SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure 1. MICAL-L1 interacts with EHD1 and EHD3 but displays only weak interactions with EHD4 and no binding to EHD2. (**A**) The *S. cerevisae* yeast strain AH109 was co-transformed with the indicated GAL4 binding domain (GAL4bd) fusion constructs and Galbd-p53 (control), together with the indicated GAL4 transcription activation (GAL4ad) fusion products: Gal4ad-MICAL-L1 or GAL4ad-SV40 Large T-antigen (control). Co-transformants were assayed for their growth on non-selective (+HIS) and selective (–HIS) media. (**B**) Bacterially expressed, purified recombinant GST-only, GST-EH-domain of EHD1 (GST-EH1), GST-EH-domain of EHD2 (GST-EH2), GST-EH-domain of EHD3 (GST-EH3) and GST-EH-domain of EHD4 (GST-EH4) were incubated with lysates from untreated HeLa cells. The bound proteins were resolved by 8% reducing SDS-PAGE, transferred to nitrocellulose, and immunoblotted with mouse anti-MICAL-L1 followed by anti-mouse-HRP conjugated antibodies. Enhanced chemiluminescence (ECL) was used for detection. The lane denoted "Lysate" depicts 1% of the lysate input. Purified protein bands shown in bottom panel were visualized with Coomassie blue dye staining.

Supplemental Figure 2. Dynamics of MICAL-L1 and EHD1 association with membranes. GFP–MICAL-L1 and Tomato-EHD1 were subjected to fluorescence recovery after photobleaching (FRAP) analysis. HeLa cells were transiently transfected with GFP–MICAL-L1 and Tomato-EHD1 constructs, and examined by live confocal image analysis 24 h later. Two short tubular structures decorated with EHD1 and MICAL-L1 were photobleached (indicated in rectangular region on interest box in the pre-bleach panel). GFP–MICAL-L1 and Tomato-EHD1 recovery to the bleached area was monitored every 10 s (see Supplemental Video 1). Recovery is depicted in the insets at the indicated times.

Supplemental Figure 3. Depletion of EHD proteins does not affect localization of endogenous MICAL-L1 to tubular membranes. (**A-E**) HeLa cells on coverslips (**A-D**) or on 35 mm plates (**E**) were Mock-treated (**A, C and E**) or treated either with both EHD1 and EHD3 SiRNA (**B and E**) or with both EHD1 and EHD4 SiRNA (**D and E**). 48 h later, both mock- and EHD1 and EHD3 SiRNA-treated cells were transfected with GFP-Myc-EHD3 (**A, B and E**). After an additional 24 h, cells on coverslips were fixed and incubated with mouse anti-MICAL-L1 antibodies followed by staining with Alexa Fluor 568-conjugated goat anti-mouse antibody (**A-D**), Bar, 10 µm. (**E**) Similarly, Mock- and SiRNA-treated cells on 35 mm plates were harvested after an additional 24 h. Cells were then lysed and proteins separated by 8% SDS–PAGE were subjected to immunoblotting with anti-EHD1, anti-EHD4 and anti-GFP antibodies (top and middle panel) and monoclonal anti-actin antibody to validate equal protein loading (bottom panel), followed by anti-rabbit and anti-mouse–HRP-conjugated antibodies. ECL was used for detection.

Supplemental Figure 4. Depletion of MICAL-L1 has a moderate effect on tubular localization of EHD3 but prevents association of EHD4 with tubular membranes. (**A-L**) HeLa cells on coverslips were Mock treated (**A-C and G-I**) or treated with MICAL-L1-SiRNA (**D-F and J-L**). After 48 h, both mock and SiRNA-treated cells were transfected with either Myc-EHD3 (**A-F**) or with HA-EHD4 (**G-L**). After an additional 24 h, the cells were fixed and incubated with rabbit anti-Myc antibody (**A-F**) or rabbit anti-EHD4 (**G-L**) and mouse anti-MICAL-L1 antibodies (**A-L**) followed by staining with Alexa Fluor 568-conjugated goat anti-rabbit and 488-conjugated goat anti-mouse antibody. Bar, 10 µm.

Supplemental Figure 5. MICAL-L1 depletion causes mis-sorting of EHD1 to early endosomes and Rab8a to a TfR-containing ERC region. **(A-L**) HeLa cells on coverslips were Mock treated (**A-C and**

G-I) or treated with MICAL-L1-SiRNA (**D-F and J-L**). After 48 h, both mock and SiRNA-treated cells were transfected with Myc-EHD1 (**A-F**) or with Cherry-Rab8a (**G-L**). After an additional 24 h, the cells were fixed and incubated with rabbit anti-Myc and mouse anti-EEA1 antibodies (**A-F**) or mouse anti-TfR antibodies (**G-L**) followed by staining with Alexa Fluor 568-conjugated goat anti-rabbit and 488-conjugated goat anti-mouse antibody. Bar, 10 μm.

Supplemental Figure 6. Loss of MICAL-L1 does not affect association of EHD2, GFP-H-Ras or endogenous Rab11 with tubular membranes. (**A-F**) HeLa cells on coverslips were Mock-treated (**A,C and E**) or treated with MICAL-L1-SiRNA (**B, D and F**). 48 h later, the cells were transfected with Myc-EHD2 (**A-B**) or GFP fused to the double palmitoylated and farnesylated C-terminal tail of H-Ras (GFP-H-Ras) (**E-F**) or left untransfected (**C-D**). After an additional 24 h, cells were fixed and incubated with rabbit anti-Myc antibody (**A-B**) or rabbit anti-Rab11 antibodies (**C-D**) followed by staining with Alexa Fluor 568-conjugated goat anti-rabbit antibodies. (**G-J**) HeLa cells on coverslips were triple transfected with Myc-EHD1 (**G**), GFP-H-Ras (**H**) and HA-MICAL-L1 (**I**). After 24 h, cells were fixed and incubated with rabbit anti-Myc antibody (**A**) and mouse anti-HA antibodies (**I**) followed by staining with Alexa Fluor 568-conjugated goat anti-rabbit and 405-conjugated goat anti-mouse antibodies. Bar, 10 μm.

Supplemental Figure 7. The MICAL-L1 coiled-coil domain stabilizes Rab8a on tubular membranes. (**A-G**) HeLa cells on coverslips were transfected with Cherry-Rab8a only or co-transfected with HA-MICAL-L1 wt coiled-coils (HA-wt-CC, **B-D**), or HA-MICAL-L1 coiled-coils: site 1+ site 2 mutated to alanine residues (HA-wt-CC Site 1+2 mut., **E-G**). After 24 h, cells were fixed and directly analyzed or incubated with mouse anti-HA antibody followed by the appropriate secondary antibodies. Bar, 10 μm.

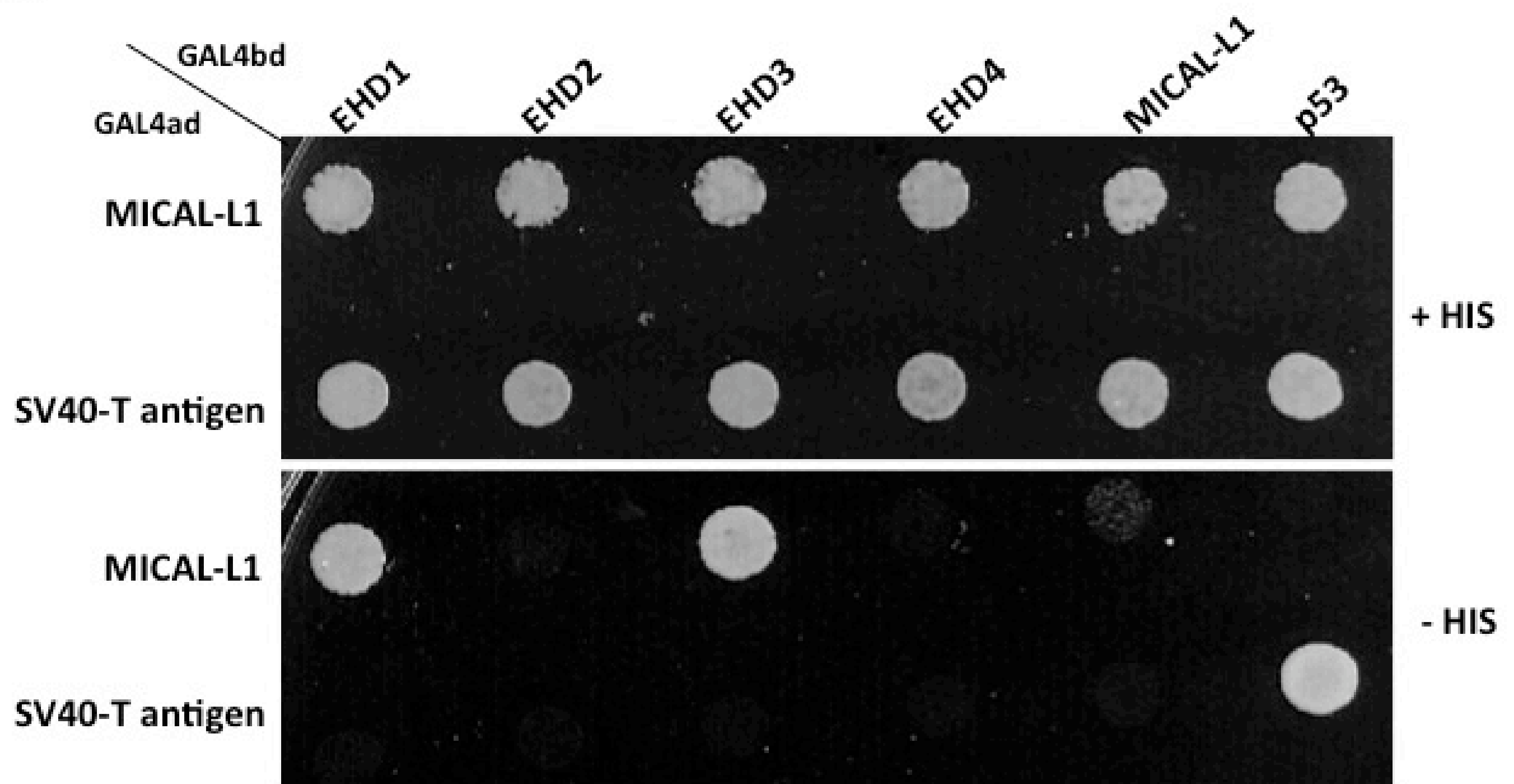
Supplemental Figure 8. Internalized transferrin receptor partially localizes to GFP-MICAL-L1 containing tubular membranes in live cells. HeLa cells were transiently transfected with GFP–MICAL-L1 construct, and 24 h later the cells were serum-starved for 30 min and pulsed with Tf-568 for 10 min. Live cells were then analyzed by confocal time-lapse microscopy during ‘chase’ in complete medium for the indicated times. Representative images from one such time-lapse video are shown in the series of panels. Yellow arrows indicate internalized transferrin on tubular MICAL-L1-decorated membranes. Insets show continuous co-localization of transferrin with MICAL-L1.

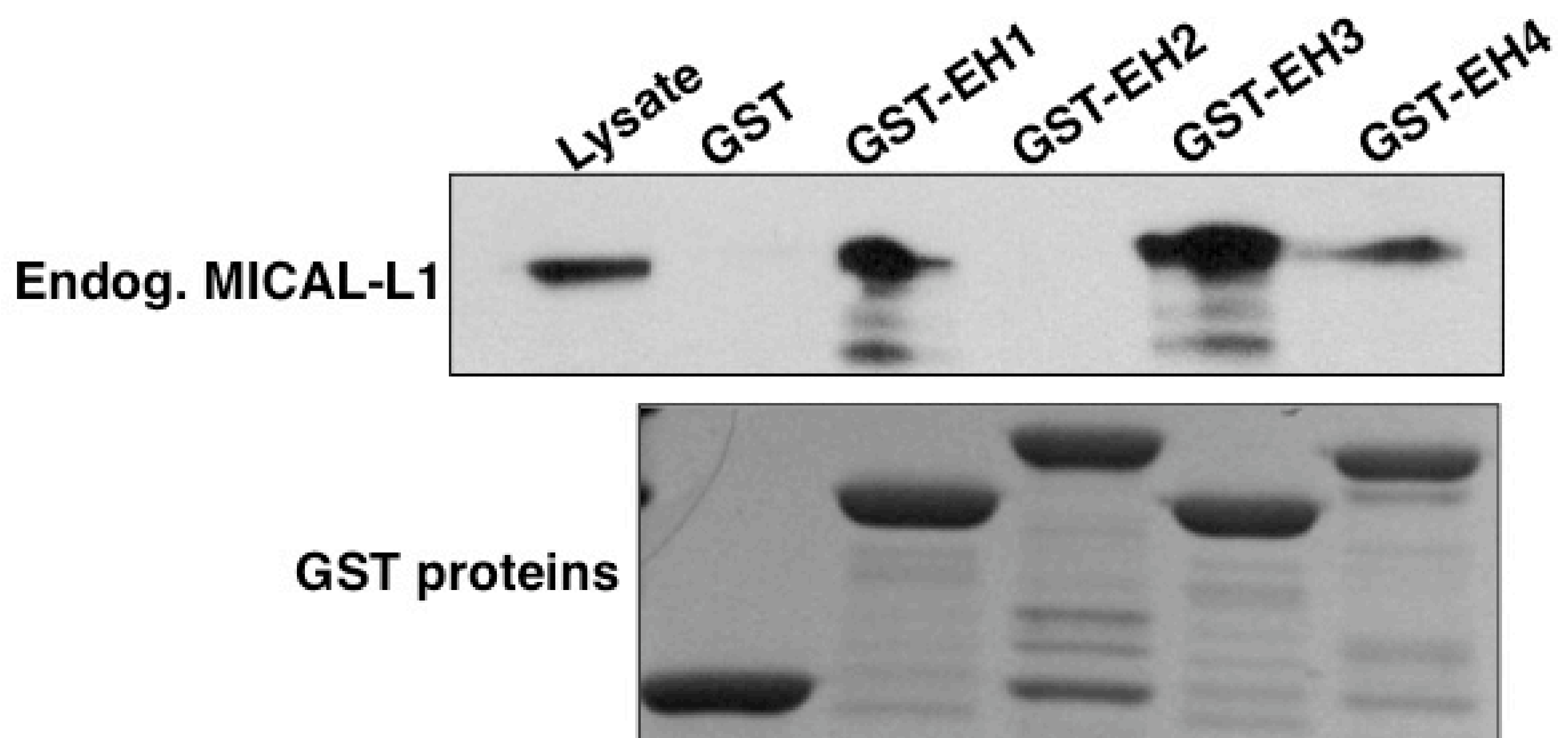
Supplemental Figure 9. SiRNA-mediated depletion of Rab8 causes a modest delay in recycling of β1 integrins. HeLa cells on coverslips were Mock-treated or co-treated with Rab8a and Rab8b-SiRNA for 72 h. Cells were then starved and pulsed with anti-β1 integrin antibody for 1 h and fixed, or pulsed for 1 h and 'chased' in complete media for 3 additional hours at 37°C prior to fixation. Quantitative analysis of the percentage of β1 integrins remaining following 3 h of chase was done. Approximately 80 cells from three independent experiments were counted for each set of treatments. Error bars show standard error. Significance ($p<0.05$) was determined by the t-test for independent samples.

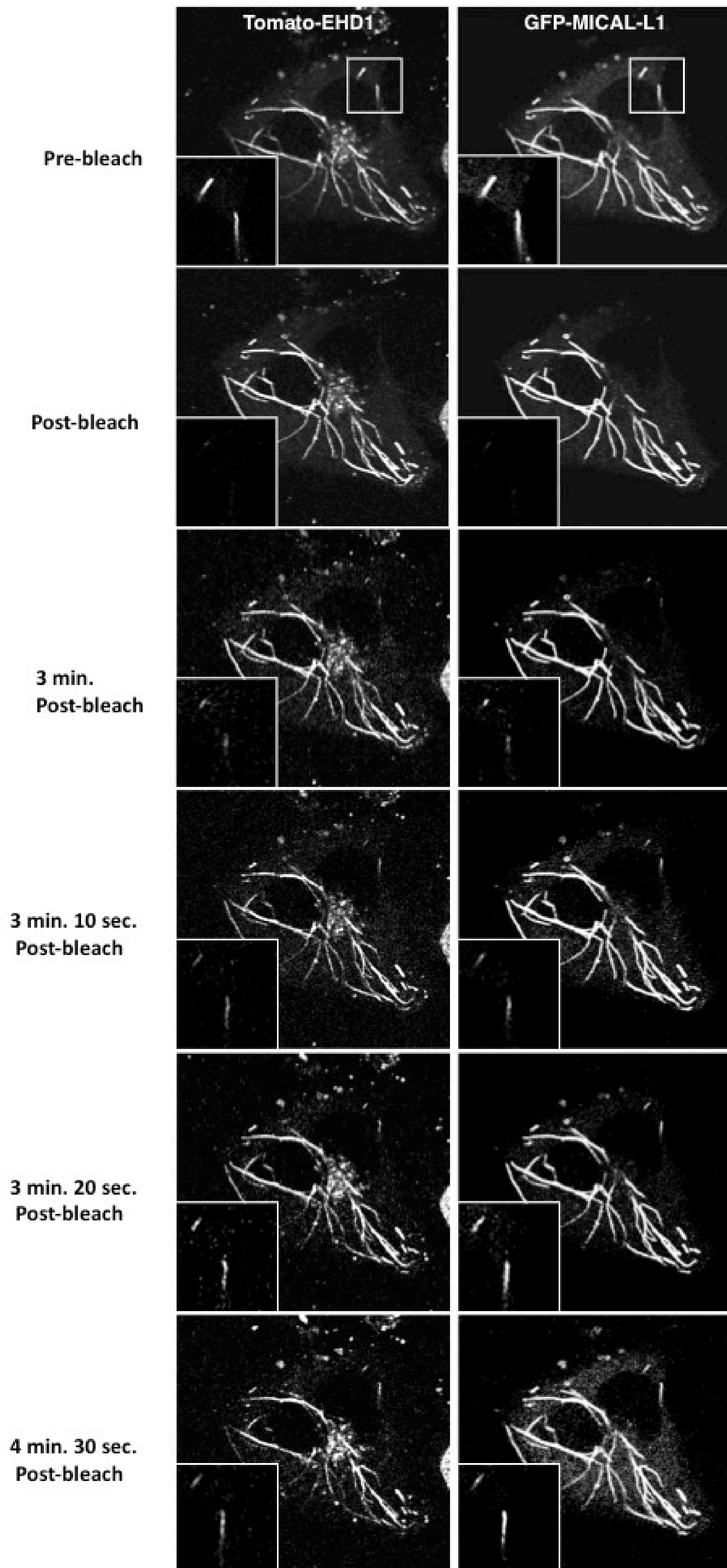
Supplementary Video 1. Dynamics of MICAL-L1 and EHD1 association with membranes. The video shows fluorescence recovery after photobleaching (FRAP) of GFP–MICAL-L1 and Tomato-EHD1. HeLa cells were transiently transfected with GFP–MICAL-L1 and Tomato-EHD1 constructs, and examined by live confocal image analysis 24 h later. Two independent and unconnected tubular structures with EHD1 and MICAL-L1 were photobleached (as indicated in rectangular box in the pre-

bleach panel). GFP–MICAL-L1 and Tomato-EHD1 recovery to the bleached area was monitored every 10 s. The movie was compiled from 47 captured images.

Supplementary Video 2. Live image analysis of MICAL-L1 and EHD1 containing tubular membranes. HeLa cells were transiently transfected with GFP–MICAL-L1 and Tomato-EHD1 constructs, and examined by live confocal image analysis 24 h later. The video shows representative movement of GFP-MICAL-L1- and Tomato EHD1-containing tubular membranes and vesicles in the cell. Note that the smaller tubules appear to be more dynamic than the longer. The stacks were acquired every 10 seconds over a period of 8 minutes.


A
GAL4bd
GAL4ad
EHD1
EHD2
EHD3
EHD4
MICAL-L1
p53
MICAL-L1
SV40-T antigen
+ HIS
MICAL-L1
SV40-T antigen
- HIS


B
Lysate
GST
GST-EH1
GST-EH2
GST-EH3
GST-EH4
Endog. MICAL-L1
GST proteins



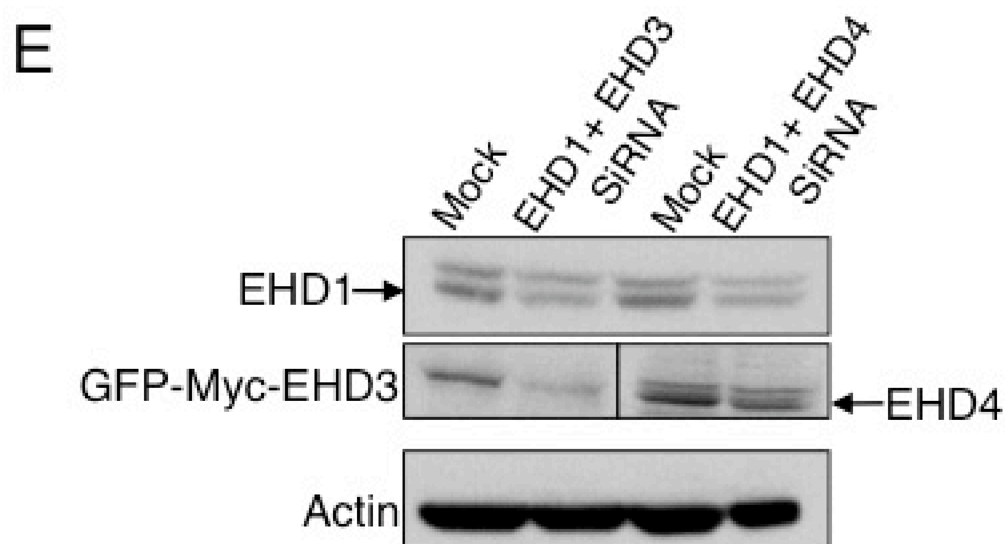
Tomato-EHD1
GFP-MICAL-L1
Pre-bleach
Post-bleach
3 min.
Post-bleach
3 min. 10 sec.
Post-bleach
3 min. 20 sec.
Post-bleach
4 min. 30 sec.
Post-bleach

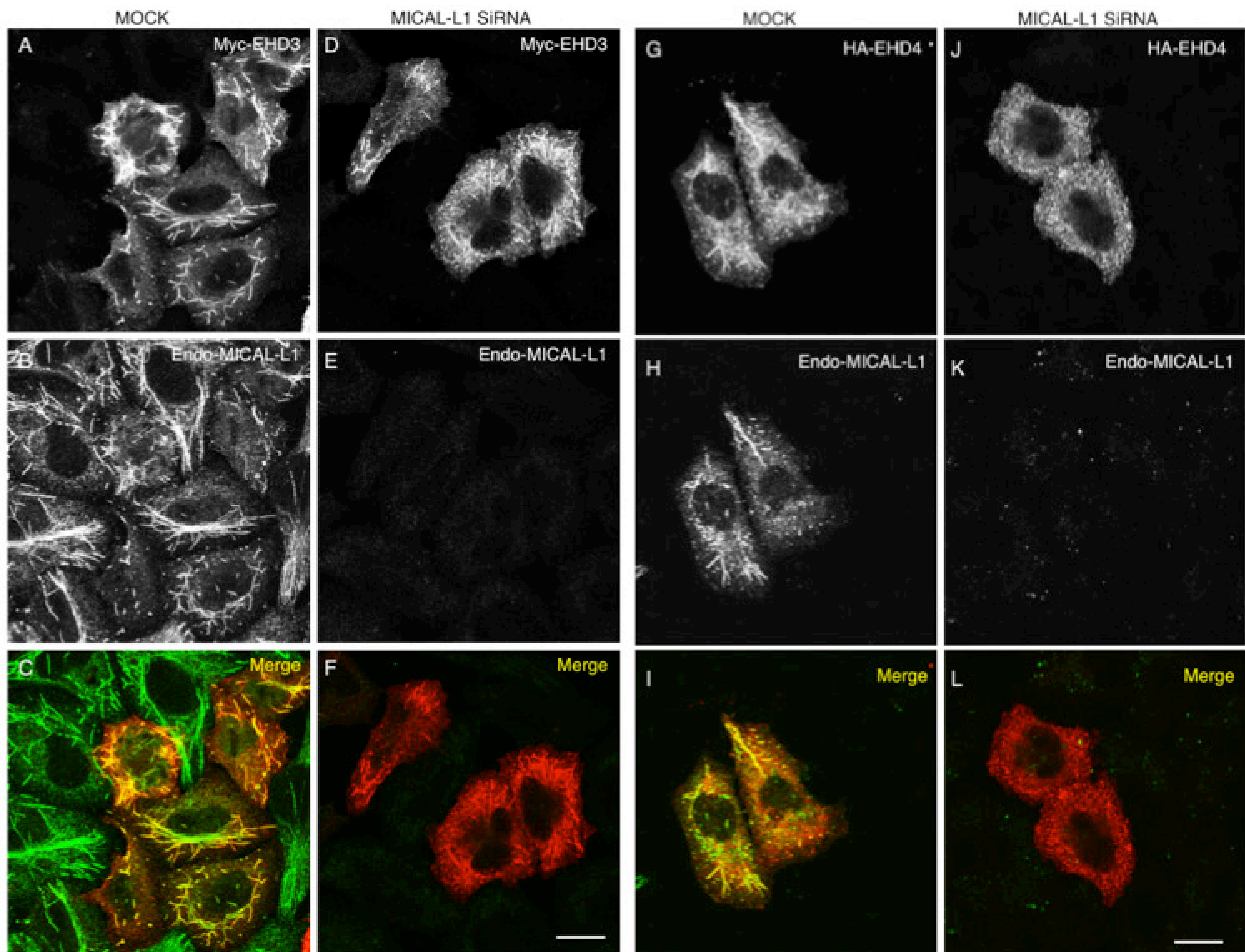
A Endo-MICAL-L1
Mock

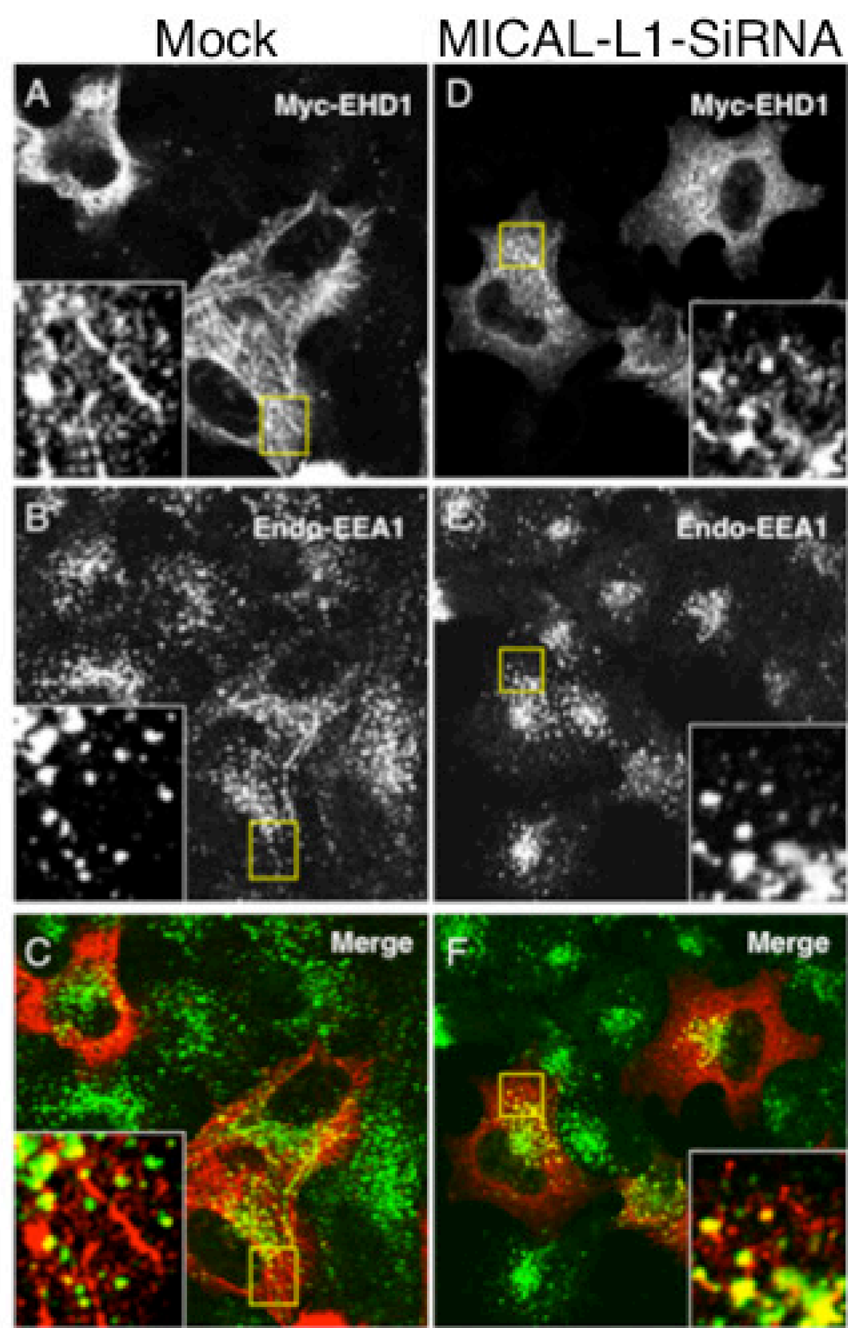
B Endo-MICAL-L1
EHD1+EHD3-RNAi

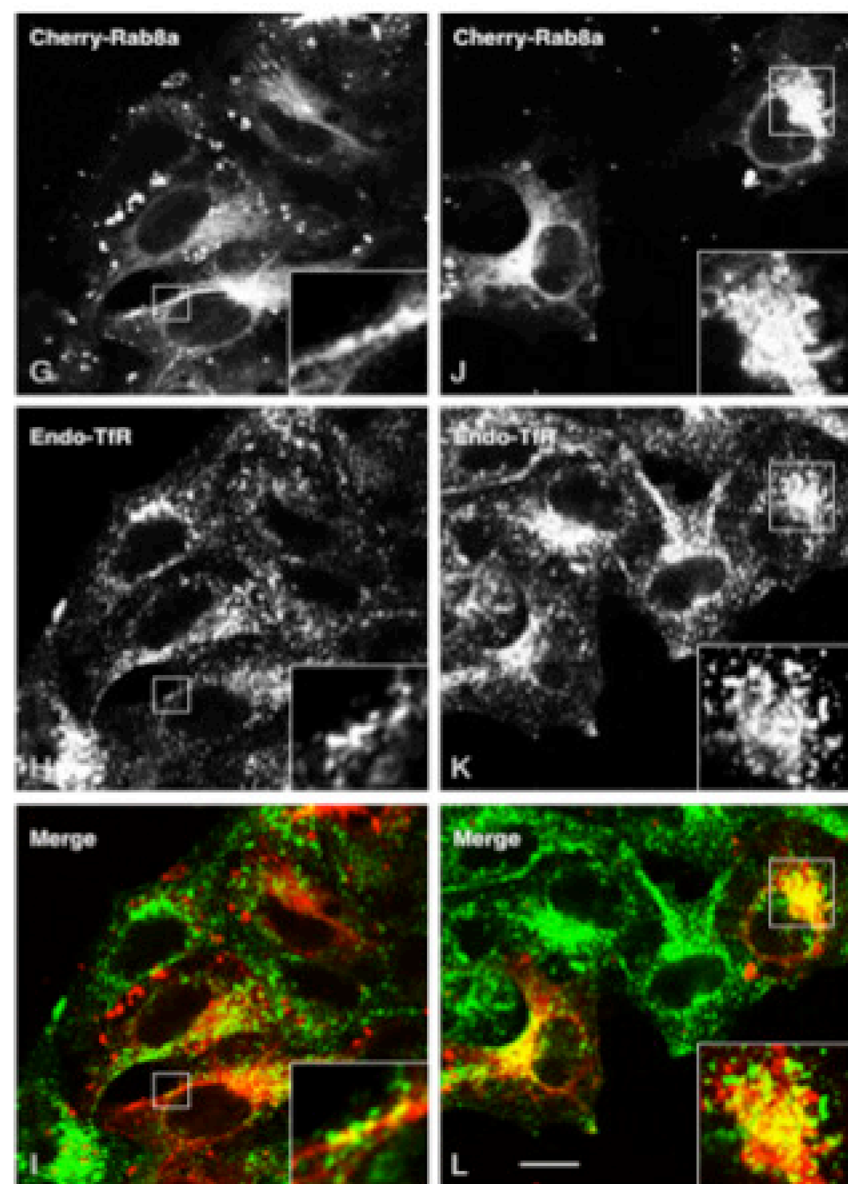
C Endo-MICAL-L1
Mock

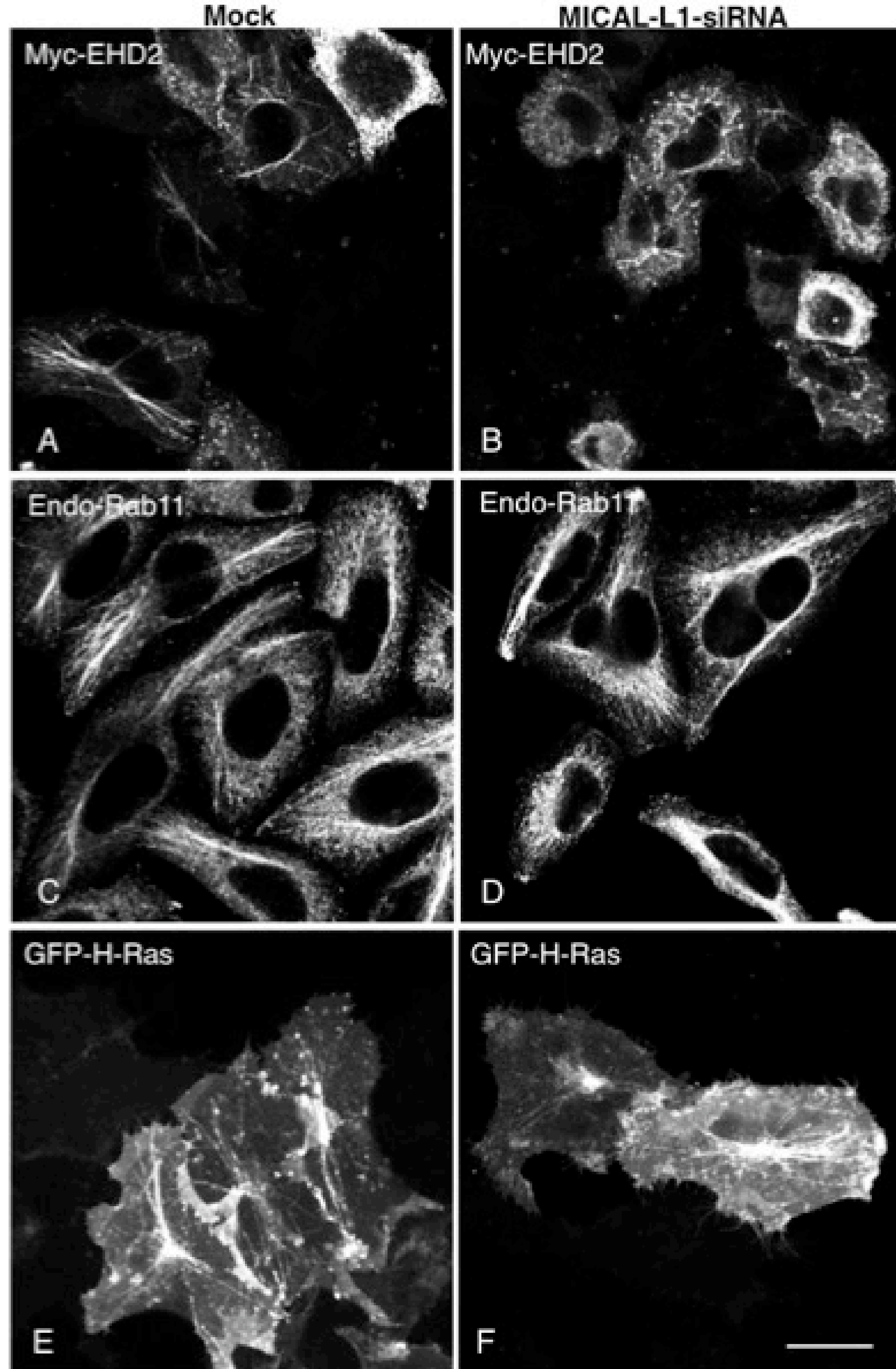
D Endo-MICAL-L1
EHD1+EHD4-SiRNA



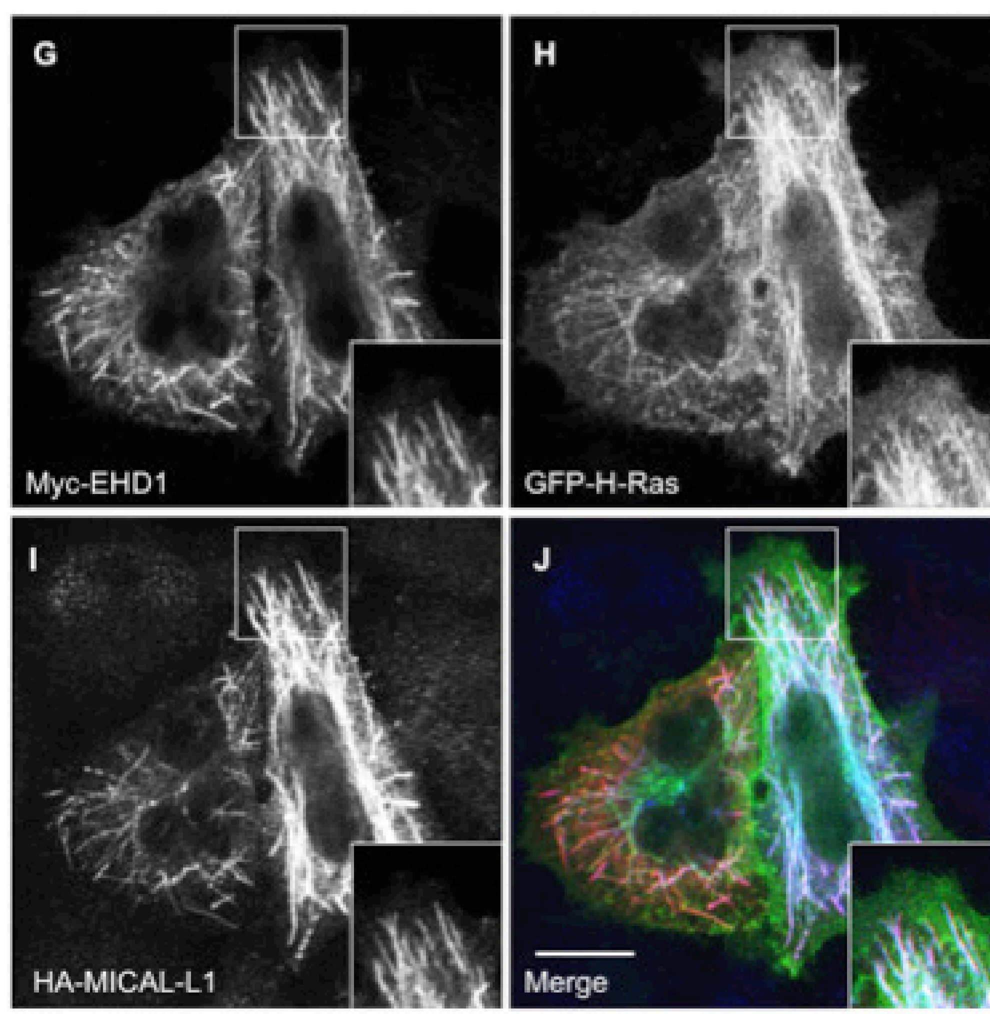

MOCK
MICAL-L1 SiRNA
MOCK
MICAL-L1 SiRNA
A
Myc-EHD3
D
Myc-EHD3
G
HA-EHD4
J
HA-EHD4
B
Endo-MICAL-L1
E
Endo-MICAL-L1
H
Endo-MICAL-L1
K
Endo-MICAL-L1
C
Merge
F
Merge
I
Merge
L
Merge


Mock
MICAL-L1-SiRNA
A
Myc-EHD1
D
Myc-EHD1
B
Endo-EEA1
E
Endo-EEA1
C
Merge
F
Merge


Cherry-Rab8a
G
Cherry-Rab8a
J
Endo-TfR
H
Endo-TfR
K
Merge
I
Merge
L

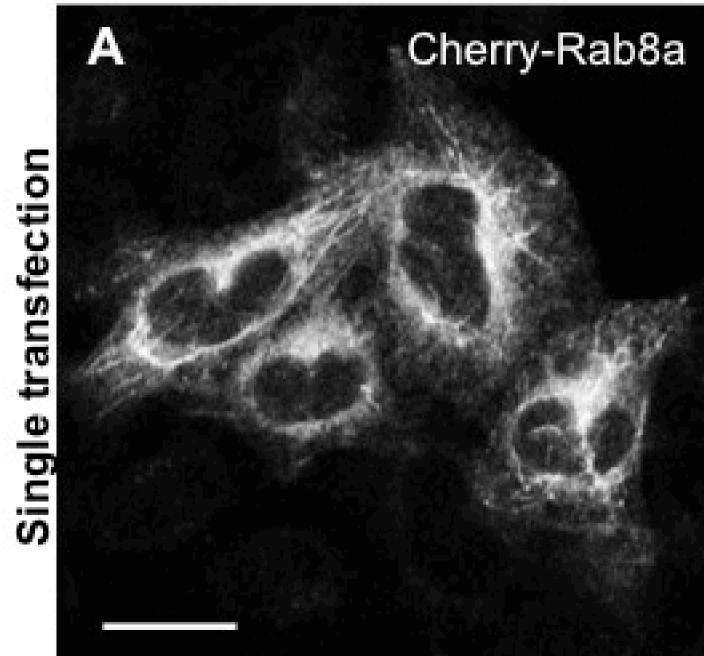


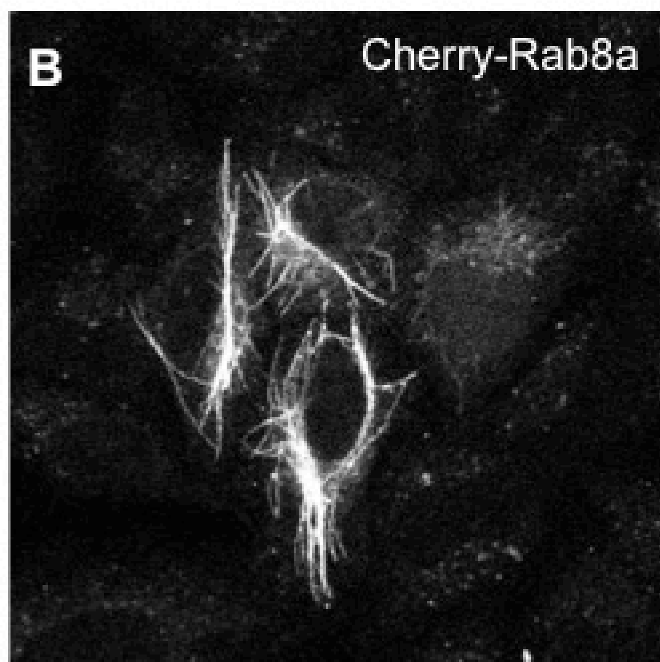
Mock
MICAL-L1-siRNA
Myc-EHD2
Myc-EHD2
A
B
Endo-Rab11
Endo-Rab11
C
D
GFP-H-Ras
GFP-H-Ras
E
F

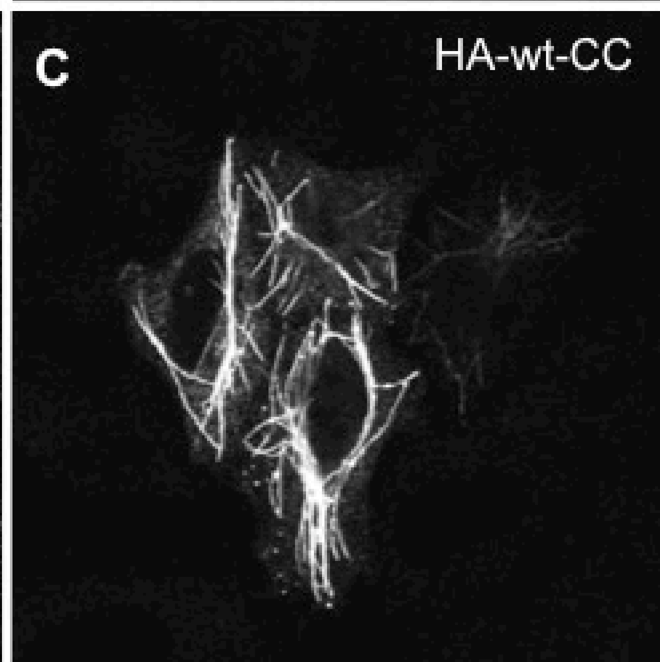


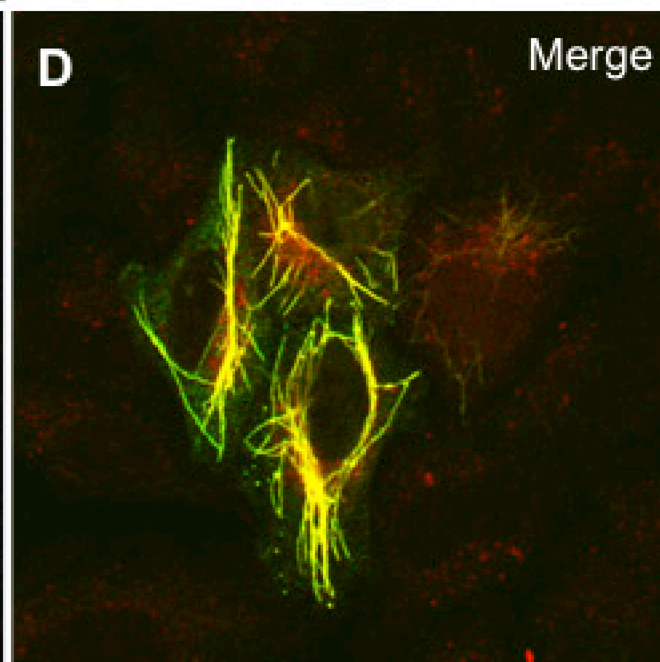
G
H
Myc-EHD1
GFP-H-Ras
I
J
HA-MICAL-L1
Merge

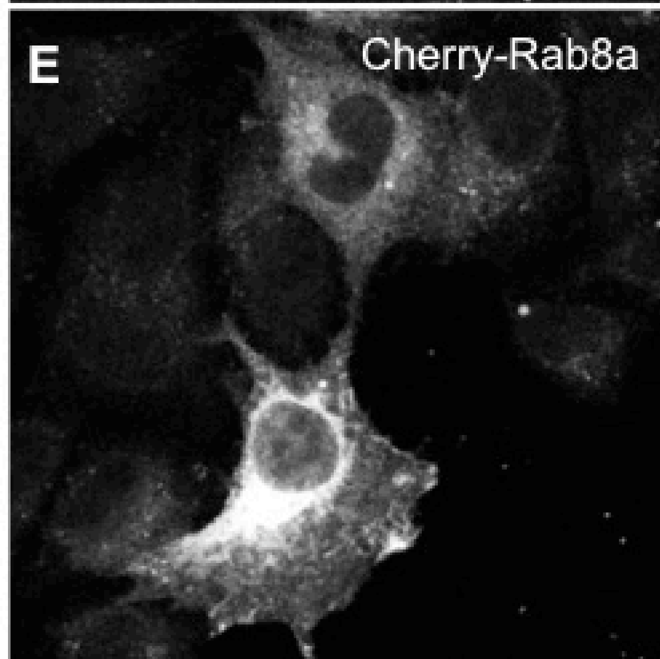
Single transfection

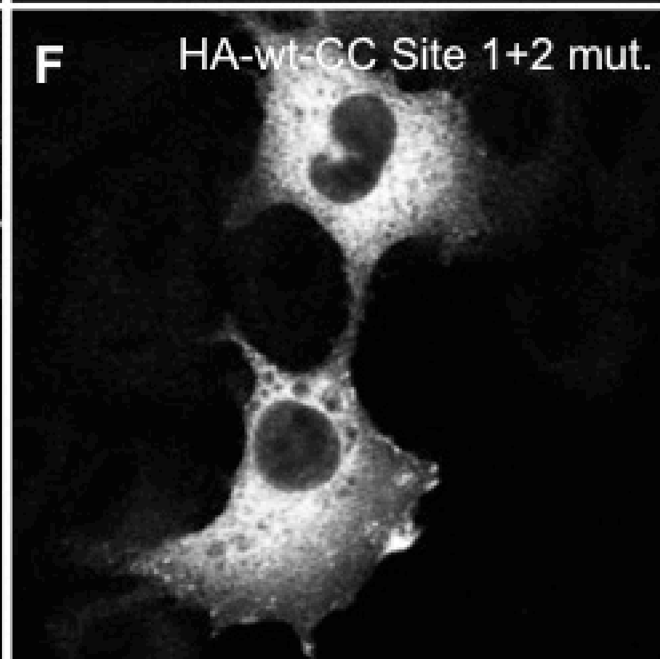


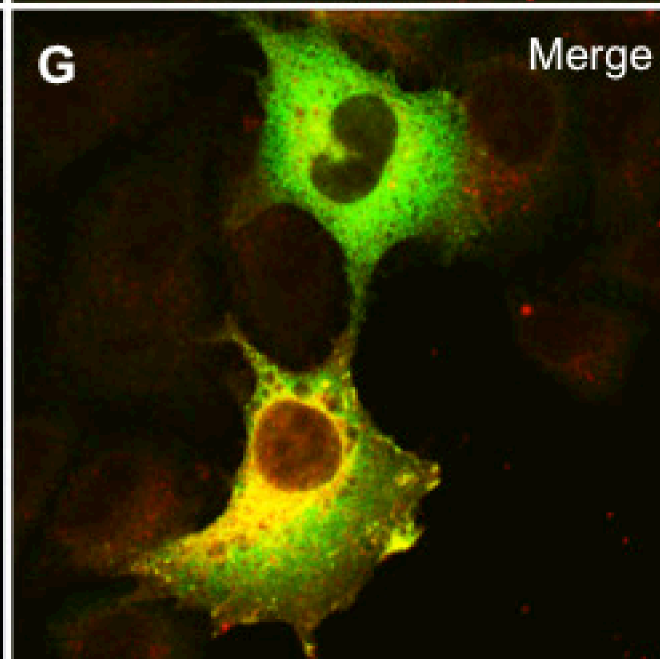


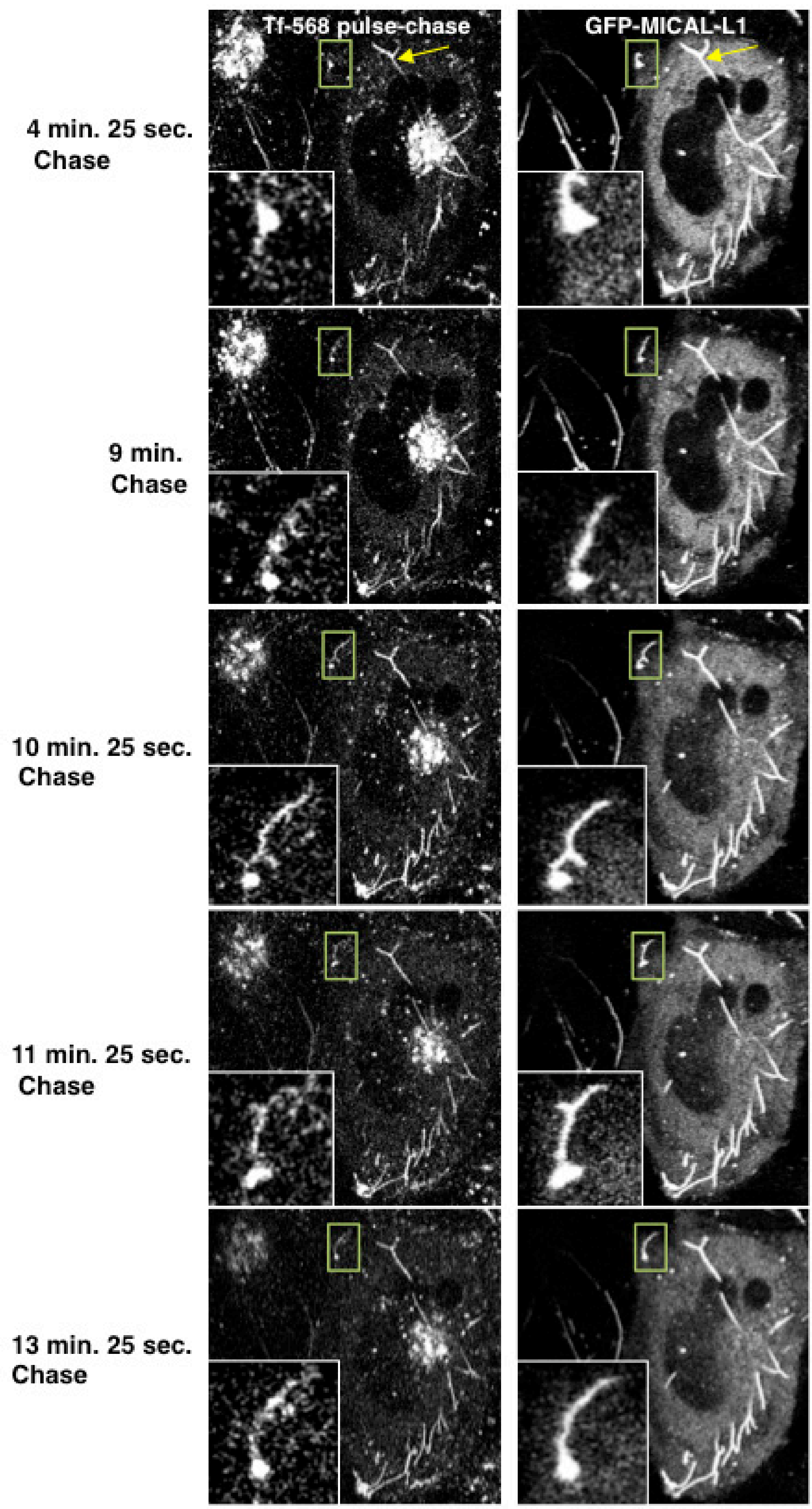


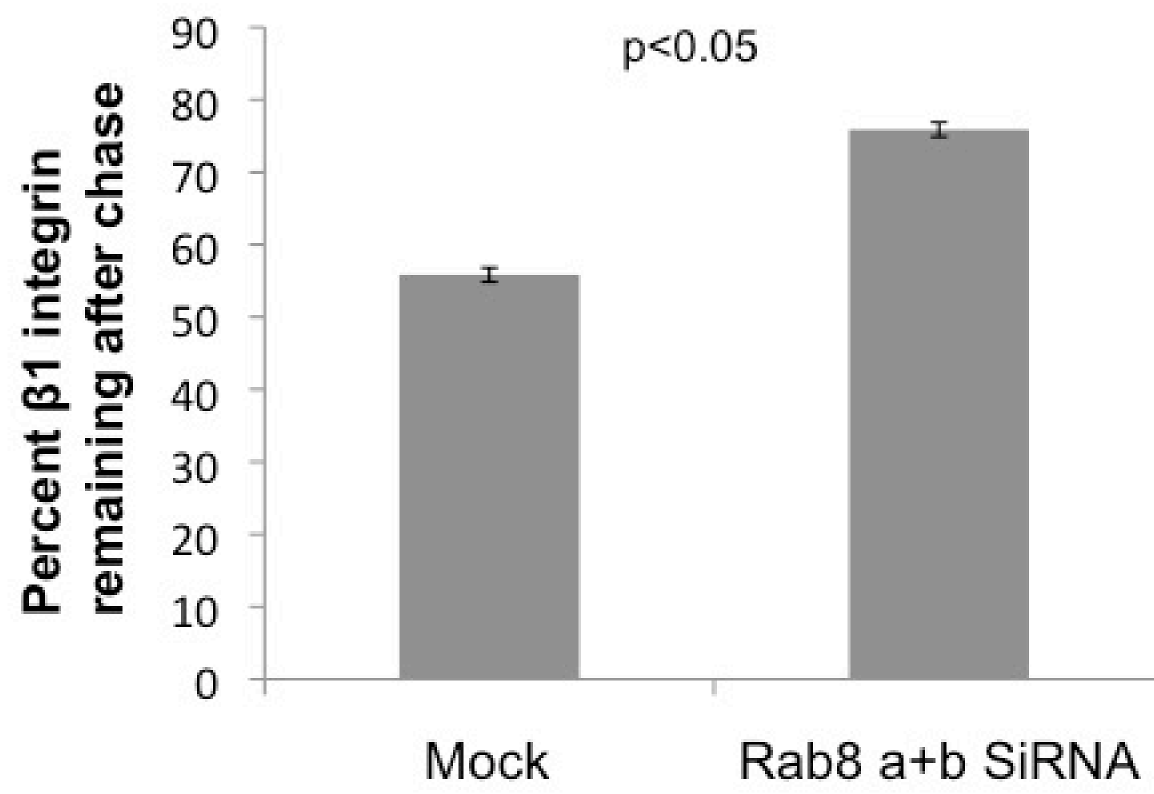









Tf-568 pulse-chase
GFP-MICAL-L1
4 min. 25 sec.
Chase
9 min.
Chase
10 min. 25 sec.
Chase
11 min. 25 sec.
Chase
13 min. 25 sec.
Chase


Percent β1 integrin remaining after chase
p<0.05
90
80
70
60
50
40
30
20
10
0
Mock
Rab8 a+b SiRNA