Supporting Information

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Supplemental figure legends

Figure S1. DGKα lipid kinase assay at the TCR complex. RNAi was used to silence DGKα and DGKζ; at 72 h post-treatment, cells (10^7 cells/ml) were starved as in Figure 1B and stimulated with anti-CD3/CD28 beads for various times. TCR-bound samples were used as an enzyme source for the DAG kinase assay, using optimum conditions to measure DGKα activity (assay II), as described in method section. PA production is represented as arbitrary units. The TCL fraction was analyzed by WB to confirm RNAi efficiency and activation (B).

Figure S2. Ectopically expressed DGKζ is recruited to the TCR complex after triggering and does not alter recruitment of endogenous DGKζ. (A) GFP-DGKζ chimera or GFP alone were transiently expressed in Jurkat T cells. At 24 h post-transfection, cells were collected, serum-starved, and TCR-bound complex isolated using anti-CD3/CD28-coated beads. Associated proteins were analyzed by WB and transfected constructs detected using anti-GFP antibody (top). Ectopic DGKζ expression was measured in parallel in total cell lysate (TCL). Ectopic DGKζ was enriched in the activated TCR immune complex compared to controls, at a level similar to that of endogenous DGKζ. GFP-DGKζ overexpression did not compete with endogenous DGKζ, which was still recruited to the active TCR. As an additional control, GFP alone did not associate to the pulldown complex in any conditions tested. (B,C) Jurkat T cells were transiently transfected with the GFP-DGKζ fusion protein and stimulated either with CD3/CD28-coated beads or with SEE-loaded antigen presenting cells (B and C respectively). (B) Bead-treated cells were incubated for 20 minutes and fixed with paraformaldehyde for immunofluorescence analysis. GFP-DGKζ was monitored and compared to actin cytoskeleton (Phaloidin-Rhodamine, red). Representative images are shown, Bar= 5 μm. (C) For cells activated with SEE-loaded APC, translocation dynamics were monitored by time-lapse microscopy (see Fig. 3B).

Figure S3. GFP-DGKζ translocates to the PM following T cell/APC presentation and MARCKS domain phosphorylation is required. (A) Structure and graphic representation of DGKζ fused to GFP and the mutants used in assays. (B) Jurkat T cells were transiently transfected with the fusion protein GFP-DGKζ WT or mutated in the MARCKS domain (SD or SA, SA-ΔAnk). At maximal construct expression, T cells were collected in HBSS/2% FBS and DGKζ translocation was followed by time-lapse fluorescence microscopy after stimulation with Raji B cells alone or pulsed with 1 μg/ml SEE (*). After 15 min incubation with APC, live images were captured on an Olympus confocal microscope. Representative images are shown. Bar = 5 μm. Truncation of the C-terminal region reverses the negative restriction caused by Ser-to-Ala mutation, and the SA-ΔAnk mutant translocated to the PM in response to SEE presentation. (C) GFP-DGKζ

(green) was transiently transfected into Jurkat T cells and translocation tracked in live cells presented to SEE-pulsed Raji B cells (blue). When stated, Jurkat T cells were preincubated with 50 or 100 nM BIM or 6 μM rottlerin. Representative images are shown (left); graph showing subcellular distribution (right); PM: plasma membrane, V: vesicular, C: cytosol, C/V: cytosol and vesicular, PM/V: plasma membrane and vesicular.

Figure S4. Co-expression of Cherry-C1ab and different DGK constructs in basal condition, prior to antigen presentation. Jurkat T cells were transfected with the tandem C1 domain of PKCθ fused to Cherry (Cherry-C1ab), alone or cotransfected with GFP-tagged constructs of DGKα or ζ wild type (WT) or mutated. Representative images are shown prior to antigen presentation. Bar = 5 μm.

Supplemental Movie legends

Movie 1. Time-lapse videomicroscopy of GFP-DGKζ translocation after antigen presentation by SEE-pulsed B cells.

Movie 2 (a, b): Time-lapse videomicroscopy of GFP-DGKζ and actin-cherry translocation after antigen presentation by SEE-pulsed B cells.

Movie 3. Time-lapse videomicroscopy of the PKCθ C1 tandem domain (C1ab) fused to the cherry fluorescent protein (Cherry C1ab). DAG pool formation (red) and accumulation at the IS after antigen presentation by SEE-pulsed B cells.

Movie 4. Time-lapse videomicroscopy of Jurkat T cells cotransfected with Cherry-C1ab (DAG pool, red) and GFP-DGKζ (green) after antigen presentation by SEE-pulsed B cells.

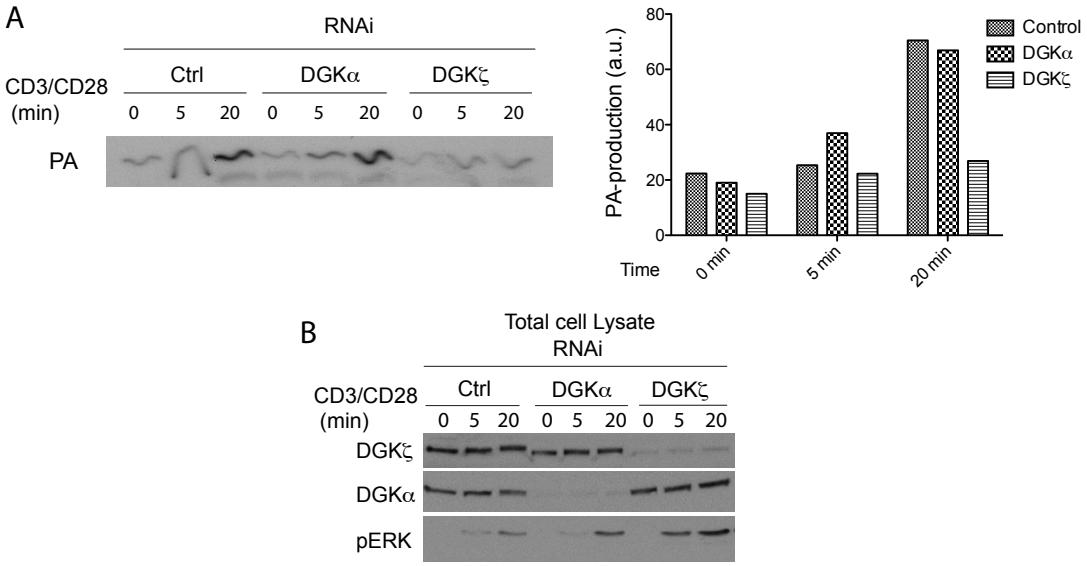
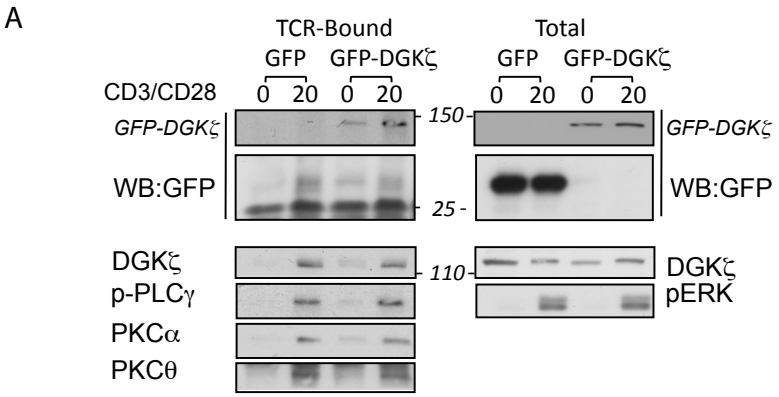

A
RNAi
CD3/CD28
(min)
Ctrl
DGKα
DGKζ
0 5 20 0 5 20 0 5 20
PA
PA-production (a.u.)
80
60
40
20
0
Time
0 min
5 min
20 min
Control
DGKα
DGKζ
B
Total cell Lysate
RNAi
CD3/CD28
(min)
Ctrl
DGKα
DGKζ
0 5 20 0 5 20 0 5 20
DGKζ
DGKα
pERK

Figure S1


A
TCR-Bound
Total
GFP
GFP-DGKζ
CD3/CD28
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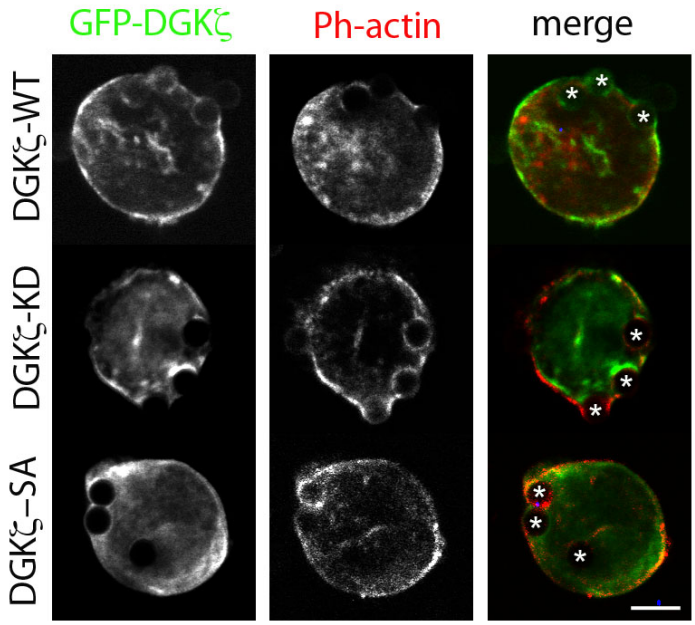
150
25
110
GFP-DGKζ
WB:GFP
DGKζ
p-PLCγ
PKCα
PKCθ
pERK


B
GFP-DGKζ
Ph-actin
merge
DGKζ-WT
DGKζ-KD
DGKζ–SA

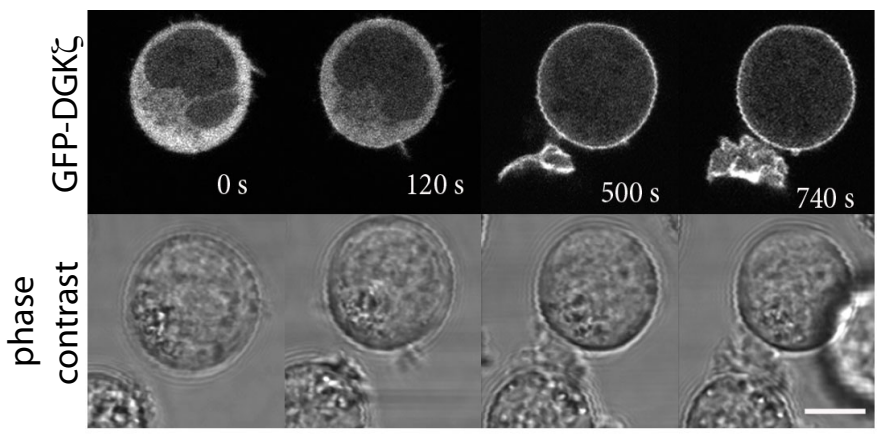

C
GFP-DGKζ
0 s
120 s
500 s
740 s
phase contrast

Figure S2

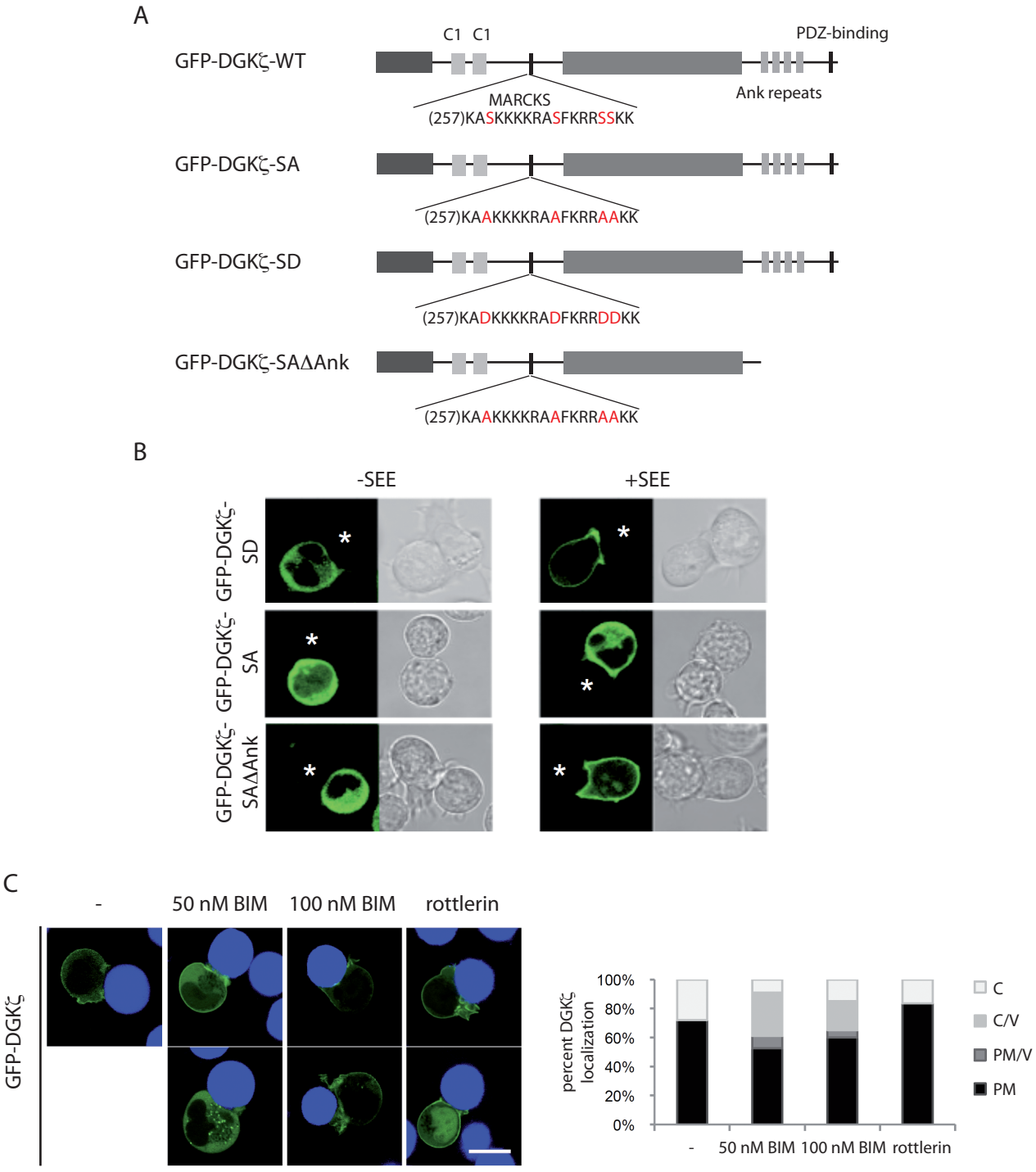

A
C1 C1
PDZ-binding
GFP-DGKζ-WT
MARCKS
(257)KASKKKKRASFKRRSSKK
Ank repeats
GFP-DGKζ-SA
(257)KAAKKKKRAAFKRRAAKK
GFP-DGKζ-SD
(257)KADKKKKRADFKRRDDKK
GFP-DGKζ-SAΔAnk
(257)KAAKKKKRAAFKRRAAKK
B
-SEE
+SEE
GFP-DGKζ-SD
GFP-DGKζ-SA
GFP-DGKζ-SAΔAnk
C
-
50 nM BIM
100 nM BIM
rottlerin
GFP-DGKζ
percent DGKζ localization
100%
80%
60%
40%
20%
0%
C
C/V
PM/V
PM

Figure S3

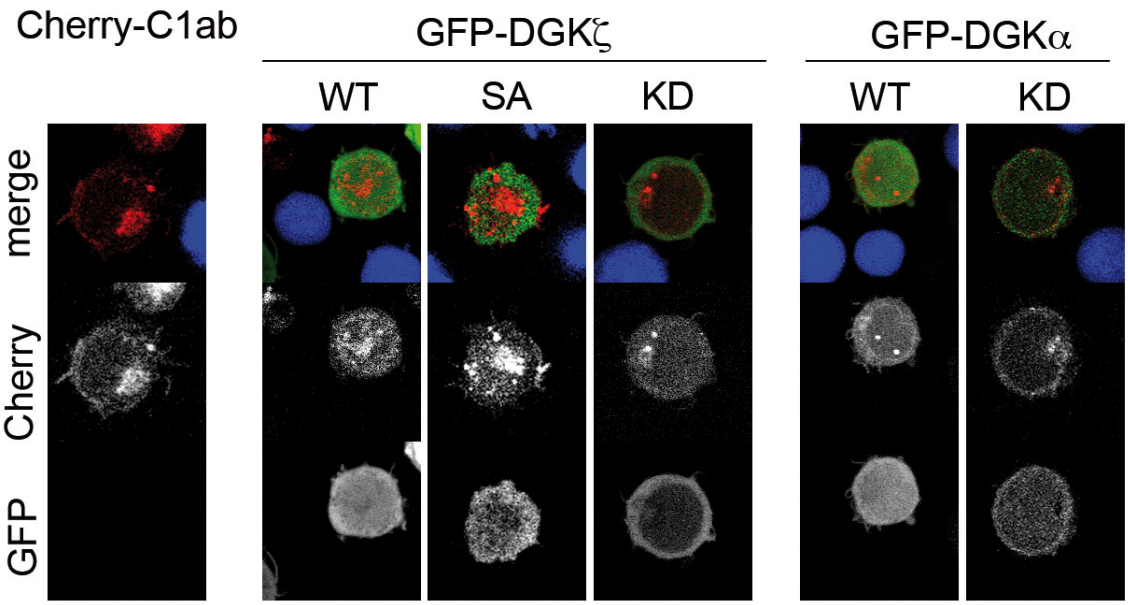


Figure S4