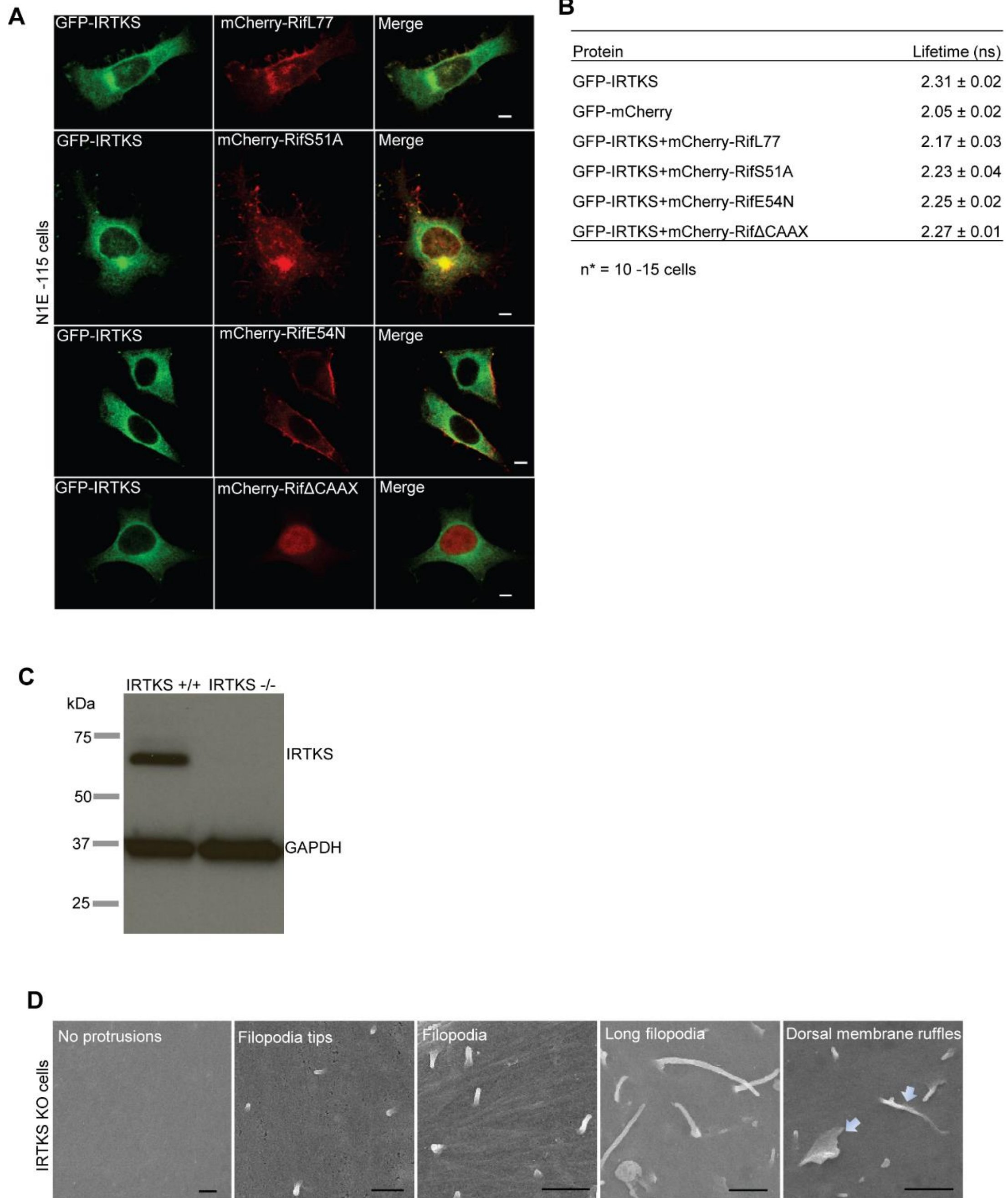
SUPPLEMENTARY FIGURES



Protein	Lifetime (ns)
GFP-IRTKS	2.31 ± 0.02
GFP-mCherry	2.05 ± 0.02
GFP-IRTKS+mCherry-RifL77	2.17 ± 0.03
GFP-IRTKS+mCherry-RifS51A	2.23 ± 0.04
GFP-IRTKS+mCherry-RifE54N	2.25 ± 0.02
GFP-IRTKS+mCherry-RifΔCAAX	2.27 ± 0.01

n* = 10 -15 cells

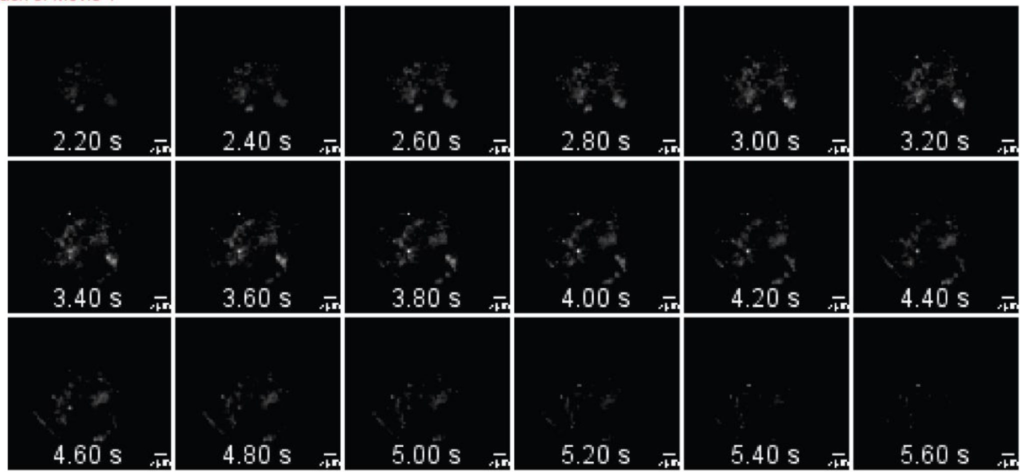
Figure S1. IRTKS Western blot and SEM image of observed structures. (A). N1E 115 cells were transfected with DNA encoding GFP-IRTKS and mCherry-RifL77 or Rif mutants, S51A or E54N or ΔCAAX deletion. Scale bar 5 µm. (B) Lifetime measurements were made of cells similar to those presented in (A) of GFP-IRTKS in the presence of mCherry-RifL77, or Rif S51A, or RifE54N, or RifΔCAAX mutant. The lifetime of GFP-IRTKS and GFP-mCherry controls for minimum and maximum FRET, respectively, are also presented. (C) Western blot of wildtype MEF (left lane) and IRTKS KO MEF cells (right lane). Total protein lysates were prepared from MEFs at E12.5. Western blot was performed with rabbit polyclonal anti-IRTKS and HRP-conjugated goat anti-rabbit IgG. (D) SEM analysis of cell morphologies. SEM images of structures scored in Fig 2, 3 and 4, (left to right) background morphology (no protrusions), filopodia tips, filopodia, long filopodia and dorsal membrane ruffles (arrowhead). Scales bar, 1µm.

A

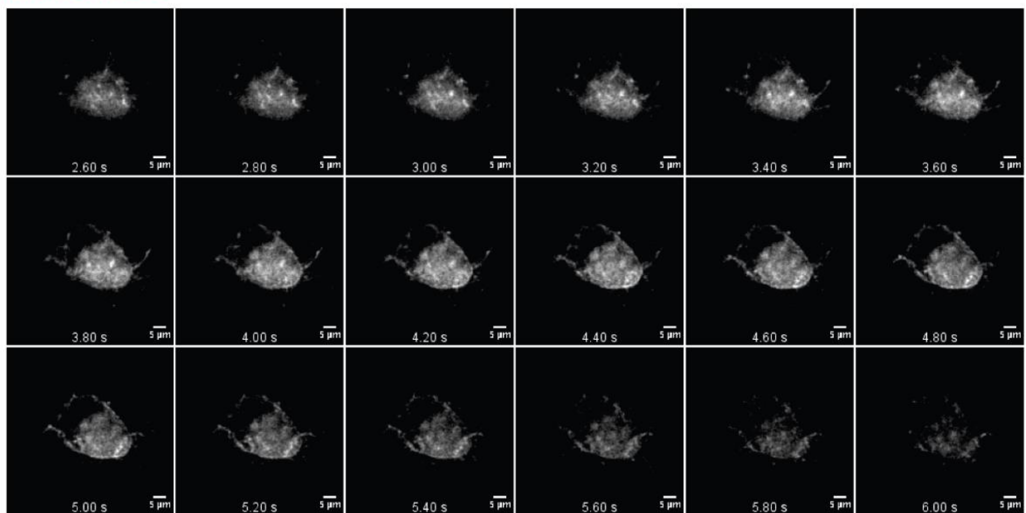
Movie 1

Movie 2

Selected stack of Movie 1



Selected stack of Movie 2



Journal of Cell Science • Supplementary information

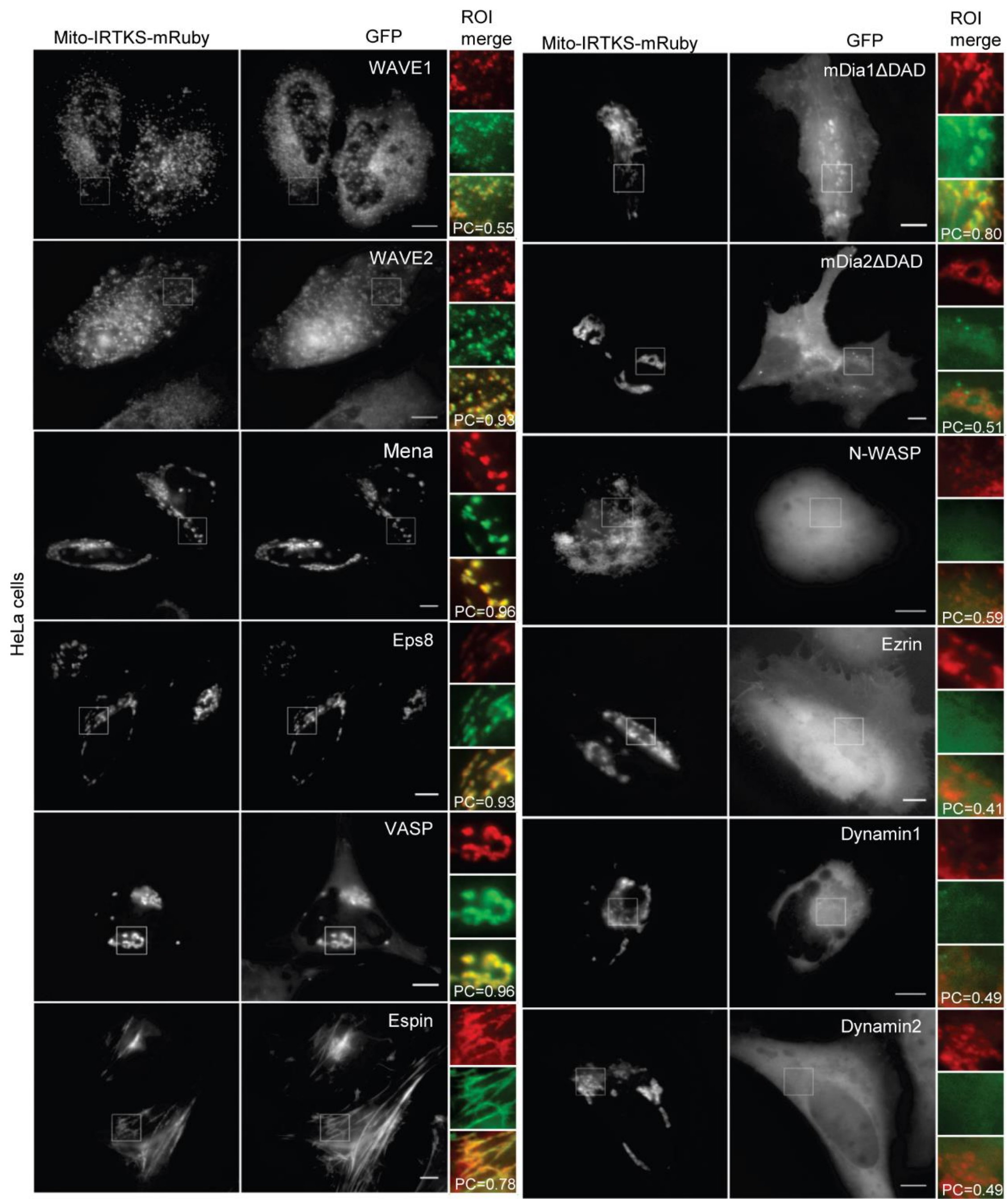
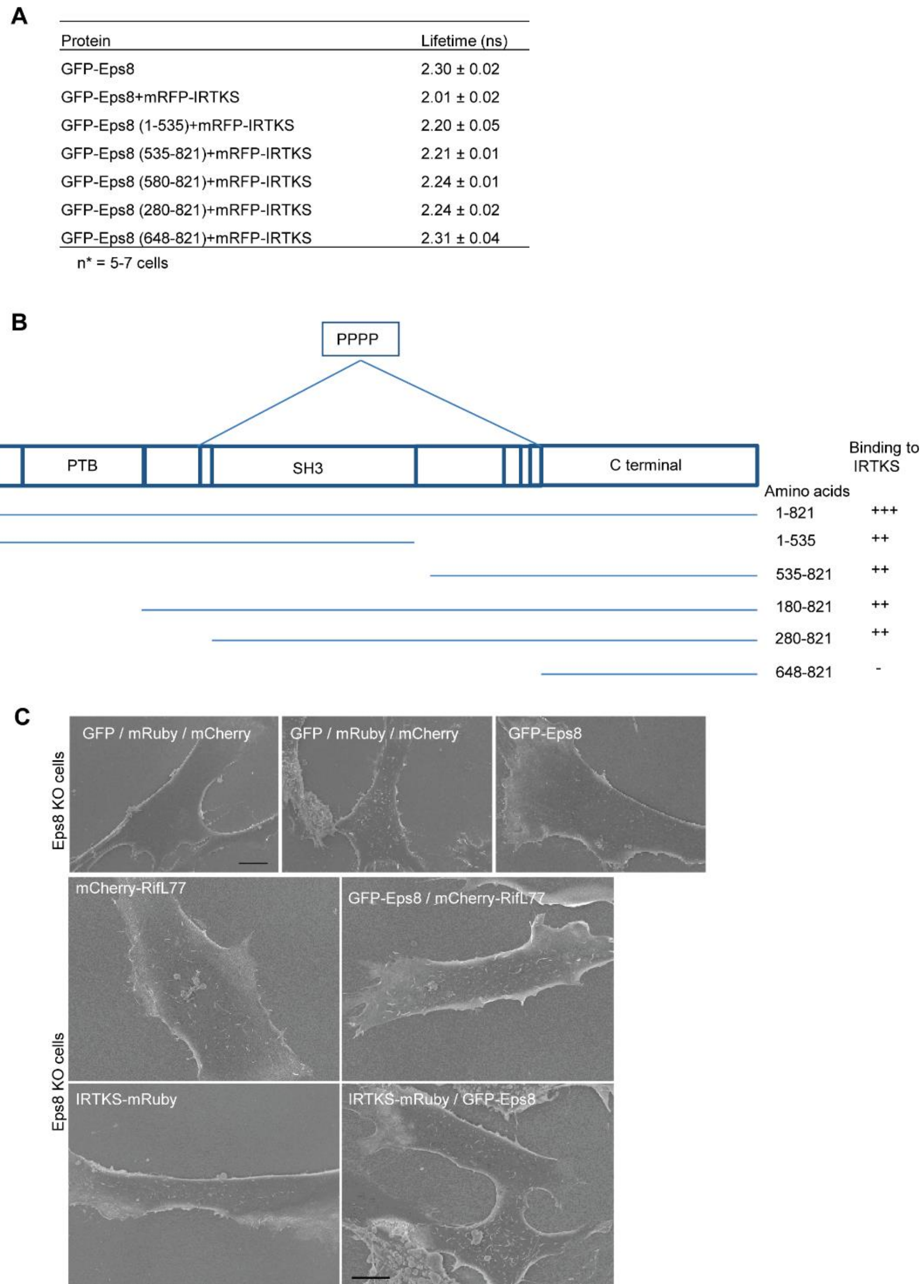

B
Mito-IRTKS-mRuby
GFP
ROI merge
WAVE1
PC=0.55
WAVE2
PC=0.93
Mena
PC=0.96
HeLa cells
Eps8
PC=0.93
VASP
PC=0.96
Espin
PC=0.78
mDia1ΔDAD
PC=0.80
mDia2ΔDAD
PC=0.51
N-WASP
PC=0.59
Ezrin
PC=0.41
Dynamin1
PC=0.49
Dynamin2
PC=0.49

Figure S2. 3D imaging of wildtype MEF cells grown in a 3D matrix and colocalisation analysis of SH3 domain interactors in HeLa cells.

(A). Wildtype MEF cells were transfected with GFP-CAAX^{Rif} (plasma membrane localisation CAAX motif from the C-terminal end of Rif) and grown in a 3D matrix as the control (movie 1). Wildtype MEF cells were transfected with GFP-RifL77 and grown in a 3D matrix (collagen type I) to check for protrusions in a physiologically relevant environment (movie 2). The movies presented are representative of 7-10 cells each for GFP-CAAX^{Rif} and GFP-RifL77. A selected region of each movie is also shown as a stack image. Scales bar, 5 µm. (B) Hela cells were cotransfected with of Mito-IRTKS-mRuby and GFP-tagged potential SH3 domain-binding partners. Scales bar, 10 µm. Colocalisation analysis was carried out on the ROI as described in the Materials and Methods and PC values are presented in Fig3.



Protein	Lifetime (ns)
GFP-Eps8	2.30 ± 0.02
GFP-Eps8+mRFP-IRTKS	2.01 ± 0.02
GFP-Eps8 (1-535)+mRFP-IRTKS	2.20 ± 0.05
GFP-Eps8 (535-821)+mRFP-IRTKS	2.21 ± 0.01
GFP-Eps8 (580-821)+mRFP-IRTKS	2.24 ± 0.01
GFP-Eps8 (280-821)+mRFP-IRTKS	2.24 ± 0.02
GFP-Eps8 (648-821)+mRFP-IRTKS	2.31 ± 0.04

n* = 5-7 cells

Figure S3. Eps8 binding site in IRTKS and SEM analysis of Eps8 KO cells.

(A). Hela cells were transfected with mRFP-IRTKS and GFP-tagged deletion mutants of Eps8 and the interaction of the two proteins measured by FLIM. (B) Schematic of Eps8 deletion mutants used in (A) with corresponding (does not contain) amino acid numbers. The binding efficiency is represented as + and – representing binding and not binding respectively. (C) SEM images of the Eps8 KO cells in Fig.3D, showing the entire cell: (top panel) GFP/mRuby/mCherry control (left and middle), and GFP-Eps8 rescue (right); (middle panel) mCherry-RifL77 (left), and GFP-Eps8/ mCherry-RifL77 (right); (bottom panel) IRTKS-mRuby and IRTKS-mRuby/GFP-Eps8 (right). Scale bar, 10 µm.

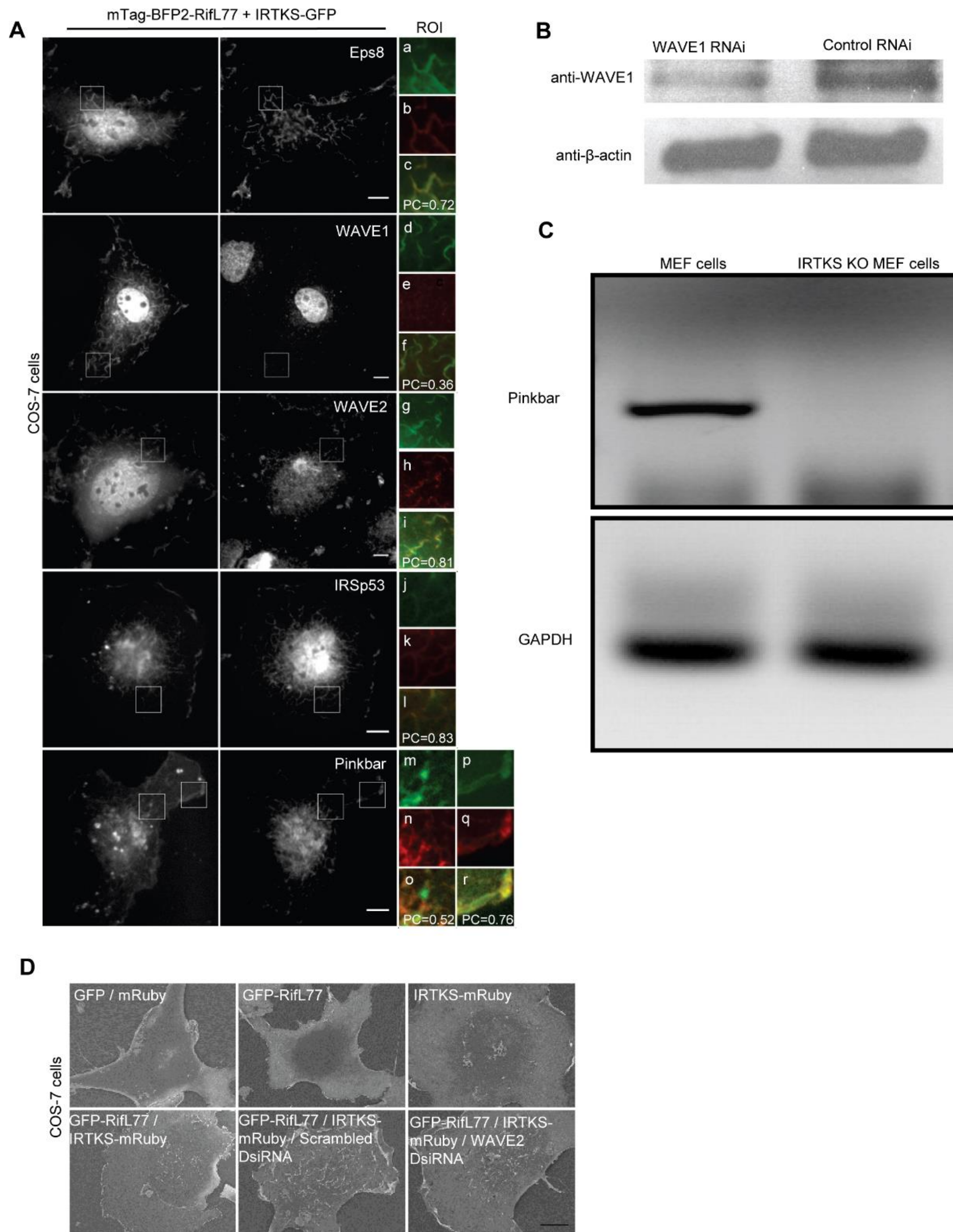

A
mTag-BFP2-RifL77 + IRTKS-GFP
ROI
Eps8
WAVE1
WAVE2
IRSp53
Pinkbar
COS-7 cells
PC=0.72
PC=0.36
PC=0.81
PC=0.83
PC=0.52
PC=0.76
B
WAVE1 RNAi
Control RNAi
anti-WAVE1
anti-β-actin
C
MEF cells
IRTKS KO MEF cells
Pinkbar
GAPDH
D
GFP / mRuby
GFP-RifL77
IRTKS-mRuby
GFP-RifL77 / IRTKS-mRuby
GFP-RifL77 / IRTKS-mRuby / Scrambled DsiRNA
GFP-RifL77 / IRTKS-mRuby / WAVE2 DsiRNA
COS-7 cells

Figure S4. Endogenous protein colocalisation and Pinkbar detection. (A). mTag-BFP2-RifL77 and IRTKS-GFP were expressed in COS-7 cells and stained endogenous proteins using Alexa568-conjugated secondary antibodies. ROIs used for colocalisation analysis are shown in the following panels: Eps8 (a-c), WAVE1 (d-f), WAVE2 (g-i), IRSp53 (j-l) and Pinkbar (m-o and p-r). IRTKS-GFP (a, d, g, j, m, p), endogenous proteins (b, e, h, k, n, q) and merge (c, f, i, l, o, r). Two ROIs are shown for Pinkbar –cytoplasm (m, n and o; denoted as Pinkbar1 in Fig.4B) and edge ruffling (p, q and r; denoted as Pinkbar 2 in Fig. 4B). PC values derived from the colocalisation analysis are presented in the merge panels. (B). Western blot showing specificity of anti-WAVE1 using transfected WAVE1 RNAi or non-targeting control RNAi protein levels in N1E 115 cells 36 h after transfection with loaded extracts of equal quantity. Anti-β-actin is used as control for western blot. (C) RT-PCR to detect endogenous Pinkbar gene expression in wildtype MEF cells and IRTKS KO MEF cells. GAPDH amplification was used control. Scales bar, 10 µm. (D). SEM images COS-7 cell in Fig. 4D, showing the entire cell: (upper panel, left to right) GFP / mRuby, GFP-RifL77 and IRTKS-mRuby; (lower panel, left to right) GFP-RifL77 / IRTKS-mRuby, Scrambled DsiRNA with GFP-RifL77 / IRTKS-mRuby, and WAVE2 DsiRNA with GFP-RifL77 / IRTKS-mRuby. Scale bar, 10 µm.

Journal of Cell Science • Supplementary information

Supplementary movie 1: 3D Images (Figure S2 A) of MEF-WT cells expressing GFP-CAAXRif (plasma membrane localisation CAAX motif from the C-terminal end of Rif) was generated by having a slice thickness of 1.1µm per slice with the sampling speed of 4 µs per pixel. The selected ROI (20 frames) were converted into movie and played back at 5 frames per second.

Supplementary movie 2: 3D Images (Figure S2 A) of MEF-WT cells expressing GFP-RIF was generated by having a slice thickness of 1.1µm per slice with the sampling speed of 4 µs per pixel. The selected ROI (18 frames) were converted into movie and played back at 5 frames per second.