Figure S1

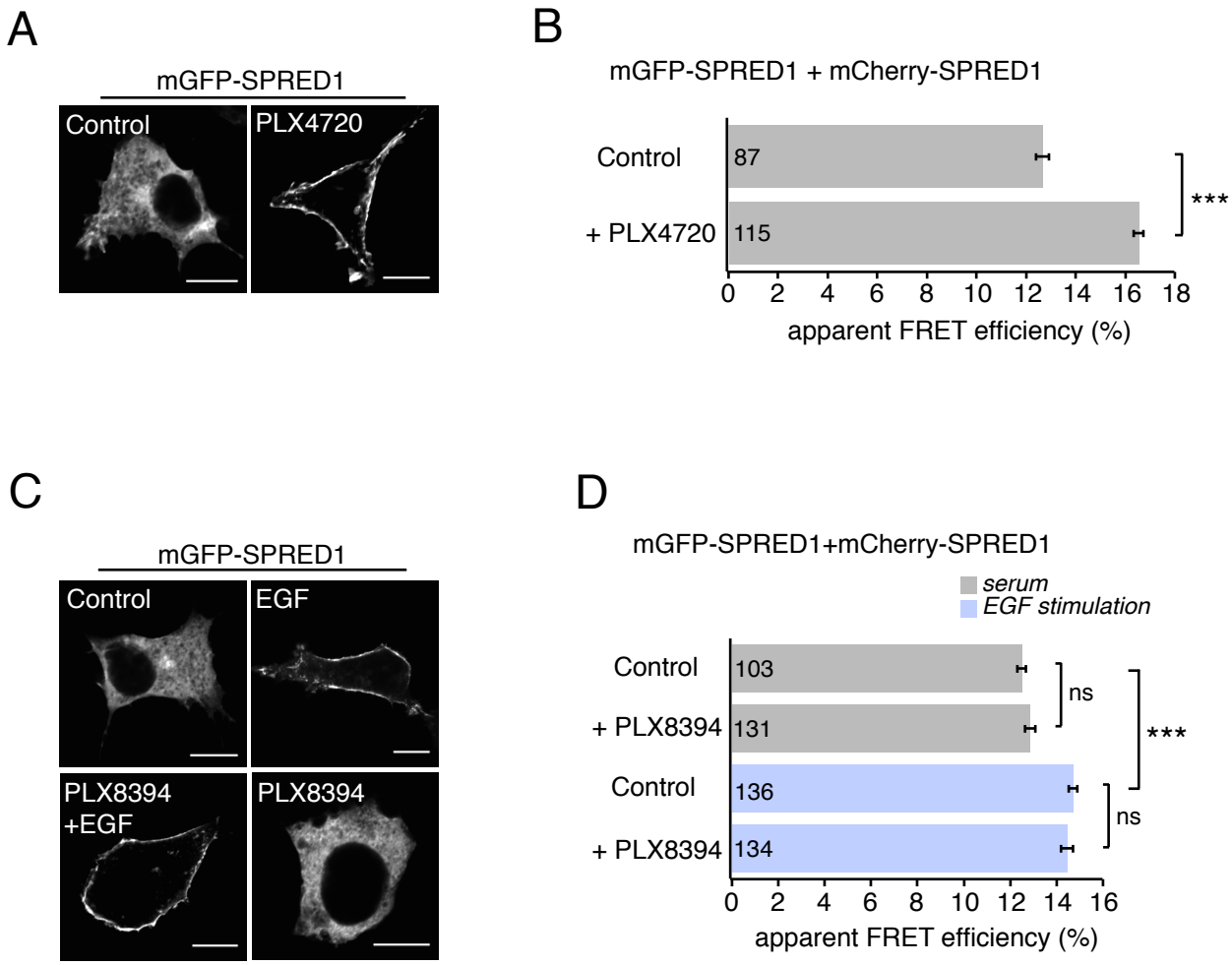

A
mGFP-SPRED1
Control
PLX4720
B
mGFP-SPRED1 + mCherry-SPRED1
Control
+ PLX4720
87
115
***
0 2 4 6 8 10 12 14 16 18
apparent FRET efficiency (%)
C
mGFP-SPRED1
Control
EGF
PLX8394 +EGF
PLX8394
D
mGFP-SPRED1+mCherry-SPRED1
serum
EGF stimulation
Control
+ PLX8394
Control
+ PLX8394
103
131
136
134
ns
***
ns
0 2 4 6 8 10 12 14 16
apparent FRET efficiency (%)

Figure S2

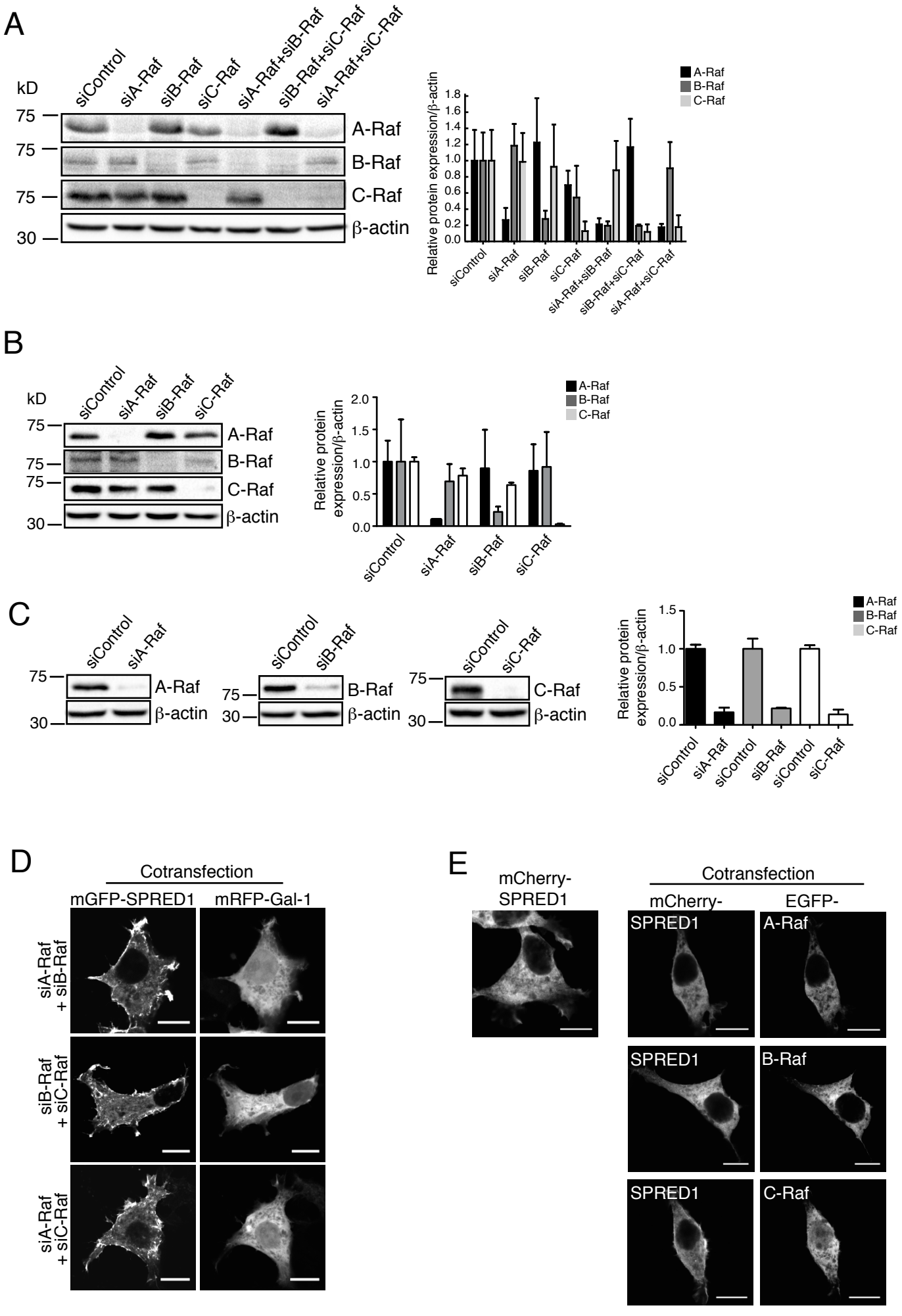
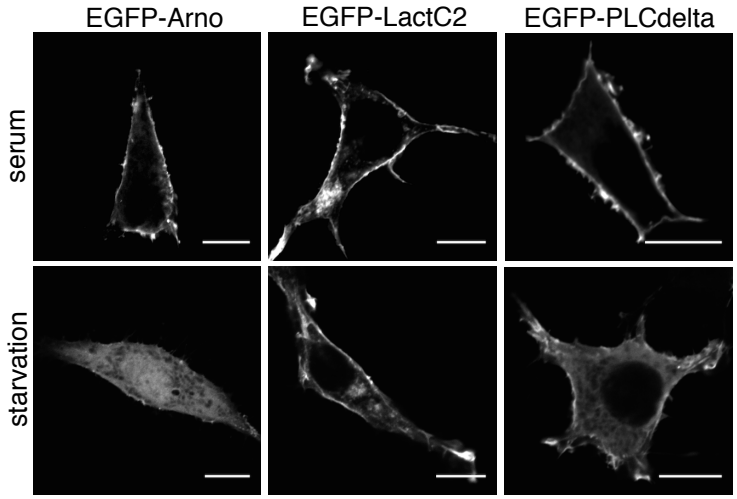

A
siControl
siA-Raf
siB-Raf
siC-Raf
siA-Raf+siB-Raf
siB-Raf+siC-Raf
siA-Raf+siC-Raf
kD
75
75
75
30
A-Raf
B-Raf
C-Raf
β-actin
Relative protein expression/β-actin
A-Raf
B-Raf
C-Raf
B
C
D
Cotransfection
mGFP-SPRED1
mRFP-Gal-1
siA-Raf + siB-Raf
siB-Raf + siC-Raf
siA-Raf + siC-Raf
E
mCherry-SPRED1
Cotransfection
mCherry-SPRED1
EGFP-A-Raf
EGFP-B-Raf
EGFP-C-Raf

Figure S3



Supplementary Figure legends

Figure S1. Effect of Raf inhibitors on SPRED1 plasma membrane translocation. (A) Confocal images of HEK cells transiently transfected with mGFP-SPRED1 for 24 h, followed by control treatment (0.1% DMSO in growth medium) for 1 h, or PLX4720 treatment (10 μM, 1 h) in normal serum conditions. Scale bars, 10 μm. (B) SPRED1 membrane translocation FRET-assay was conducted in HEK cells transiently coexpressing mGFP-/mCherry-SPRED1 for 24 h, following treatments as in (A) and fixed. Numbers inside the bars correspond to the total number of cells studied in each case. Error bars indicate the s.e.m. ***, $p<0.001$. (C) HEK cells were transiently transfected with mGFP-SPRED1 for 24 h, followed by control treatment (0.1% DMSO in growth medium) for 1 h, or serum-starvation for 5 h and stimulation with EGF (100 ng/ml, 10 min), or serum-starvation for 5 h and PLX8394 treatment (10 μM, 1 h) followed by EGF stimulation (100 ng/ml, 10 min), or PLX8394 treatment alone (10 μM, 1 h), as indicated. Scale bars, 10 μm. (D) SPRED1 membrane translocation FRET-assay was conducted in HEK cells transiently coexpressing mGFP-/mCherry-SPRED1. After transfection of 24 h, the cells were treated as in (C) and fixed. *Grey bars*: control samples in normal serum conditions; *blue bars:* EGF-stimulated samples. Numbers inside the bars correspond to the total number of cells studied in each case. Error bars indicate the s.e.m. ***, $p<0.001$; ns, non significant.

Figure S2. Effect of B-Raf or C-Raf downmodulation on Gal-1-induced SPRED1 translocation. (A) Representative Western blot images of HEK cells transfected with specific single siRNAs or double siRNAs targeting indicated Raf isoforms. β-actin was used as a loading control. Quantification of the protein expression levels relative

to β-actin shows 80-90% knock-down of A-Raf, B-Raf and/ or C-Raf (two independent biological repeats). Error bars indicate the s.e.m. (B) Representative Western blots of HEK cells transfected with specific siRNAs targeting indicated Raf isoforms. β-actin was used as a loading control. Quantification of the protein expression levels relative to β-actin shows 80-90% knock-down of A-Raf, B-Raf or C-Raf (three independent biological repeats). Error bars indicate the s.e.m. (C) Representative Western blots of HEK cells transfected with specific siRNAs targeting indicated Raf isoforms. Quantification of the protein expression levels relative to loading control β-actin shows 80-90% knock-down of A-Raf, B-Raf or C-Raf (three independent biological repeats). Error bars indicate the s.e.m. (D) A-Raf, B-Raf and C-Raf were first silenced as pairs (as shown on the figure) with specific siRNAs in HEK cells for 24 h. The cells were then cotransfected with mGFP-SPRED1 and mRFP-Gal-1 for further 24 h and imaged with confocal microscope. Scale bars, 10 μm. (E) Confocal images of HEK cells transfected with mCherry-tagged SPRED1 alone (left image) or together with EGFP-tagged A-Raf, B-Raf or C-Raf for 24 h. Scale bars, 10 μm.

Figure S3. Localization of specific lipid probes under serum or in serum-free conditions. Confocal images of HEK cells transfected with EGFP-Arno (a probe for $PI(3,4,5)P_3$), EGFP-LactC2 (phosphatidylserine, PS) or EGFP-PLCdelta ($PI(4,5)P_2$) for 24 h in normal serum conditions (upper panels), or transfected for 24 h in normal serum conditions followed by starvation for 5 h (lower panels). Scale bars, 10 μm.