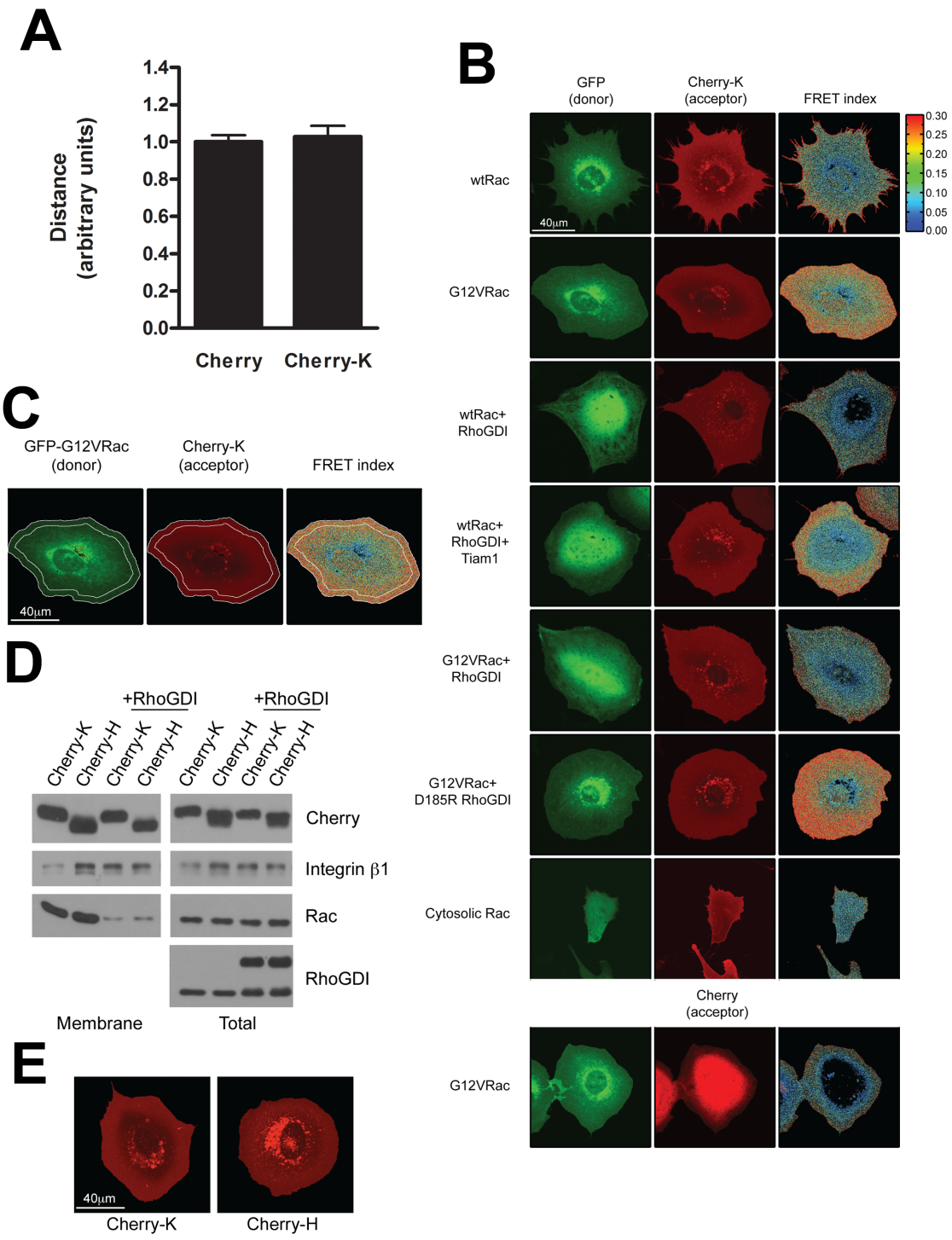

A
Distance (arbitrary units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cherry
Cherry-K
B
GFP (donor)
Cherry-K (acceptor)
FRET index
0.30
0.25
0.20
0.15
0.10
0.05
0.00
wtRac
40μm
G12VRac
wtRac+ RhoGDI
wtRac+ RhoGDI+ Tiam1
G12VRac+ RhoGDI
G12VRac+ D185R RhoGDI
Cytosolic Rac
Cherry (acceptor)
G12VRac
C
GFP-G12VRac (donor)
Cherry-K (acceptor)
FRET index
40μm
D
+RhoGDI
+RhoGDI
Cherry-K
Cherry-H
Cherry-K
Cherry-H
Cherry-K
Cherry-H
Cherry-K
Cherry-H
Cherry
Integrin β1
Rac
RhoGDI
Membrane
Total
E
40μm
Cherry-K
Cherry-H

Fig. S1. Development of a FRET biosensor for Rac membrane targeting

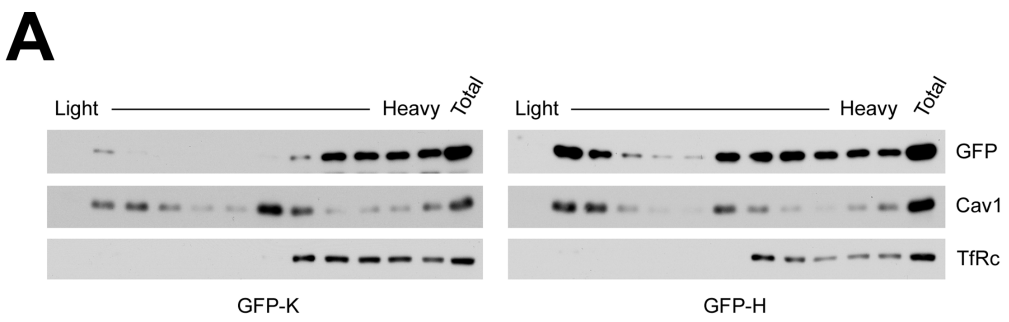
(**A**) Random migration assay of mouse embryo fibroblasts expressing Cherry (n=96) or Cherry-K (n=67). Values are mean distance migrated ± s.e.m.

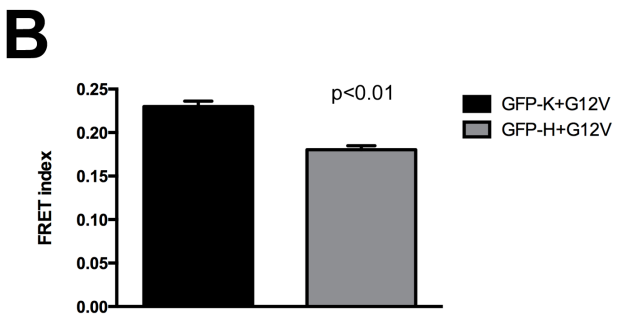
(**B**) Representative images of donor (GFP), acceptor (Cherry-K or Cherry) and FRET index of cells expressing the indicated constructs. Saturated pixels in the donor or acceptor images were artificially set to 0 in the FRET index images.

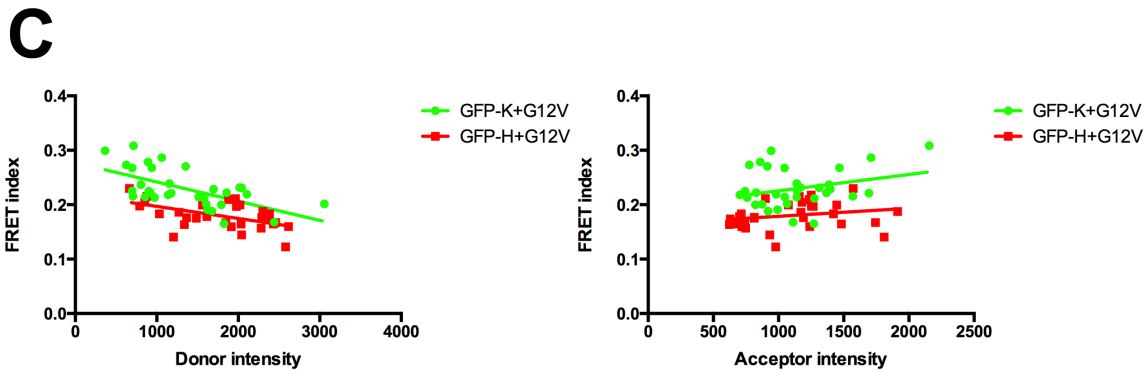
(**C**) Example of the automated cell edge identification (for details see Materials and Methods). A 4-6 μm-wide cell edge was identified using the acceptor (Cherry-K) image. This area was used to create a mask (area between the white lines) that was applied to the corresponding donor and FRET index images.

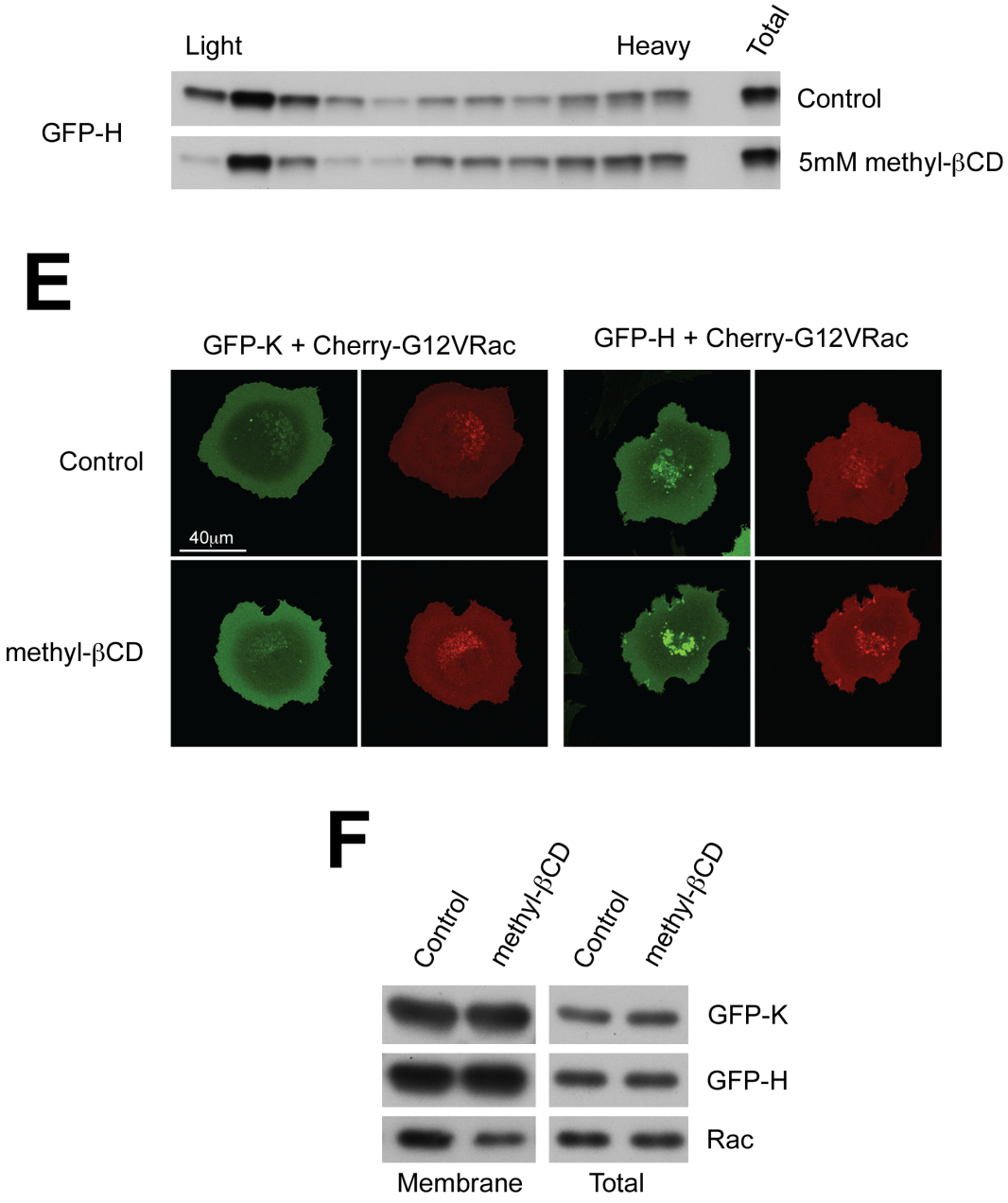
(**D**) Test for the effect of RhoGDI over-expression on the partitioning of Cherry-K and Cherry-H FRET acceptors. NIH3T3 cells expressing the indicated constructs were subjected to sub-cellular fractionation. Particulate fractions and post-nuclear material (total) were immunoblotted for Cherry, integrin β1, Rac and RhoGDI. Membrane localization of Cherry-K and Cherry-H is resistant to RhoGDI over-expression, whereas Rac is depleted from the membrane fraction.

(E) Representative images of cells expressing Cherry-K or Cherry-H.


A
Light
Heavy
Total
Light
Heavy
Total
GFP
Cav1
TfRc
GFP-K
GFP-H


B
p<0.01
GFP-K+G12V
GFP-H+G12V
FRET index
0.25
0.20
0.15
0.10
0.05
0.00


C
GFP-K+G12V
GFP-H+G12V
FRET index
Donor intensity
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
GFP-K+G12V
GFP-H+G12V
FRET index
Acceptor intensity
0.4
0.3
0.2
0.1
0.0
0
500
1000
1500
2000
2500


D
Light
Heavy
Total
GFP-H
Control
5mM methyl-βCD
E
GFP-K + Cherry-G12VRac
GFP-H + Cherry-G12VRac
Control
methyl-βCD
40μm
F
Control
methyl-βCD
Control
methyl-βCD
GFP-K
GFP-H
Rac
Membrane
Total

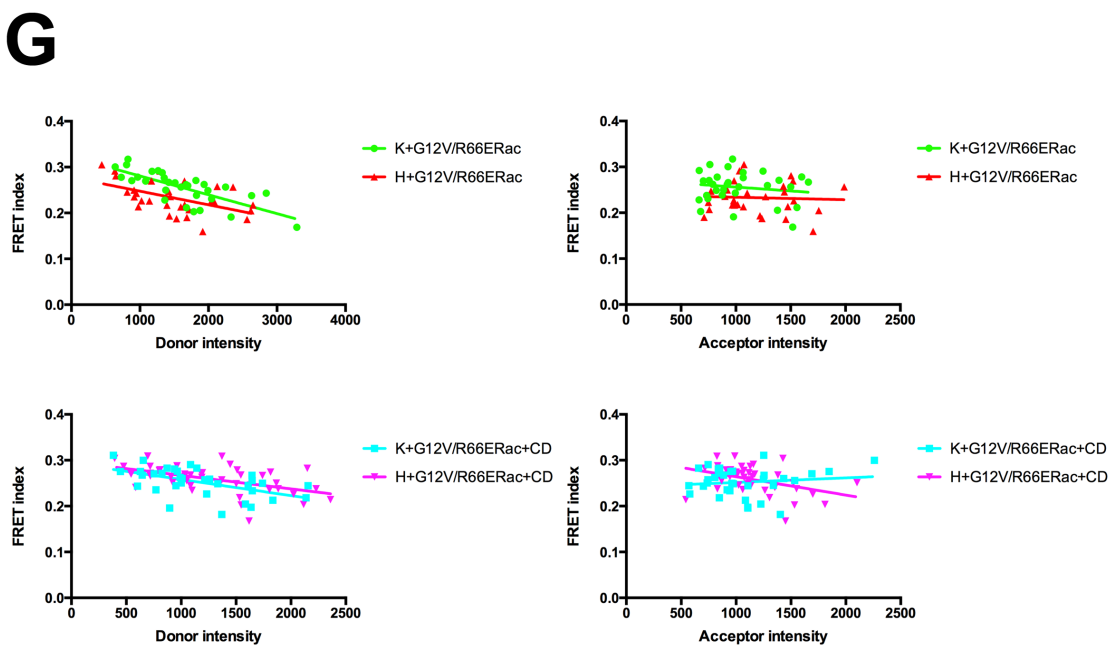


Fig. S2. Rac domain localization in cells

(**A**) NIH3T3 cells expressing GFP-K or GFP-H were extracted with 0.05% TX-100 and extracts separated on sucrose gradients. Fractions were immunoblotted for GFP, caveolin1 and transferrin receptor. Results are representative of three independent experiments.

(**B**) FRET index of cells expressing the indicated constructs. Values are means ± s.e.m. n=32 cells in each group.

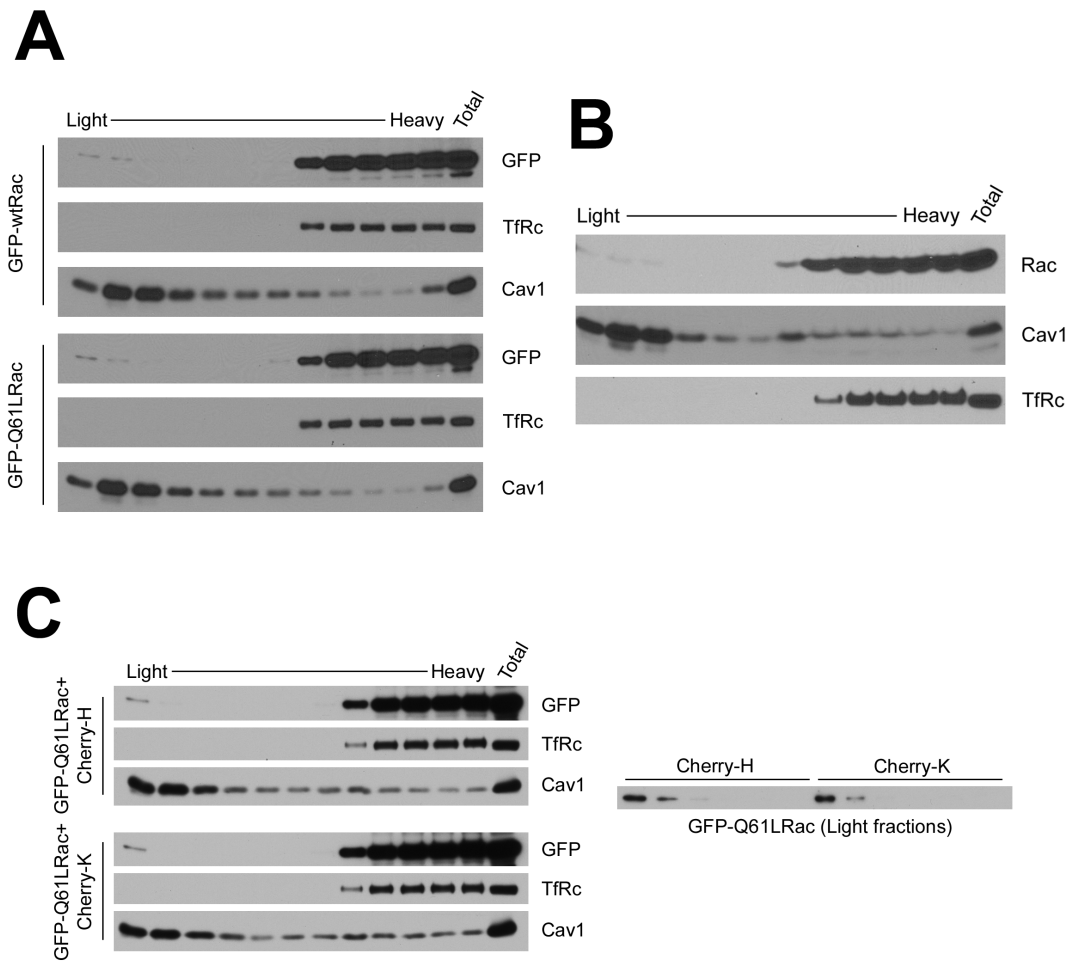
(C) Scatter plots of FRET index relative to donor (left) or acceptor intensity (right) in cells expressing the indicated constructs. Lines represent linear regression of the corresponding data points.

(D) Representative immunoblot of the distribution of GFP-H in sucrose density fractions in control or methyl-β cyclodextrin-treated cells.

(**E**) Examples of control and methyl-β cyclodextrin-treated cells expressing the indicated constructs.

(**F**) Representative immunoblot of the distribution of GFP-K, GFP-H and endogenous Rac in the particulate fraction (Membrane) and post-nuclear material (Total) in control or methyl-β cyclodextrin-treated cells.

(G) Scatter plots of FRET index relative to donor (left) or acceptor intensity (right) in cells expressing the indicated constructs with (bottom) or without 5mM β-methyl cyclodextrin (top), described in Fig. 2D. Lines represent linear regression of the corresponding data points.


A
Light
Heavy
Total
GFP-wtRac
GFP
TfRc
Cav1
GFP-Q61LRac
GFP
TfRc
Cav1
B
Light
Heavy
Total
Rac
Cav1
TfRc
C
Light
Heavy
Total
GFP-Q61LRac+ Cherry-H
GFP
TfRc
Cav1
GFP-Q61LRac+ Cherry-K
GFP
TfRc
Cav1
Cherry-H
Cherry-K
GFP-Q61LRac (Light fractions)

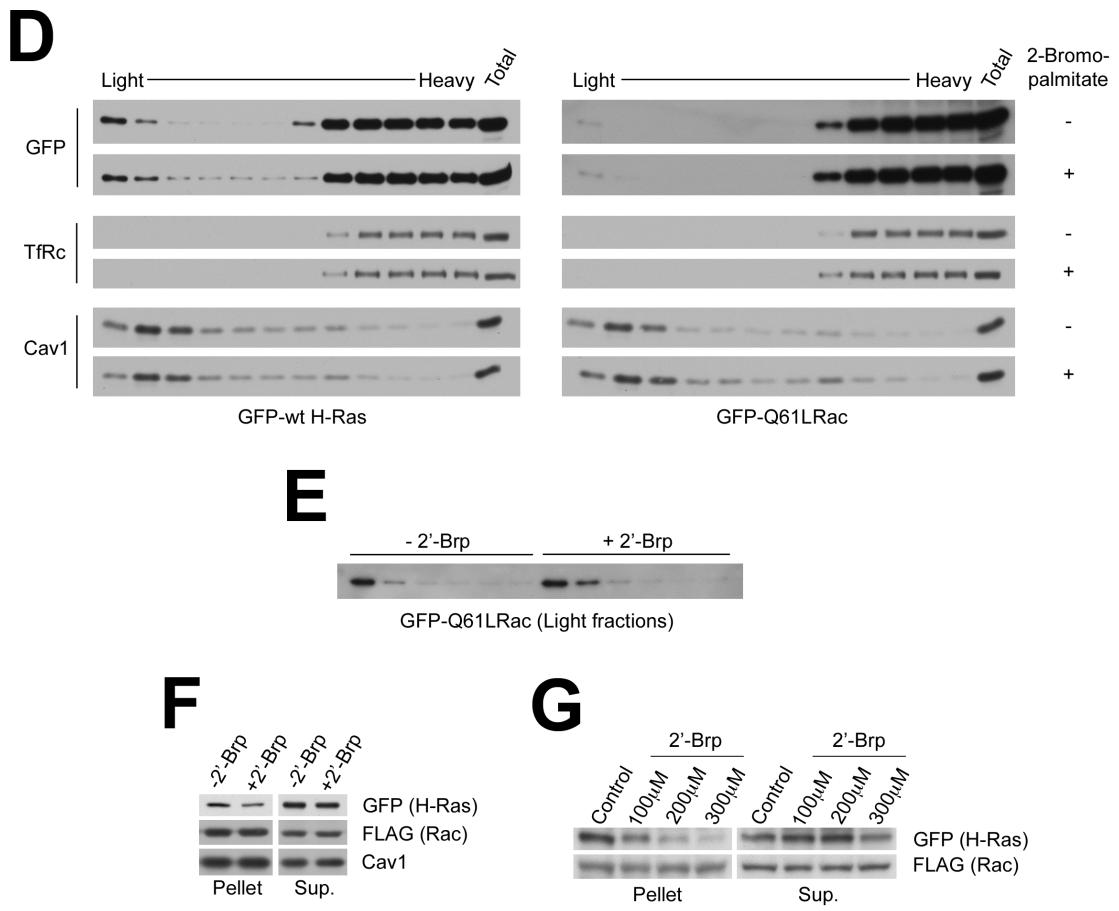


Fig. S3. Distribution of Rac on density gradients

(**A**) NIH3T3 cells expressing GFP-wtRac or GFP-Q61LRac were extracted with 0.1% TX-100 and extracts separated on sucrose gradients. Fractions were immunoblotted for GFP, transferrin receptor and caveolin1. Results are representative of three independent experiments.

(**B**) Distribution of endogenous Rac on density gradients. Membranes of NIH3T3 cells were isolated following non-detergent cell lysis, then solubilized with 0.1% TX-100 and separated on sucrose gradients. Fractions were immunoblotted for Rac, caveolin1 and transferrin receptor.

(**C**) NIH3T3 cells expressing GFP-Q61LRac together with Cherry-H or Cherry-K were analyzed as in A. Similar results were obtained in two experiments. Right panel shows the first six light fractions using longer exposure.

(**D**) Effect of inhibiting palmitoylation. NIH3T3 cells expressing GFP-wt H-Ras (left) or GFP-Q61LRac (right) were treated with 100μM 2'-bromopalmitate or DMSO for 1hour, and 0.1% TX-100 extracts separated on sucrose gradients. Fractions were immunoblotted for GFP, transferrin receptor and caveolin1. Similar results were obtained in two experiments.

(**E**) The first six light fractions of the GFP-Q61LRac distribution in D are shown using a longer exposure.

(**F, G**) NIH3T3 cells co-expressing GFP-wt H-Ras and FLAG-Cherry-Q61LRac were treated with 100μM 2'-bromopalmitate or DMSO for 1hour (F) or the indicated doses for 16hours (G) and extracted with 0.1% TX-100. Triton-resistant pellets and supernatants (Sup.) were analyzed by immunoblotting for GFP, FLAG and Cav1. Similar results were obtained in two experiments.

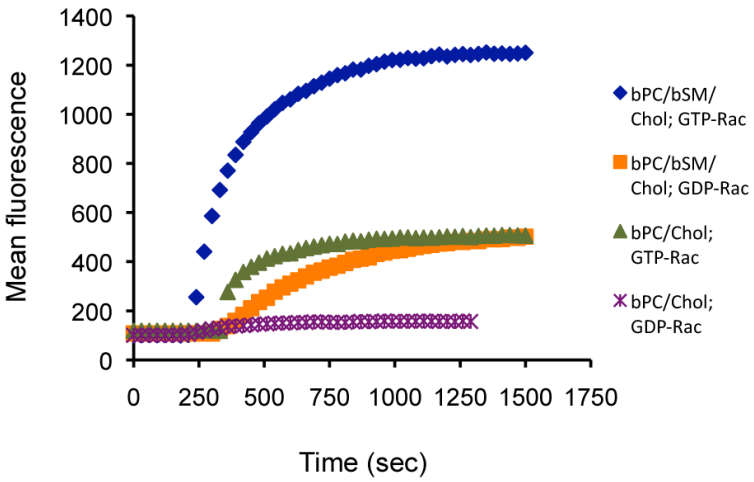


Fig. S4. Binding of GFP-Rac to supported lipid bilayers

Representative binding curves of GFP-Rac, loaded with GTP or GDP, to supported bilayers witrh the indicated lipid compositions. Rac was added at 210 seconds and the mean fluorescence of the entire observed membrane area was recorded.