similar results.

Figure S6. Ca^{2+} responses to spiradoline alone and its synergistic response with UTP are PTx-sensitive. NIH-3T3 cells stably expressing Ro2 receptor were treated for 18 h with buffer (A) or 50 ng/ml PTx (B), and Ca^{2+} assays were performed to assess synergy responses. (C) Peak-offsets of responses were measured and synergy ratios of 2.6 and 1.2 were calculated for buffer and PTx treatments, respectively.

Figure S7. Levels of PLCβ isoform knockdown and YFP-PLCβ expression in NIH-3T3 cells. (A) siRNA-mediated KD of PLCβ isoforms was performed in NIH-3T3-C5aR cells using PLCβ isoform specific siRNA versus LacZ control siRNA. After 72 h, RNA samples were taken in parallel with analyses of Ca^{2+} responses. PLCβ isoform mRNA levels were measured by qRT-PCR and expressed as percent change vs LacZ control siRNA samples. (B) NIH-3T3 cells were stably transfected with YFP or YFP-tagged murine PLCβ isoforms and analyzed by FACS to measure YFP-fusion protein expression. (C) NIH-3T3 cells were stably transfected with YFP or YFP-tagged bovine PLCβ4 (wild-type or C-terminal mutant (ΔCT)) and analyzed by FACS to measure YFP-fusion protein expression. (D) PLCβ4 inhibition of synergy is dependent on its Gαq binding region. NIH-3T3 cells were stably transfected with YFP-bPLCβ4, YFP-bPLCβ4ΔCT (C-terminal mutant), or YFP vector control and assayed for Ca^{2+} response synergy to UTP + C5a. Synergy ratios were calculated for peak offset values. Values are mean+/-SEM from 2-3 assays using 2 independently derived cell lines per target, *$p<0.05$ versus YFP.

Figure S8. Levels of PLCβ3 expression and knockdown in NIH-3T3 cells. siRNA-mediated knockdown of mPLCβ3 was performed in NIH-3T3-C5aR cells stably expressing YFP or YFP-tagged PLCβ constructs. After 72 h, total protein and RNA samples were taken in parallel with analyses of Ca^{2+} responses. (A) Western blotting for mPLCβ3 in YFP or YPF-hPLCβ3-expressing cells treated with mPLCβ3 isoform-specific siRNA versus LacZ control siRNA. Arrows indicate endogenous mPLCβ3 and introduced YFP-hPLCβ3. (B) mPLCβ3 mRNA was measured by qRT-PCR. Levels are expressed as percent change vs LacZ control siRNA samples.

Figure S9. Transfection of PLCβ isoforms and mutants into NIH-3T3 cells. NIH-3T3 cells were stably transfected with YFP or YFP-tagged PLCβ constructs including: intact hPLCβ3; C-terminal

mutant, hPLCβ3ΔCT; Y-box mutant, hPLCβ3ΔYB, C-terminal mutant hPLCβ3ΔYBΔCT, intact mPLCβ2, and a C-terminal deletion mutant of mPLCβ2 (PLCβ2–CT). (A) Cells were analyzed by FACS to measure YFP-fusion protein expression. (B) Cells were then subjected to RNAi using murine-specific anti-PLCβ3 or LacZ control siRNA. After 72 h, RNA samples were taken in parallel with analyses of Ca^{2+} responses. mPLCβ3 mRNA was measured by qRT-PCR. Levels are expressed as percent change vs LacZ siRNA control samples. (C) C5a+UTP synergy ratios were calculated for each cell line and treatment (see Figs. 7,8) and the changes in synergy ratio (differences in synergy ratio between LacZ control and mPLCβ3-specific siRNA) for each cell line were calculated to determine the delta with knockdown of mPLCβ3. A delta of zero (0) reflects no change in synergy with mPLCβ3 KD, and thus the ability of the introduced construct to substitute for endogenous mPLCβ3. Values are mean+/-SEM from n=6-15 assays using 2-7 independently derived cell lines per target. (D) PLCβ2 can substitute for PLCβ3 to mediate Ca^{2+} synergy in NIH-3T3 cells. NIH-3T3 cells were stably transfected with YFP or YFP-tagged PLCβ constructs including: intact mPLCβ2 or a C-terminal deletion mutant of mPLCβ2 (PLCβ2–CT). Cells were then subjected to RNAi using murine-specific anti-PLCβ3 or LacZ control siRNA. Synergy ratios were calculated for each cell line and treatment and the changes in synergy ratio for each cell line were calculated to determine the delta with knockdown of mPLCβ3. Although intact mPLCβ2 maintained synergy (y=0 shows no change in synergy with KD of mPLCβ3), the C-terminal mutant did not, *$p<0.05$. Values are mean+/-SEM from n=3-7 assays using 2-3 independently derived cell lines per target.

Supplements

Figure S1

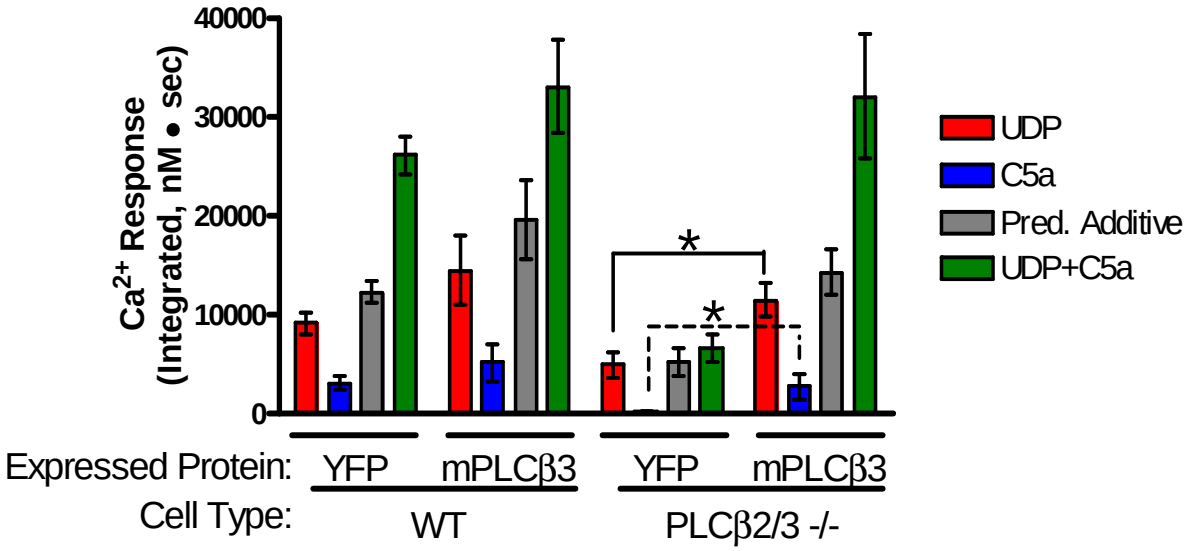


Figure S2

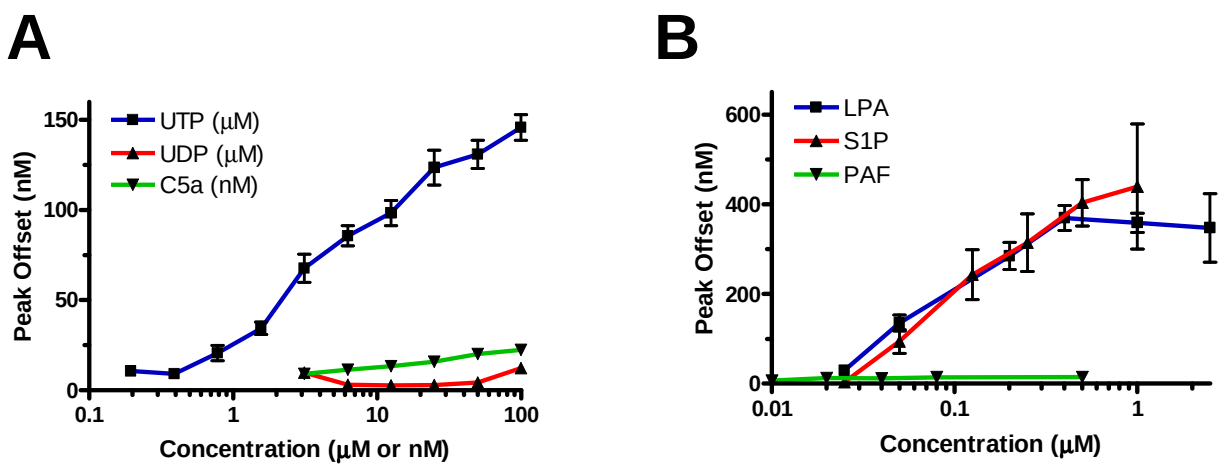

A
B
UTP (µM)
UDP (µM)
C5a (nM)
Peak Offset (nM)
Concentration (µM or nM)
LPA
S1P
PAF
Concentration (µM)

Figure S3

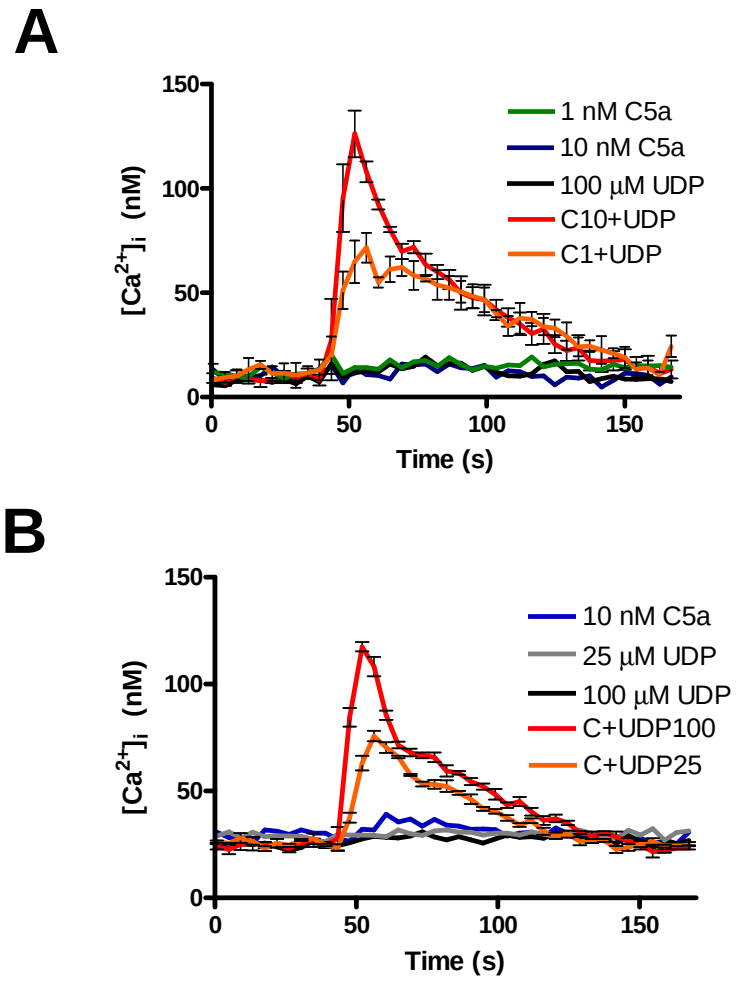

A
150
100
50
0
0
50
100
150
Time (s)
[Ca2+]i (nM)
1 nM C5a
10 nM C5a
100 µM UDP
C10+UDP
C1+UDP
B
150
100
50
0
0
50
100
150
Time (s)
[Ca2+]i (nM)
10 nM C5a
25 µM UDP
100 µM UDP
C+UDP100
C+UDP25

Figure S4

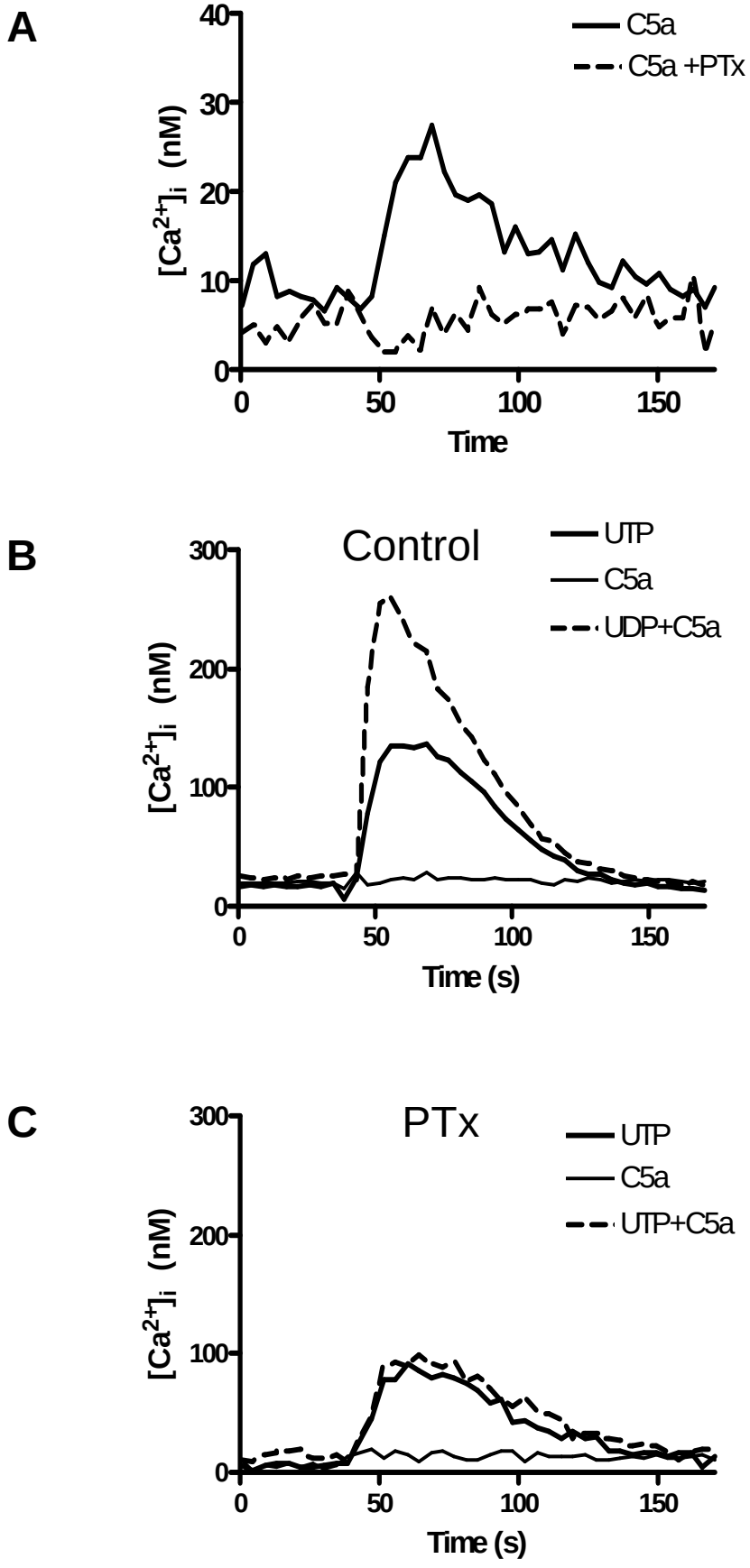

A
C5a
C5a +PTx
[Ca2+]i (nM)
Time
B
Control
UTP
C5a
UDP+C5a
[Ca2+]i (nM)
Time (s)
C
PTx
UTP
C5a
UTP+C5a
[Ca2+]i (nM)
Time (s)

Figure S5

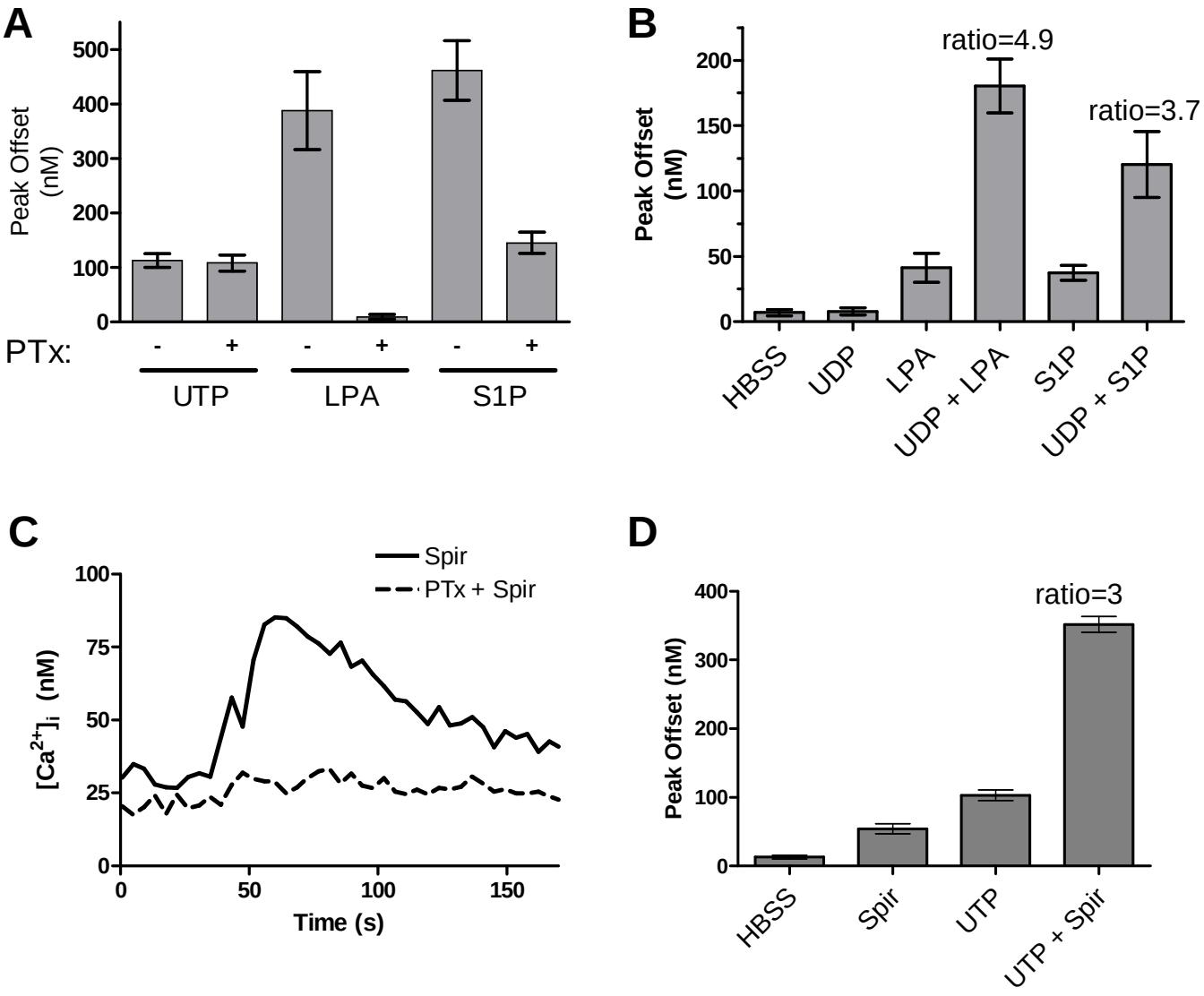

A
Peak Offset (nM)
500
400
300
200
100
0
PTx:
-
+
-
+
-
+
UTP
LPA
S1P
B
Peak Offset (nM)
200
150
100
50
0
ratio=4.9
ratio=3.7
HBSS
UDP
LPA
UDP + LPA
S1P
UDP + S1P
C
Spir
PTx + Spir
[Ca2+]i (nM)
100
75
50
25
0
0
50
100
150
Time (s)
D
Peak Offset (nM)
400
300
200
100
0
ratio=3
HBSS
Spir
UTP
UTP + Spir

Figure S6

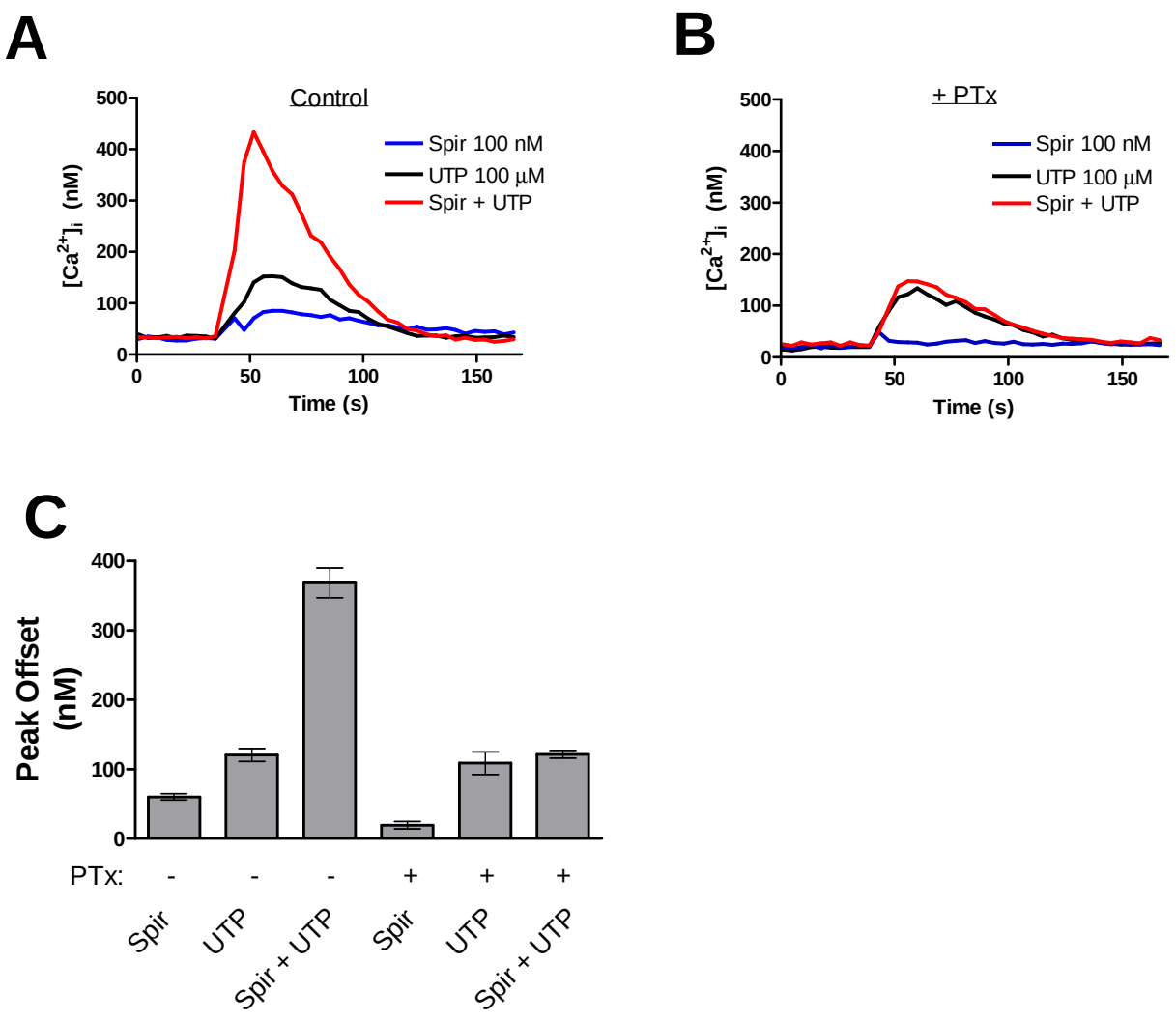

A
Control
Spir 100 nM
UTP 100 μM
Spir + UTP
[Ca2+]i (nM)
Time (s)
B
+ PTx
Spir 100 nM
UTP 100 μM
Spir + UTP
[Ca2+]i (nM)
Time (s)
C
Peak Offset (nM)
PTx:
Spir
UTP
Spir + UTP
Spir
UTP
Spir + UTP

Figure S7

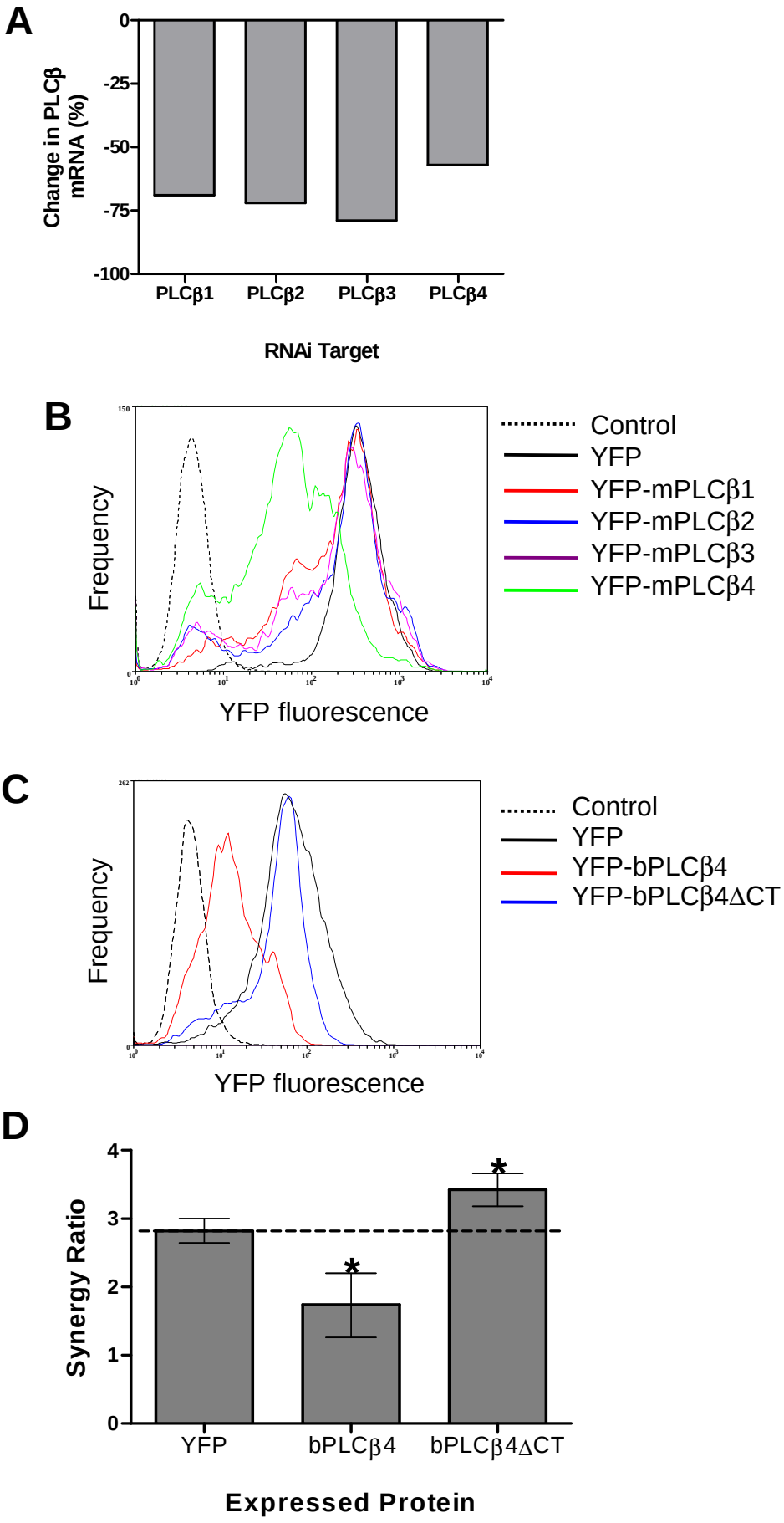
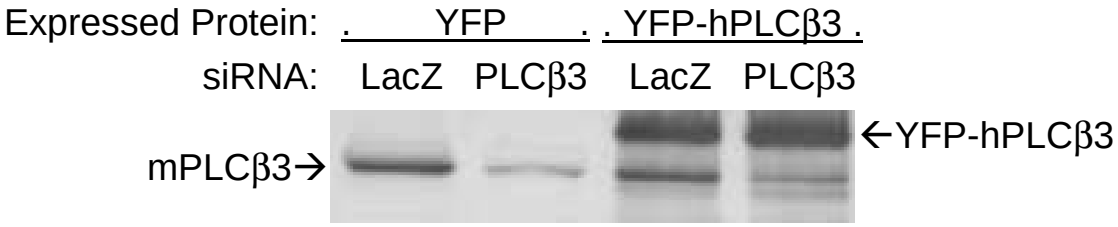

A
0
-25
-50
-75
-100
Change in PLCβ mRNA (%)
PLCβ1
PLCβ2
PLCβ3
PLCβ4
RNAi Target
B
Frequency
YFP fluorescence
Control
YFP
YFP-mPLCβ1
YFP-mPLCβ2
YFP-mPLCβ3
YFP-mPLCβ4
C
Frequency
YFP fluorescence
Control
YFP
YFP-bPLCβ4
YFP-bPLCβ4ΔCT
D
Synergy Ratio
4
3
2
1
0
*
*
YFP
bPLCβ4
bPLCβ4ΔCT
Expressed Protein

Figure S8

A


Expressed Protein: . YFP . . YFP-hPLCβ3 .
siRNA: LacZ PLCβ3 LacZ PLCβ3
mPLCβ3→
←YFP-hPLCβ3

B

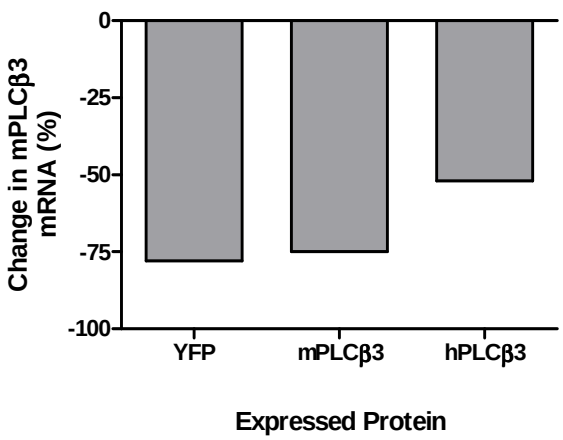
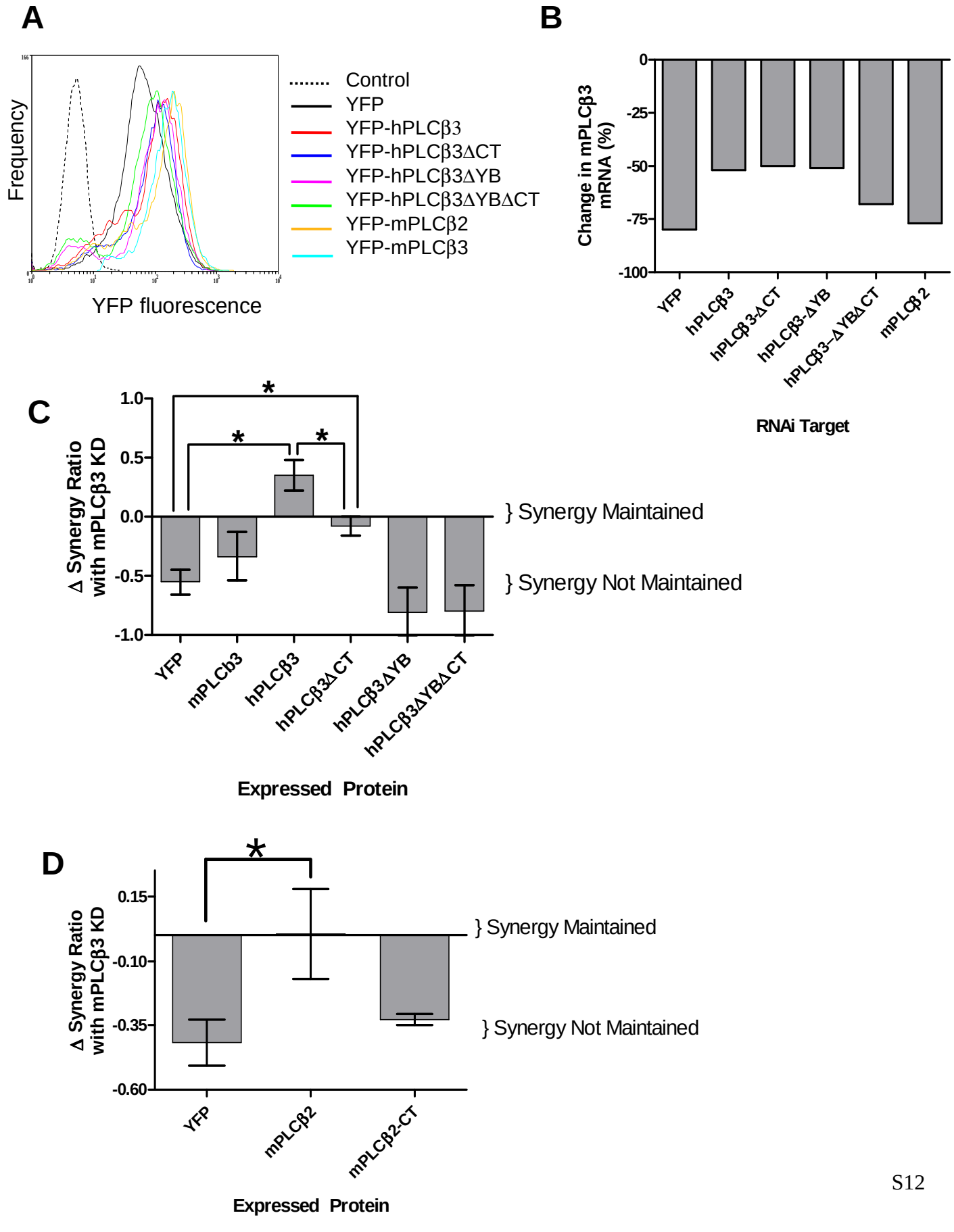

Change in mPLCβ3 mRNA (%)
0
-25
-50
-75
-100
YFP
mPLCβ3
hPLCβ3
Expressed Protein

Figure S9


A
Frequency
YFP fluorescence
Control
YFP
YFP-hPLCβ3
YFP-hPLCβ3ΔCT
YFP-hPLCβ3ΔYB
YFP-hPLCβ3ΔYBΔCT
YFP-mPLCβ2
YFP-mPLCβ3
B
Change in mPLCβ3 mRNA (%)
0
-25
-50
-75
-100
YFP
hPLCβ3
hPLCβ3-ΔCT
hPLCβ3-ΔYB
hPLCβ3-ΔYBΔCT
mPLCβ2
RNAi Target
C
Δ Synergy Ratio with mPLCβ3 KD
1.0
0.5
0.0
-0.5
-1.0
*
*
*
} Synergy Maintained
} Synergy Not Maintained
YFP
mPLCb3
hPLCβ3
hPLCβ3ΔCT
hPLCβ3ΔYB
hPLCβ3ΔYBΔCT
Expressed Protein
D
Δ Synergy Ratio with mPLCβ3 KD
0.15
-0.10
-0.35
-0.60
*
} Synergy Maintained
} Synergy Not Maintained
YFP
mPLCβ2
mPLCβ2-CT
Expressed Protein