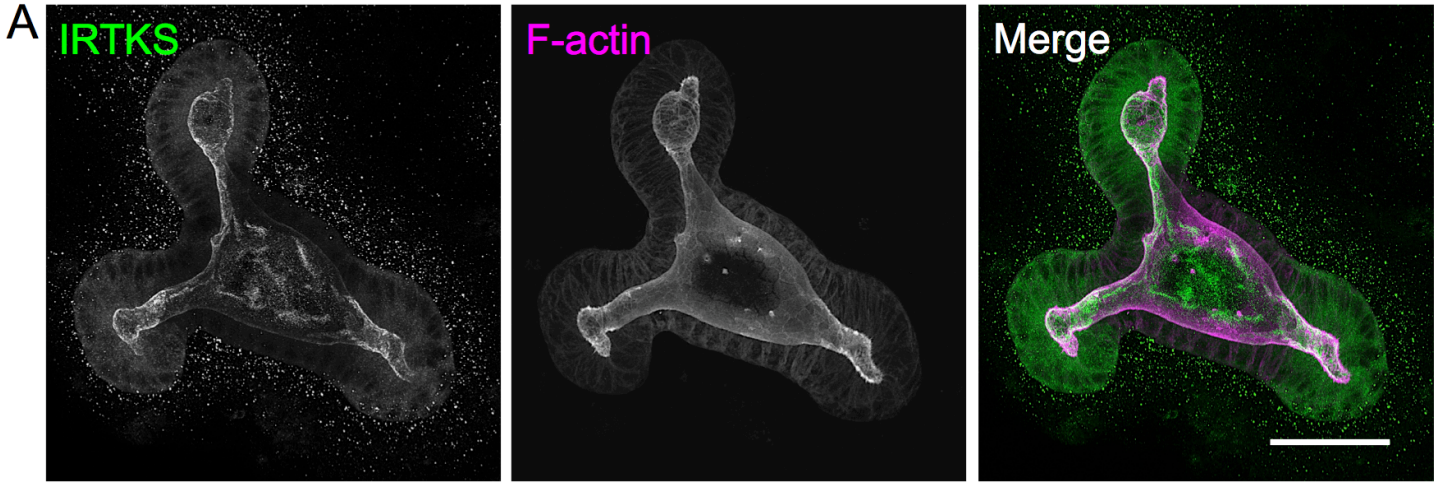
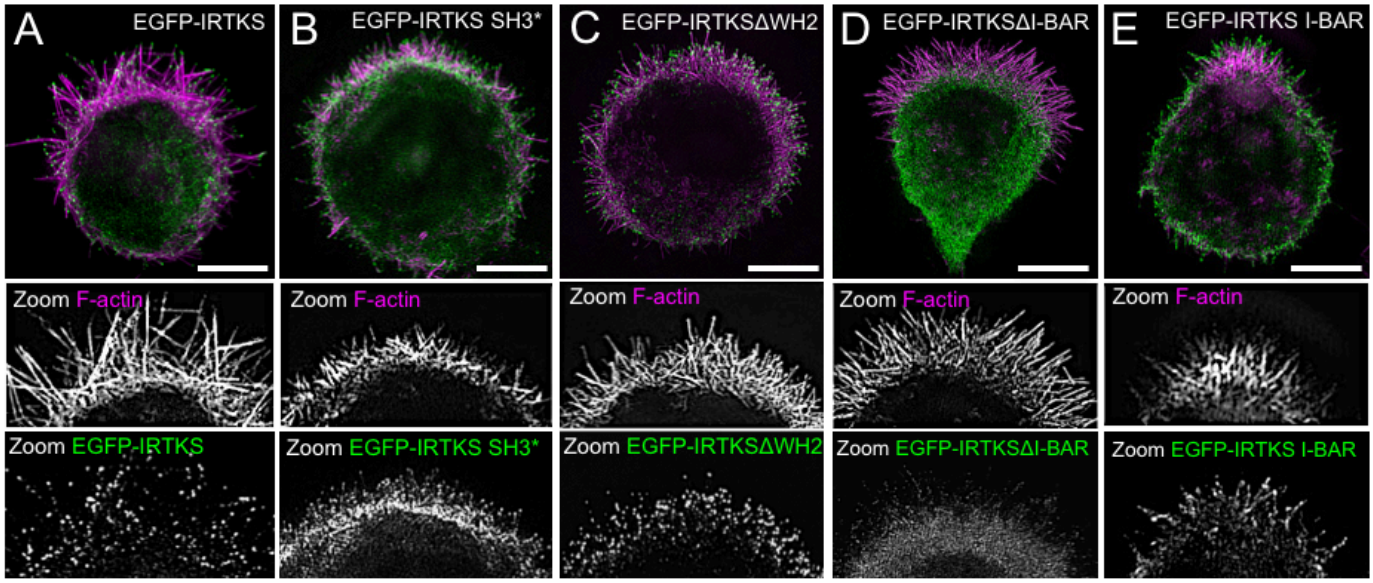
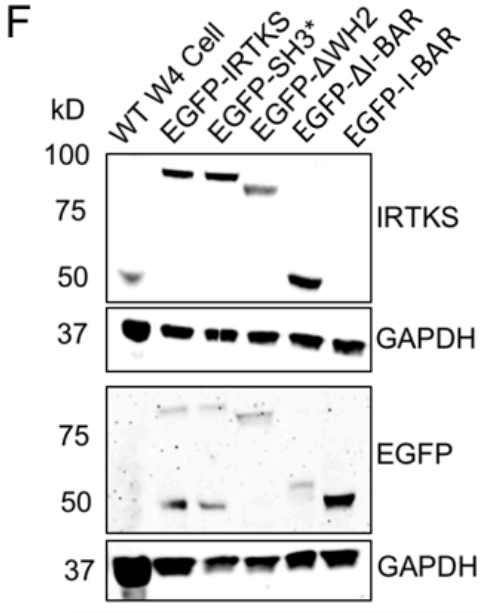
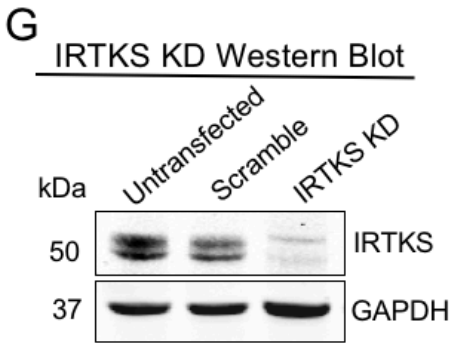
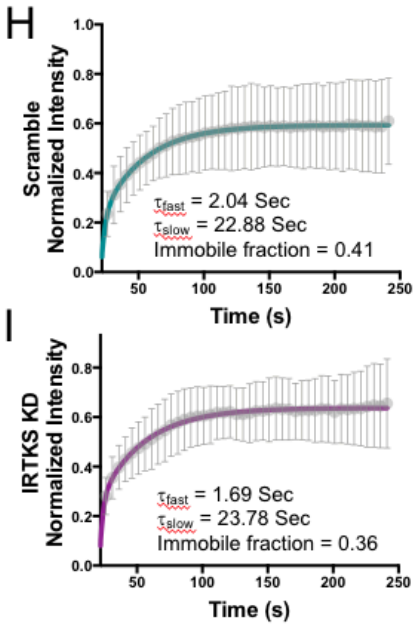


Figure S1, Related to Figure 1. IRTKS is highly expressed in the intestinal crypt domain. (**A**) Maximum intensity projection of the IRTKS stained mouse intestinal organoid used in Figure 1B. Scale bar, 40 μm.


A
EGFP-IRTKS
B
EGFP-IRTKS SH3*
C
EGFP-IRTKSΔWH2
D
EGFP-IRTKSΔI-BAR
E
EGFP-IRTKS I-BAR
Zoom F-actin
Zoom F-actin
Zoom F-actin
Zoom F-actin
Zoom F-actin
Zoom EGFP-IRTKS
Zoom EGFP-IRTKS SH3*
Zoom EGFP-IRTKSΔWH2
Zoom EGFP-IRTKSΔI-BAR
Zoom EGFP-IRTKS I-BAR


F
WT W4 Cell
EGFP-IRTKS
EGFP-SH3*
EGFP-ΔWH2
EGFP-ΔI-BAR
EGFP-I-BAR
kD
100
75
50
37
IRTKS
GAPDH
75
50
37
EGFP
GAPDH


G
IRTKS KD Western Blot
Untransfected
Scramble
IRTKS KD
kDa
50
37
IRTKS
GAPDH


H
Scramble
Normalized Intensity
τfast = 2.04 Sec
τslow = 22.88 Sec
Immobile fraction = 0.41
Time (s)
I
IRTKS KD
Normalized Intensity
τfast = 1.69 Sec
τslow = 23.78 Sec
Immobile fraction = 0.36
Time (s)

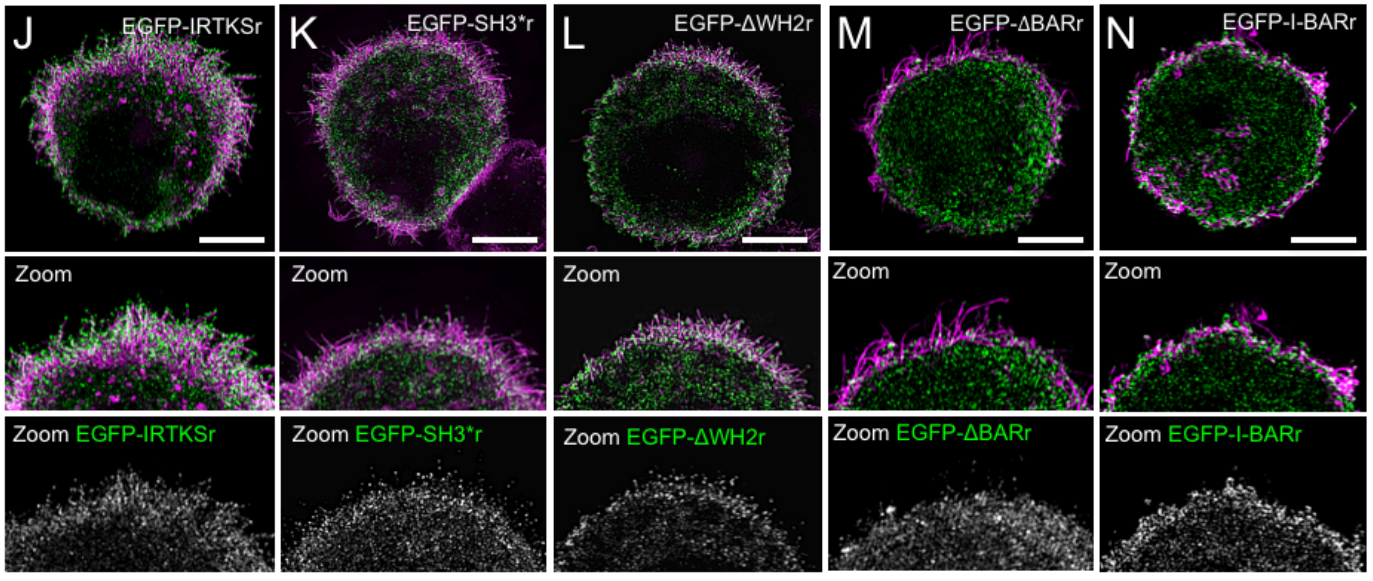

J
EGFP-IRTKSr
K
EGFP-SH3*r
L
EGFP-ΔWH2r
M
EGFP-ΔBARr
N
EGFP-I-BARr
Zoom
Zoom
Zoom
Zoom
Zoom
Zoom EGFP-IRTKSr
Zoom EGFP-SH3*r
Zoom EGFP-ΔWH2r
Zoom EGFP-ΔBARr
Zoom EGFP-I-BARr

Figure S2, Related to Figure 3. Split channel images of IRTKS overexpression and KD/rescue cells. (**A-E**) SIM projections of Ls174T-W4 cells expressing EGFP-IRTKS constructs (green) and stained with phalloidin (magenta); single channel zooms of the BB are below each image. Scale bars, 5 μm. (**F**) Western blot analysis of expression levels of EGFP-IRTKS variants in lysates from Ls174T-W4 cells. Both IRTKS and EGFP antibodies were used because the IRTKS antibody is towards a peptide sequence within the SH3 domain, thus the EGFP-I-BAR alone construct will not be targeted. GAPDH was used as a loading control. (**G**) Western blot analysis of endogenous IRTKS levels in lysates from WT Ls174T-W4 cells (untransfected), shRNA scramble control, and shRNA IRTKS KD. GAPDH was used as a loading control. (**H-I**) FRAP curves of shRNA scramble control and shRNA IRTKS KD in Ls174T-W4 cells. (**J-N**) SIM projections of IRTKS KD Ls174T-W4 cells expressing refractory EGFP-IRTKS constructs in rescue experiments. Zooms of the BB and single IRTKS channel below each image. Scale bars, 5 μm.

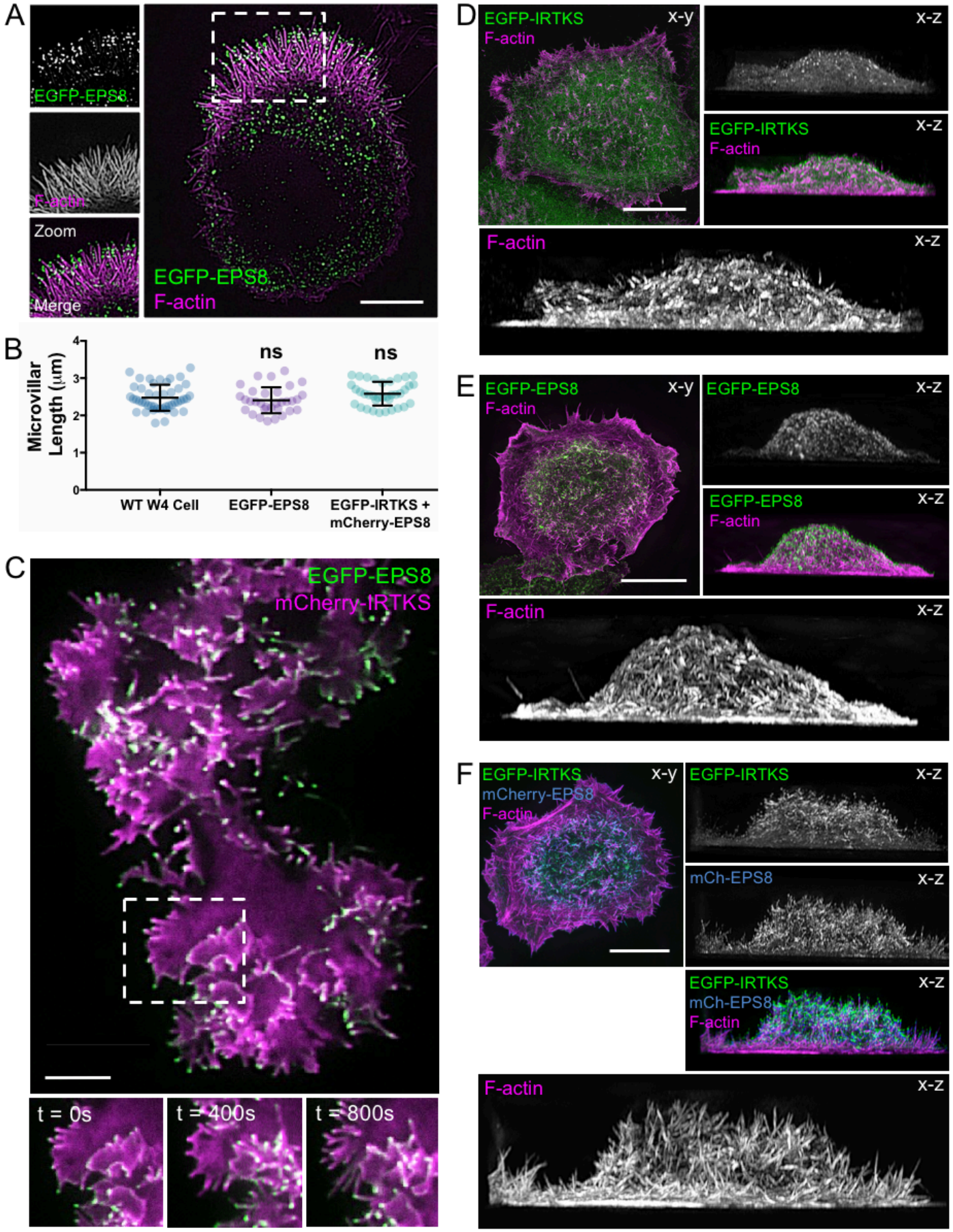

A
EGFP-EPS8
F-actin
Zoom
Merge
EGFP-EPS8
F-actin
B
Microvillar Length (μm)
0
1
2
3
4
ns
ns
WT W4 Cell
EGFP-EPS8
EGFP-IRTKS + mCherry-EPS8
C
EGFP-EPS8
mCherry-IRTKS
t = 0s
t = 400s
t = 800s
D
EGFP-IRTKS
F-actin
x-y
x-z
EGFP-IRTKS
F-actin
x-z
F-actin
x-z
E
EGFP-EPS8
F-actin
x-y
EGFP-EPS8
x-z
EGFP-EPS8
F-actin
x-z
F-actin
x-z
F
EGFP-IRTKS
mCherry-EPS8
F-actin
x-y
EGFP-IRTKS
x-z
mCh-EPS8
x-z
EGFP-IRTKS
mCh-EPS8
F-actin
x-z
F-actin
x-z

Figure S3, Related to Figure 4. The impact of EPS8 and IRTKS co-expression on the formation of actin-based protrusions. (**A**) SIM projection of a Ls174T-W4 cell expressing EGFP-EPS8. Dashed box indicates zoom; scale bar, 5 μm. (**B**) Quantitation of microvillar length comparing WT Ls174T-W4 cells, EGFP-EPS8 overexpression and EGFP-IRTKS and mCherry-EPS8 dual overexpression, which do not affect microvillar length. 25 cells/condition, 10 microvilli/cell; student's t test (*$p<0.0001$, ns, not significant) was used to determine the significance. (**C**) TIRF live-cell imaging of a B16F1 melanoma cell expressing EGFP-EPS8 (green) and mCherry-IRTKS (magenta). Dashed box indicates video frames with time in seconds. Scale bar, 10 μm. (**D-F**) *En face* (x-y) and lateral (x-z) SIM projections of HeLa cells expressing either EGFP-IRTKS (D), EGFP-EPS8 (E), or EGFP-IRTKS and mCherry-EPS8 (F) and stained with phalloidin. Scale bars, 10 μm.

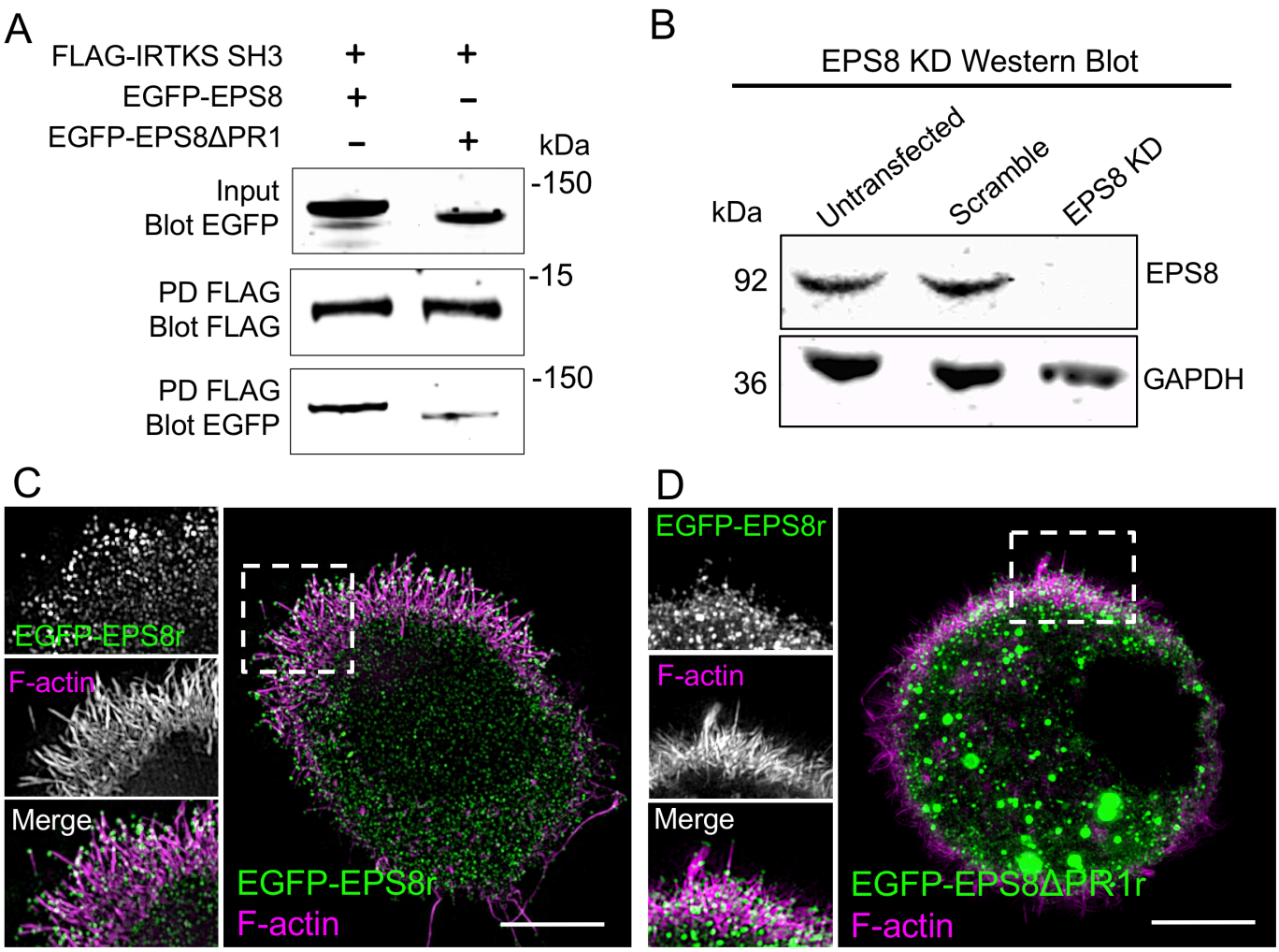


Figure S4, Related to Figures 5 & 6. Images of EPS8 KD/ rescue Ls174T-W4 cells. (**A**) Pulldown of FLAG-tagged IRTKS SH3 domain co-expressed with EGFP-tagged EPS8 or EGFP-tagged EPS8ΔPR1 reveals binding between EPS8 and the IRTKS SH3 domain. (**B**) Western blot analysis of endogenous EPS8 levels in lysates from WT Ls174T-W4 cells (untransfected), shRNA scramble control, and shRNA EPS8 KD. GAPDH was used as a loading control. (**C**) SIM projection of an EPS8 KD Ls174T-W4 cell expressing refractory EGFP-EPS8 (EGFP-EPS8r) construct in rescue experiments; dashed box indicates zoom of the BB. Scale bar, 5 μm. (**D**) SIM projection of an EPS8 KD Ls174T-W4 cell expressing refractory EGFP-EPS8ΔPR1 (EGFP-EPS8ΔPR1r) construct in rescue experiments; dashed box indicates zoom of the BB. Scale bar, 5 μm.

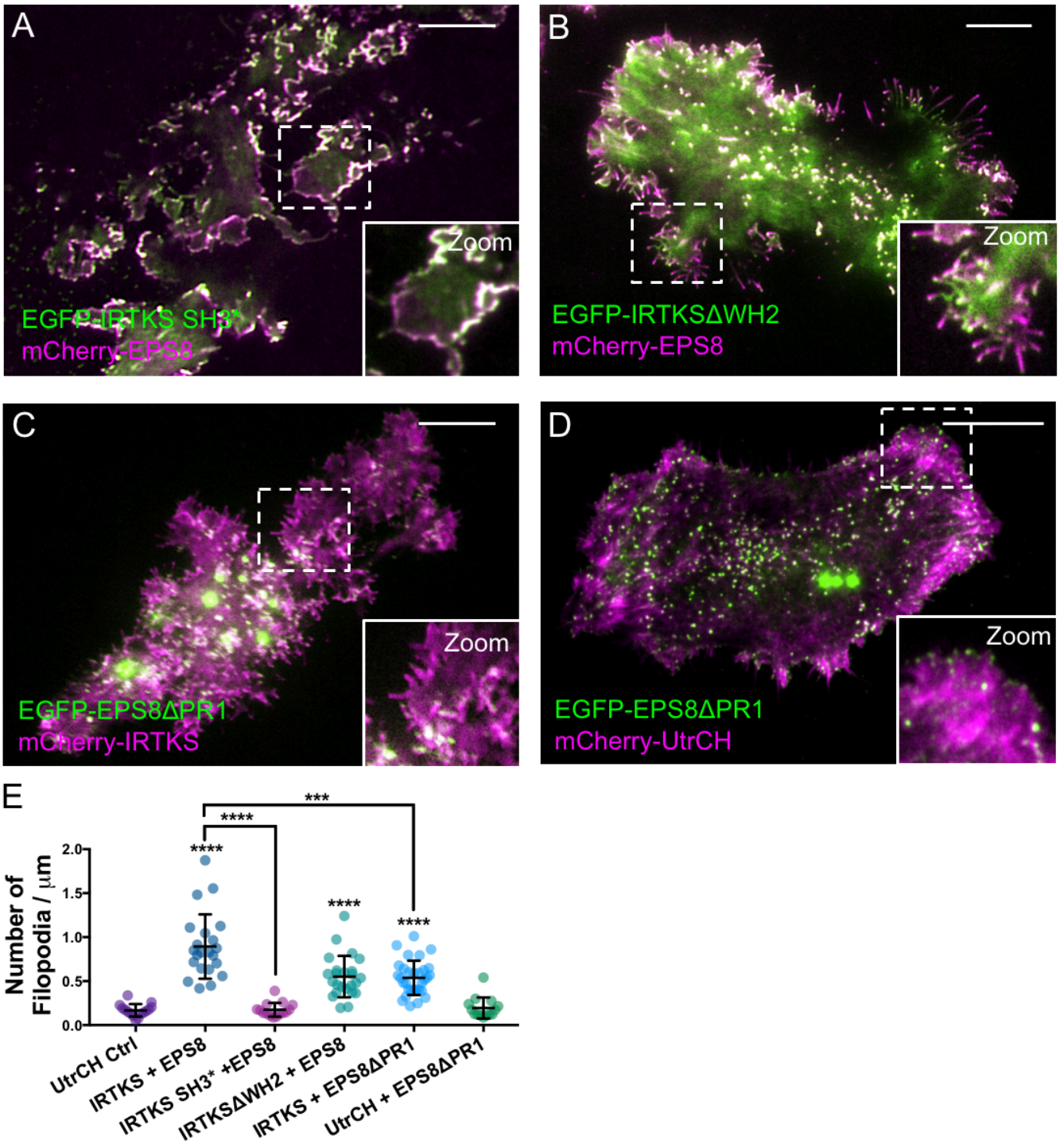


Figure S5, Related to Figure 6. Additional characterization of the IRTKS/EPS8 complex in B16F1 Melanoma Cells. (**A-D**) TIRF live-cell imaging of B16F1 melanoma cells expressing either EGFP-IRTKS SH3* (green) and mCherry-EPS8 (magenta) (A), EGFP-IRTKSΔWH2 (green) and mCherry-EPS8 (magenta) (B), or EGFP-EPS8ΔPR1 (green) and mCherry-IRTKS (magenta) (C), and EGFP-EPS8ΔPR1 (green) and mCherry-UtrCH (magenta) (D). Dashed boxes indicate zooms. Scale bars, 10 µm. (**E**) Quantitation of the number of filopodia per µm of cell perimeter in B16F1 melanoma cells from C-E; at least 15 cells/condition. All error bars indicate mean ± SD; all p values calculated using a t test (*p<0.033, **p<0.002, ***p<0.0002, ****p<0.0001).