Supplemental Materials

Molecular Biology of the Cell

Li *et al*.

SUPPLEMENTARY FIGURE LEGEND

Figure S1. Further characterizations of Grp1 recruitment.

A. Schematic of the previously proposed auto-inhibitory mechanism, and its release by the T280 phosphorylation.

B. Phosphorylation of full-length Grp1 by Akt reduces Grp1 recruitment to PIP3-containing liposomes. Grp1 constructs as indicated were incubated with PIP3-containing liposomes, followed by centrifugation to detect distribution in the pellet (P) versus supernatant (S). A representative result is shown. Quantitation is shown as mean with standard error from 3 experiments, * $p < 0.05$, Student's t-test.

C. Structure of the phosphoinositide binding pocket of the PH domain in Grp1. The structure has been previously described (PDB ID:1FGY). Key residues predicted to interact with the head group of PIP3 (shown as IP4) are highlighted. The position of the T280 residue is also shown.

D. Mutating key residues in the binding pocket of the PH domain reduces the localization of the Grp1-T280D at the recycling endosome. Adipocytes were transfected with Grp1 constructs as indicated, and then the colocalization of these constructs with internal glut4 (a marker of the recycling endosome in adipocytes) was assessed by confocal microcopy. A representative image is shown with inset providing magnified view; Grp1 constructs (red), internal glut4 (green); bar = 10 um.

Figure S2. PI4P is enriched at the recycling endosome in adipocytes.

A. Colocalization of PI4P (red) with different markers of the recycling endosome (green), as indicated, was assessed by confocal microcopy. Representative image is shown with inset providing magnified view; bar = 10 um.

B. Colocalization of PIP3 (red) with different markers of the recycling endosome (green), as indicated, was assessed by confocal microcopy. Representative image is shown with inset providing magnified view; bar = 10 um.

C. Colocalization of a PI4P biosensor (green) with internal glut4 (red) in adipocytes was assessed by confocal microcopy. Representative image is shown on left with inset providing magnified view; bar = 10 um. Quantitation is shown on right; n=10 cells examined for each experiment with 3 experiments performed.

D. Effect of wortmannin on the localization of Grp1-T280D at the recycling endosome. Adipocytes were treated with different concentrations of wortmannin as indicated, and then colocalization of Grp1-T280D (red) with internal glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

E. Effect of other inhibitors of PI4K activity on the localization of Grp1-T280D at the recycling endosome. Adipocytes were treated with different inhibitors as indicated, and then colocalization of Grp1-T280D (red) with internal glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

Figure S3. Recruitment of Grp1 to the recycling endosome depends on PI4KIIIα.

Quantitative results are shown as mean with standard error, * $p < 0.05$, Student's t-test. The number of independent experiments performed are specified below.

A. Efficiency of knocking down PI4KIIIα. Cells were treated as indicated followed by immunoblotting of whole cell lysates for proteins as indicated. A representative result along with quantitation from 3 experiments is shown.

B. Knocking down PI4KIIIα reduces the colocalization of PI4P with Rab11. Adipocytes were treated as indicated, and then the colocalization of PI4P (red) with Rab11 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

C. Knocking down PI4KIIIα reduces the colocalization of PI4P with cellubrevin. Adipocytes were treated as indicated, and then the colocalization of PI4P (red) with cellubrevin (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

D. Knocking down PI4KIIIα reduces the colocalization of PI4P with internal glut4. Adipocytes were treated as indicated, and then the colocalization of PI4P (red) with glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

E. Knocking down PI4KIIIα reduces the colocalization of Grp1-T280D with Rab11. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with Rab11 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

F. Knocking down PI4KIIIα reduces the colocalization of Grp1-T280D with cellubrevin. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with cellubrevin (green) was assessed using confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

G. Knocking down PI4KIIIα reduces the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with

glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

H. Three different siRNA sequences against PI4KIIIα were similarly effective in reducing the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated with different siRNA sequences against PI4KIIIα, and then the colocalization of Grp1-T280D with internal glut4 was quantified; n=10 cells per experiment with 3 independent experiments performed, * $p < 0.05$, Student's t-test. The sequences of the siRNAs are detailed in the Methods section.

Figure S4. Recruitment of Grp1 to the recycling endosome also depends on GRASP/IPCEF1.

A. Efficiency of knocking down ARF6. Cells were treated as indicated followed by immunoblotting of whole cell lysates. A representative result is shown. Quantitation is shown as mean with standard error from 3 experiments, * $p < 0.05$, Student's t-test.

B. Knocking down ARF6 does not affect the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with internal glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

C. Knocking down GRASP or IPCEF1, but not CASP or GRSP1, reduces the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with internal glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

D. Colocalization of endogenous GRASP or IPCEF1 with cellubrevin was assessed by confocal microscopy. Representative images are shown with inset providing magnified view; GRASP/IPCEF1 (red), cellubrevin (green); bar = 10 um.

E. Colocalization of endogenous GRASP or IPCEF1 with internal glut4 was assessed by confocal microscopy. Representative images are shown with inset providing magnified view; GRASP/IPCEF1 (red), glut4 (green); bar = 10 um.

F. Knocking down the combination of GRASP, IPCEF1, and PI4KIIIα virtually abolishes the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated as indicated, and then the colocalization of Grp1-T280D (red) with internal glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

Figure S5. Efficiency of knocking down cytohesin interactors.

Quantitative results are shown as mean with standard error from 3 independent experiments, * $p < 0.05$, Student's t-test.

A. Three different siRNA sequences against GRASP were similarly effective in reducing the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated with different siRNA sequences against GRASP, and then the colocalization of Grp1-T280D with internal glut4 was quantified; n=10 cells per experiment. The sequences of the siRNAs are detailed in the Methods section.

B. Three different siRNA sequences against IPCEF1 were similarly effective in reducing the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated with different siRNA sequences against IPCEF1, and then the colocalization of Grp1-T280D with internal

glut4 was quantified; n=10 cells per experiment. The sequences of the siRNAs are detailed in the Methods section.

C. Three different siRNA sequences against GRSP were similarly ineffective in reducing the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated with different siRNA sequences against GRSP, and then the colocalization of Grp1-T280D with internal glut4 was quantified; n=10 cells per experiment. The sequences of the siRNAs are detailed in the Methods section.

D. Three different siRNA sequences against CASP were similarly ineffective in reducing the colocalization of Grp1-T280D with internal glut4. Adipocytes were treated with different siRNA sequences against CASP, and then the colocalization of Grp1-T280D with internal glut4 was quantified; n=10 cells per experiment. The sequences of the siRNAs are detailed in the Methods section.

Figure S6. Distinguishing lipid-based versus protein-based mechanism of Grp1 recruitment.

A. Schematic of different Grp1 constructs: FL (full-length), CC-Sec7 (truncation lacking the PH domain), Sec7-PH (truncation lacking the CC domain). Constructs that contain the T280D mutation are also indicated.

B. Localization of different Grp1 constructs in cells. Adipocytes were transfected with Grp1 constructs as indicated, and then the colocalization of these constructs with internal glut4 (marking the recycling endosome in adipocytes) was assessed by confocal microcopy. A representative image is shown with inset providing magnified view; Grp1 constructs (red), internal glut4 (green); bar = 10 um.

C. Effect of knocking down different factors on the colocalization of the Sec7-PH construct with internal glut4. Adipocytes were treated as indicated, and then the colocalization of the Sec-PH construct (red) with glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

D. Effect of knocking down different factors on the colocalization of the CC-Sec7 construct with internal glut4. Adipocytes were treated as indicated, and then the colocalization of the CC-Sec7 construct (red) with glut4 (green) was assessed by confocal microscopy. Representative image is shown with inset providing magnified view; bar = 10 um.

Figure S7. GRASP and IPCEF1 can interact directly.

Quantitative results are shown as mean with standard error from 3 independent experiments, * $p < 0.05$, Student's t-test.

A. Purified IPCEF1 as a GST fusion protein was bound to beads and then incubated with purified GRASP as a soluble in a pulldown experiment. A representative result along with quantitation is shown.

B. Purified GRASP as a GST fusion protein was bound to beads and then incubated with purified IPCEF1 as a soluble in a pulldown experiment. A representative result along with quantitation is shown.

Fig S1

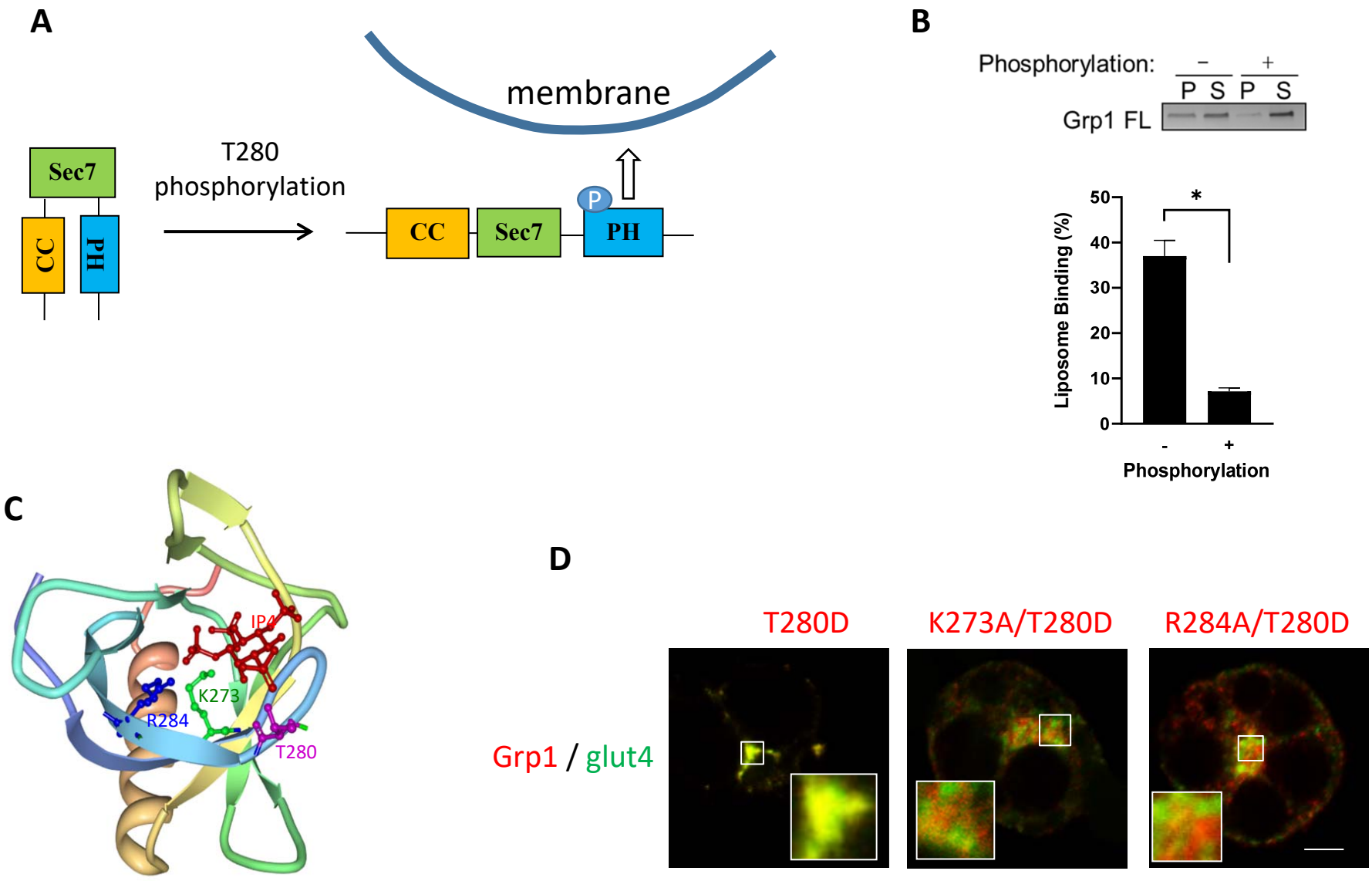
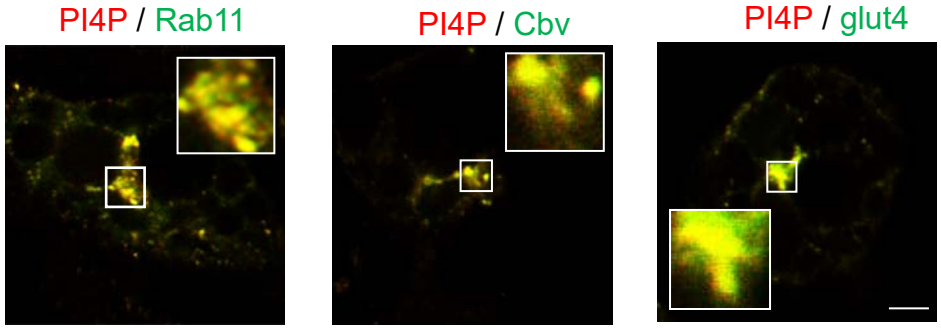
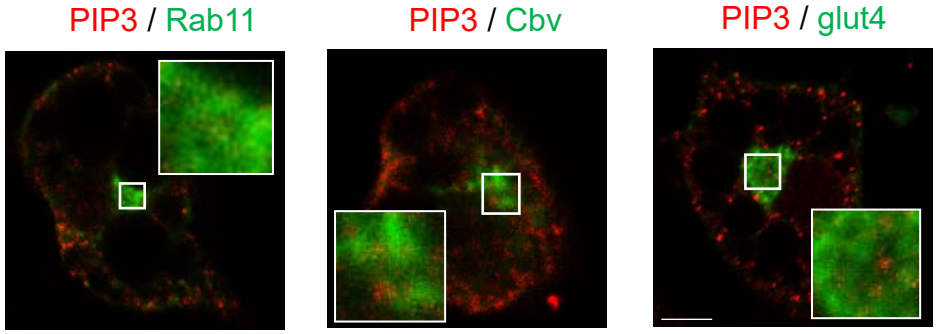

A
membrane
Sec7
CC
PH
T280
phosphorylation
CC
Sec7
P
PH
B
Phosphorylation:
−
+
P S P S
Grp1 FL
Liposome Binding (%)
50
40
30
20
10
0
*
-
+
Phosphorylation
C
IP4
K273
R284
T280
D
T280D
K273A/T280D
R284A/T280D
Grp1 / glut4

Fig S2

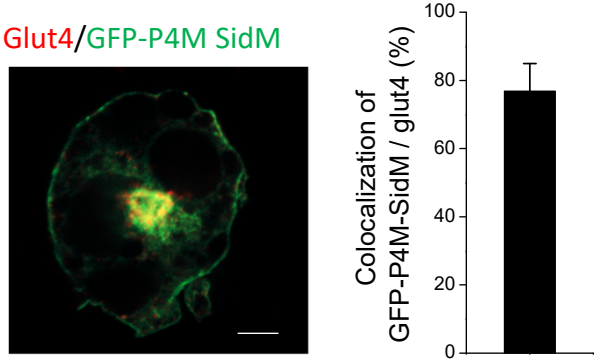
A


PI4P / Rab11
PI4P / Cbv
PI4P / glut4

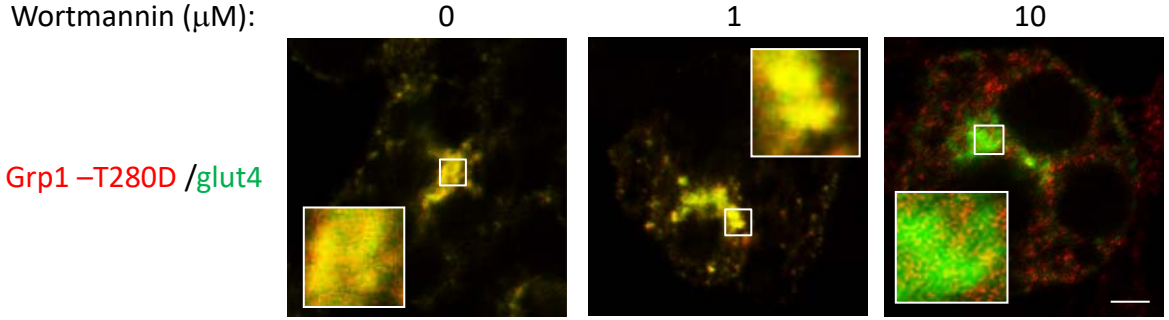
B


PIP3 / Rab11
PIP3 / Cbv
PIP3 / glut4

C


Glut4/GFP-P4M SidM
Colocalization of GFP-P4M-SidM / glut4 (%)
100
80
60
40
20
0

D


Wortmannin (μM):
0
1
10
Grp1 –T280D /glut4

E

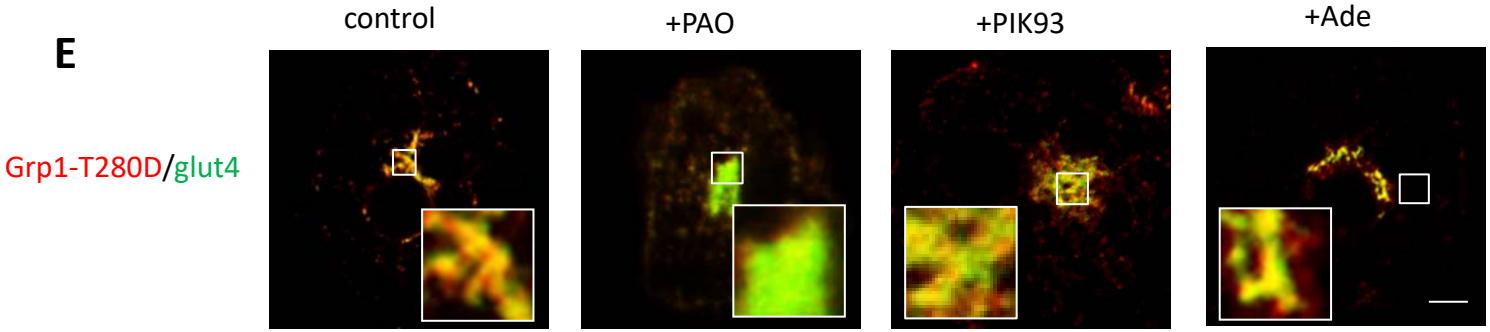

control
+PAO
+PIK93
+Ade
Grp1-T280D/glut4

Fig S3

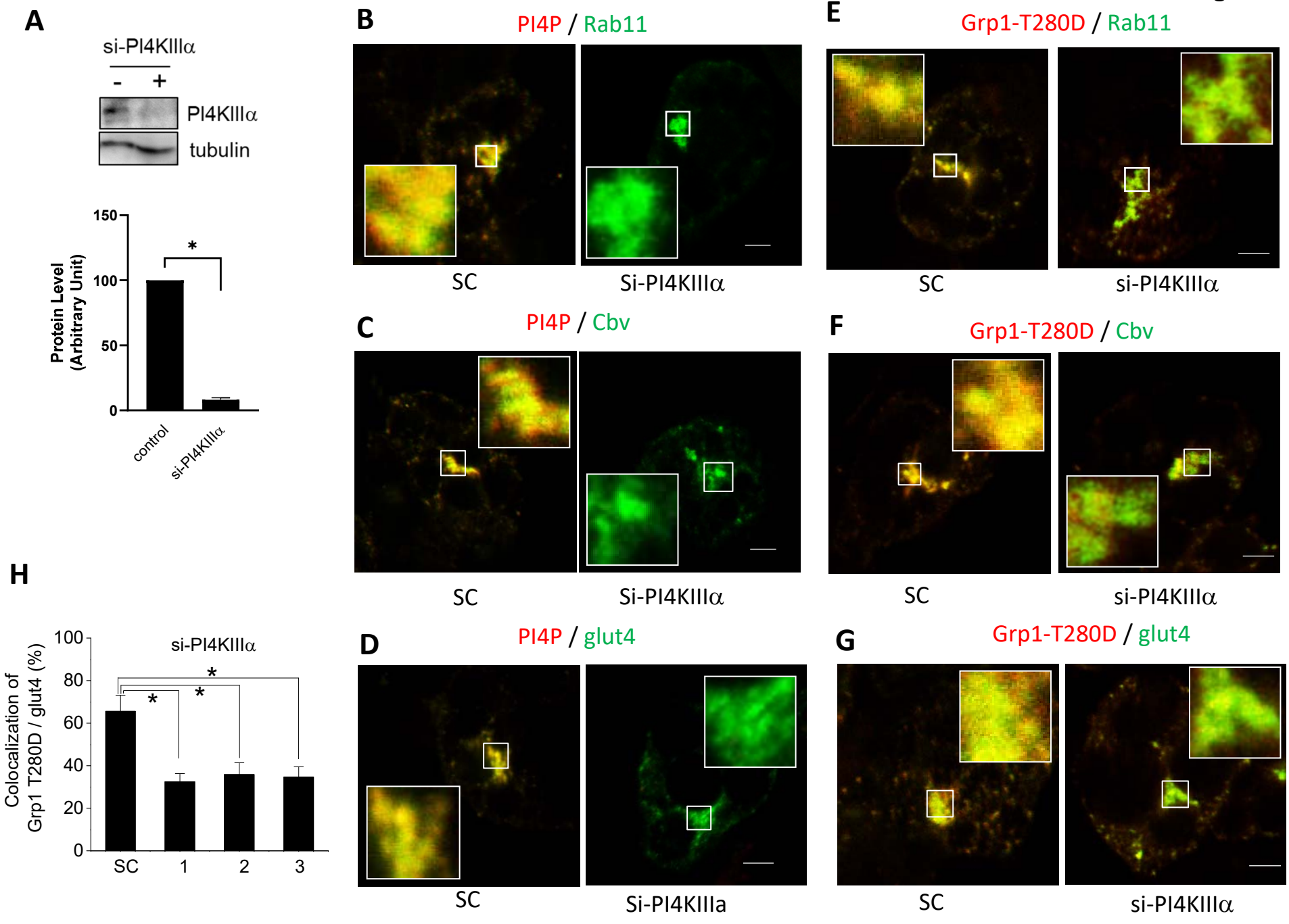

A
si-PI4KIIIα
- +
PI4KIIIα
tubulin
Protein Level (Arbitrary Unit)
150
100
50
0
*
control
si-PI4KIIIα
B
PI4P / Rab11
SC
Si-PI4KIIIα
C
PI4P / Cbv
SC
Si-PI4KIIIα
D
PI4P / glut4
SC
Si-PI4KIIIa
E
Grp1-T280D / Rab11
SC
si-PI4KIIIα
F
Grp1-T280D / Cbv
SC
si-PI4KIIIα
G
Grp1-T280D / glut4
SC
si-PI4KIIIα
H
si-PI4KIIIα
Colocalization of Grp1 T280D / glut4 (%)
100
80
60
40
20
0
*
*
*
SC
1
2
3

Fig S4

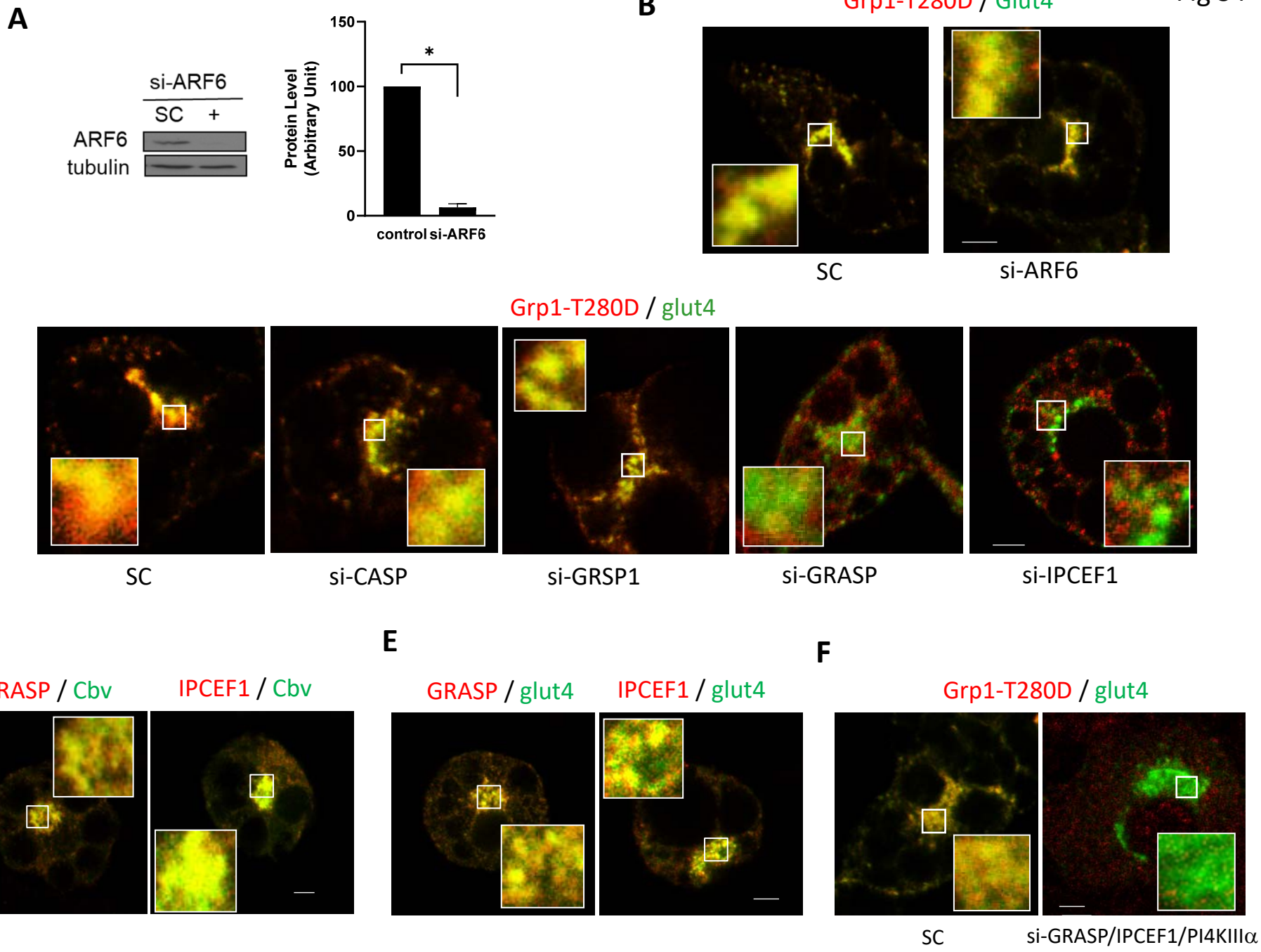

A
si-ARF6
SC +
ARF6
tubulin
Protein Level (Arbitrary Unit)
150
100
50
0
*
control si-ARF6
B
Grp1-T280D / Glut4
SC
si-ARF6
C
Grp1-T280D / glut4
SC
si-CASP
si-GRSP1
si-GRASP
si-IPCEF1
D
GRASP / Cbv
IPCEF1 / Cbv
E
GRASP / glut4
IPCEF1 / glut4
F
Grp1-T280D / glut4
SC
si-GRASP/IPCEF1/PI4KIIIα

Fig S5

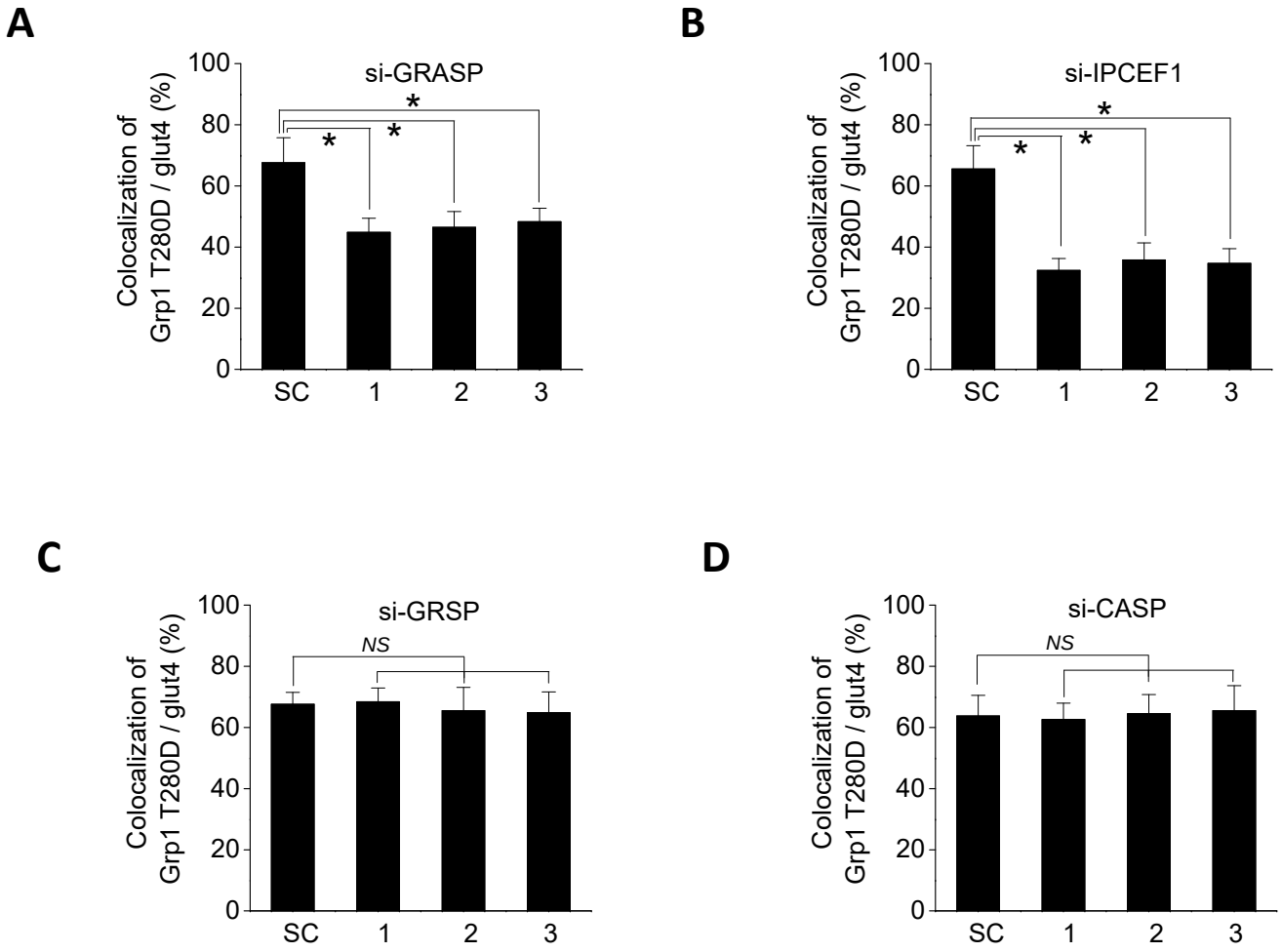
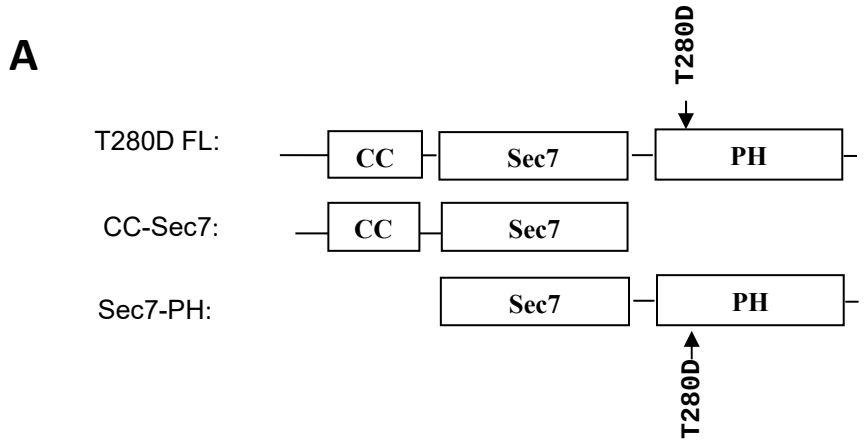
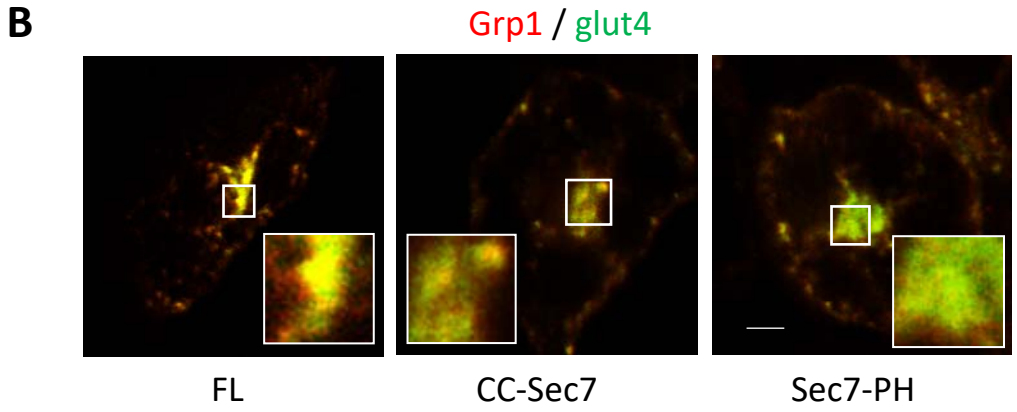
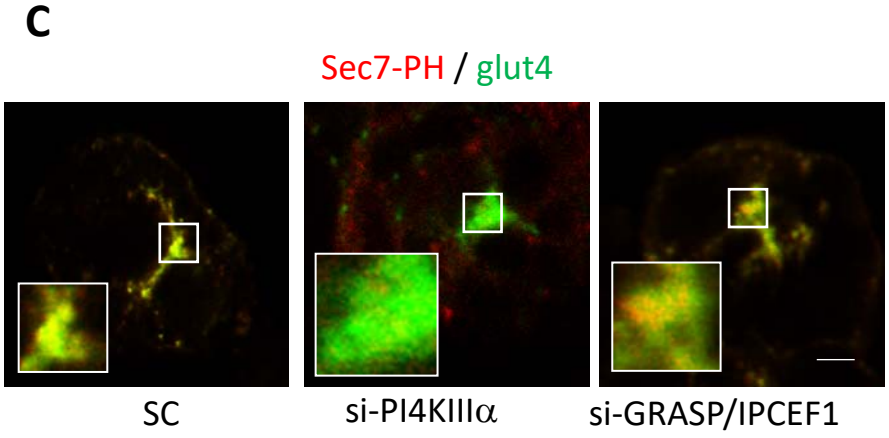

A
si-GRASP
Colocalization of Grp1 T280D / glut4 (%)
100
80
60
40
20
0
SC
1
2
3
*
*
*
B
si-IPCEF1
Colocalization of Grp1 T280D / glut4 (%)
*
*
*
C
si-GRSP
NS
Colocalization of Grp1 T280D / glut4 (%)
D
si-CASP
NS
Colocalization of Grp1 T280D / glut4 (%)

Fig S6


A
T280D
T280D FL:
CC
Sec7
PH
CC-Sec7:
CC
Sec7
Sec7-PH:
Sec7
PH
T280D


B
Grp1 / glut4
FL
CC-Sec7
Sec7-PH


C
Sec7-PH / glut4
SC
si-PI4KIIIα
si-GRASP/IPCEF1

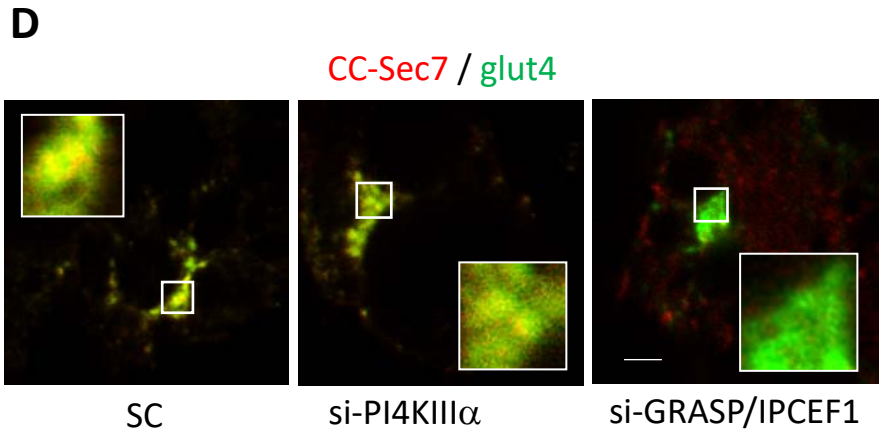
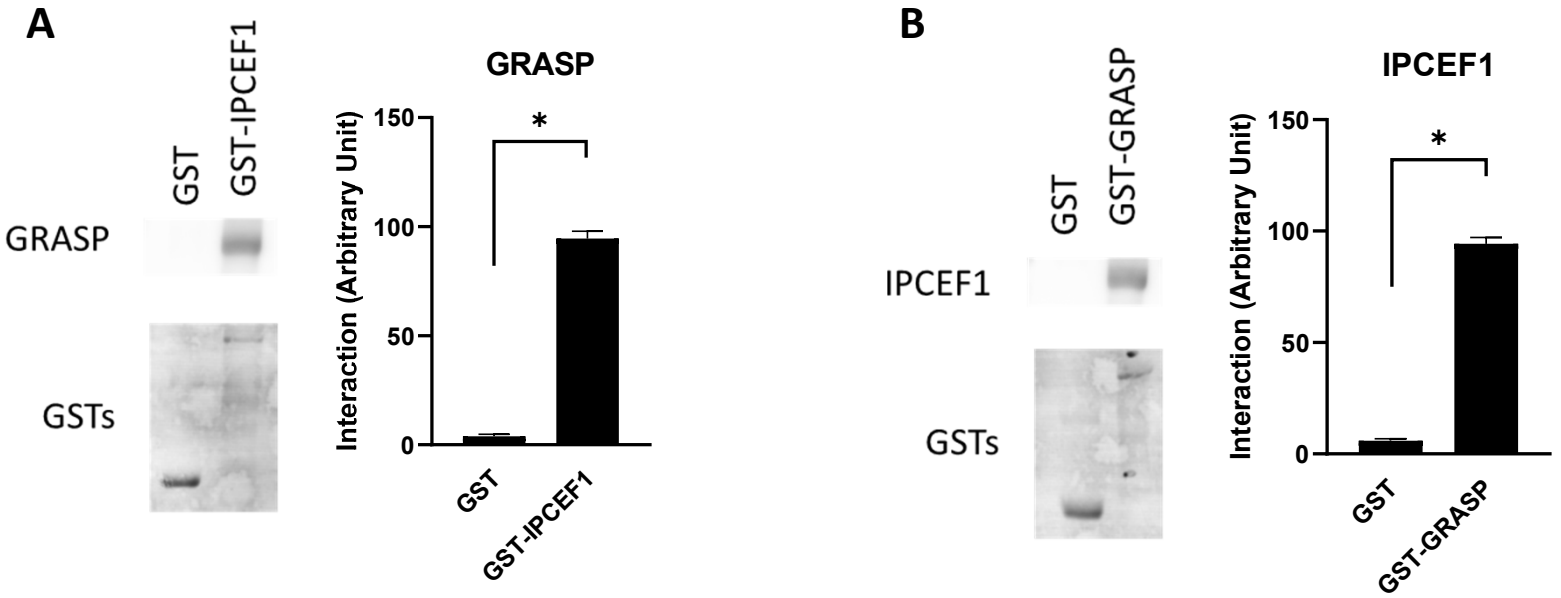

D
CC-Sec7 / glut4
SC
si-PI4KIIIα
si-GRASP/IPCEF1

Fig S7


A
GST
GST-IPCEF1
GRASP
GSTs
GRASP
Interaction (Arbitrary Unit)
150
100
50
0
*
GST
GST-IPCEF1
B
GST
GST-GRASP
IPCEF1
GSTs
IPCEF1
Interaction (Arbitrary Unit)
150
100
50
0
*
GST
GST-GRASP