Supplemental Materials

Molecular Biology of the Cell

Solis et al.

Supplemental Material

Supplemental Figure Legends

Figure S1. Characterization of reggie-tubules. (A-C) Reggie-tubules (R1-EGFP) in HeLa cells did not co-localize with markers of the endoplasmatic reticulum (PDI, A), Golgi (GM130, B) or mitochondria (CoxVI, C). Higher magnification of boxed areas in upper right. Scale bars: 10 μm. (D) Recordings of A431 cells expressing R1-EGFP revealed that reggie-tubules are highly dynamic structures (arrows in I, from boxed region in right panel) where vesicles pinch off the tubules (arrows in II, from boxed region in right panel). Scale bars: 10 μm, and 2 μm for enlarged regions.

Figure S2. Co-localization of reggie-1 and Rab11a. Sections of HeLa cells were immunolabeled using a mAb against reggie-1 (large arrowheads) and a pAb against Rab11a (small arrowheads). (A) A tubular structure, labeled for reggie-1 and Rab11a lies close to the cell surface membrane (CS). (B) Co-localization of Rab11a and reggie-1 occurs along a tubular structure aligned to putative microtubular bundles (framed) and on a vesicular profile nearby, suggesting transport along microtubular rails. (C,D) Non-transfected PC12 cells were double-labeled as HeLa cells in (A, B). A reggie-1 labeled tubule (framed; C) resides adjacent to the cell surface (CS). A large Rab11a-positive compartment (framed; D) is double-labeled with reggie-1. The specificity of labeling is supported by the absence of background. Scale bars: 0.1 μm.

Figure S3. Reggie-tubules belong to the recycling compartment. (A-E) Confocal images of HeLa cells expressing reggie-1 (R1-EGFP, R1-HA or R1-mRFP) show co-localization of reggie-tubules with the recycling-associated GTPases Arf6 (Arf6-DsRed, A), Rab8a (EGFP-Rab8a, B), but not with the retromer-regulating protein SNX1 (EGFP-SNX1, C) or the early endosomal markers EEA1 (D) and Rab4a (EGFP-Rab4a, E). Boxed areas magnified in inserts. Scale bars: 10 μm.

Figure S4. Reggie-tubules associate with components of the exocyst complex. (A-C) Immunostainings of reggie-1-EGFP (R1-EGFP) transfected HeLa cells show reggie-tubules co-localizing with the exocyst component Exo70 (A), whereas Sec5 (B) co-localized with reggie-1 at perinuclear vesicles (B). The exocyst-regulating GTPase RalA (C) was also observed at reggie-tubules. Scale bars: 10 μm. (D) Western blots analysis (WB) shows that recombinant His_6-tagged SNX4 (His-SNX4) was efficiently pulled-down by a GST-fusion construct of the head/SPFH domain of reggie-1 (GST-R1NT), but not by a GST fused to Rab11a (GST-Rab11a) or GST used as control.

Figure S5. Downregulation of reggie-1 impaired formation of Rab11a-tubules and TfR recycling. (A) HeLa cells expressing EGFP-Rab11a show the formation of Rab11a-positive tubules which are significantly reduced in reggie-1 siRNA treated (siR1, right) but not in control transfected (siGL2, left) cells. (B) Quantification of the number of cells showing Rab11a-tubules after reggie-downregulation (n=3, *$p<0.05$, paired *t*-test, error bars: SEM). (C) Western blot analysis (WB) of siRNA-transfected HeLa cells shows the efficiency of reggie-1 (R1) and reggie-2 (R2) downregulation compared to control siGL2 transfected cells. α-tubulin (α-tub) served as loading control. (D) Association of EHD1 (EGFP-EHD1) with tubules is clearly observed in control shLuc cells and persisted after reggie-1 downregulation (shR1). (E) Immunostainings of endogenous reggie-1 (R1) and TfR in a pulse-chase experiment show their co-localization at the perinuclear recycling compartment in HeLa cells (arrowheads). (F,G) A pulse-chase experiment shows the persisting accumulation of endogenous TfR (arrowheads; F) at the perinuclear compartment in shR1 cells, but less frequent in control shLuc cells. Quantification of the effect of reggie-1 downregulation on TfR recycling (G) (n=3, *$p<0.05$, paired *t*-test, error bars: SEM). (H) Immunostaining of the lysosomal marker protein Lamp-2 shows a higher degree of co-localization (arrowheads) with rhodamine-labeled Tf (Tf-rhod) in shR1 cells compared to control shLuc cells in a pulse-chase experiment (images were recorded with different acquisition settings to allow detection of the low perinuclear Tf-rhod signal observed in shLuc cells). Scale bars: 10 μm.

Figure S6. E-cadherin recycling in A431 cells. (A) Recordings of an A431 cell expressing reggie-1-mRFP (R1-mRFP) and E-cadherin-EGFP (E-cad-EGFP) during a Ca^{2+} switch experiment show both proteins at the perinuclear recycling compartment and emerging tubules (arrows) at 30 min after Ca^{2+} repletion. The image shows a cell expressing R1-mRFP and E-cad-EGFP forming contacts with non-transfected cells. Boxed area magnified in left panels. (B) Immunostaining against endogenous reggie-1 and E-cadherin confirmed their co-localization at the perinuclear compartment and tubular structures (arrowhead) emerging from this region after 30 min Ca^{2+} recovery. Boxed area magnified in right panels. (C) Immunostainings shows that internalized E-cadherin (E-cad) at 30 min after Ca^{2+} repletion co-localized with endogenous Rab11a, TfR and Lamp-2 at both the perinuclear recycling compartment and lysosomes. (D) Accumulation of E-cadherin at the recycling compartment during a Ca^{2+} switch experiment was prevented by the dynamin-blocker MiTMAB in both reggie-depleted (shR1) and control (shLuc) A431 cells. Scale bars: 10 μm, and 2 μm for enlarged regions. (E) Western blots from extracts of stably transfected A431 cells show strong reduction of reggie-1 (R1) expression levels in shR1 cells compared to control shLuc cells. The expression levels of Rab11a and SNX4 remained unaffected. α-tubulin (α-tub) was used as loading control.

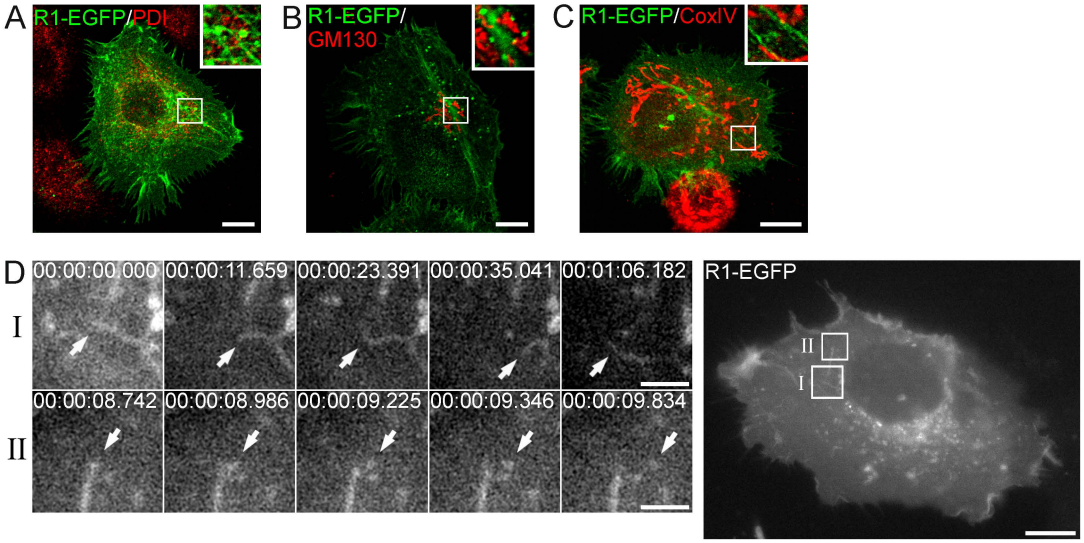
Supplemental Movie legends

Movie S1. Dynamics of reggie-tubules in HeLa cells. HeLa cells were transfected with reggie-1-EGFP and recorded using a Colibri imaging system. Tubular structures are highly dynamic and can be seen to derive from the perinuclear recycling compartment and move towards the PM as well as from the PM to the recycling compartment. Vesicles can be seen to fuse with the PM and travel back to the recycling compartment.

Movie S2. Dynamics of reggie-tubules in A431 cells. A431 cells were transfected with reggie-1-EGFP and recorded as in Movie S1. Reggie-tubules are highly dynamic and move to and from the PM. Vesicles are observed traveling through the cell to the PM and back.

Movie S3. Trafficking of E-cadherin in reggie-tubules in A431 cells. A431 cells were transfected with reggie-1-mRFP and E-cadherin-EGFP and recorded as in Movie S1 at 60 min after Ca^{2+} repletion (Ca^{2+} switch experiment). Both proteins are observed at the perinuclear recycling compartment and trafficking in tubules and vesicles.

Figure S1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R1-EGFP/PDI
B
R1-EGFP/
GM130
C
R1-EGFP/CoxIV
D
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00:00:11.659
00:00:23.391
00:00:35.041
00:01:06.182
I
00:00:08.742
00:00:08.986
00:00:09.225
00:00:09.346
00:00:09.834
II
R1-EGFP
II
I

Figure S2

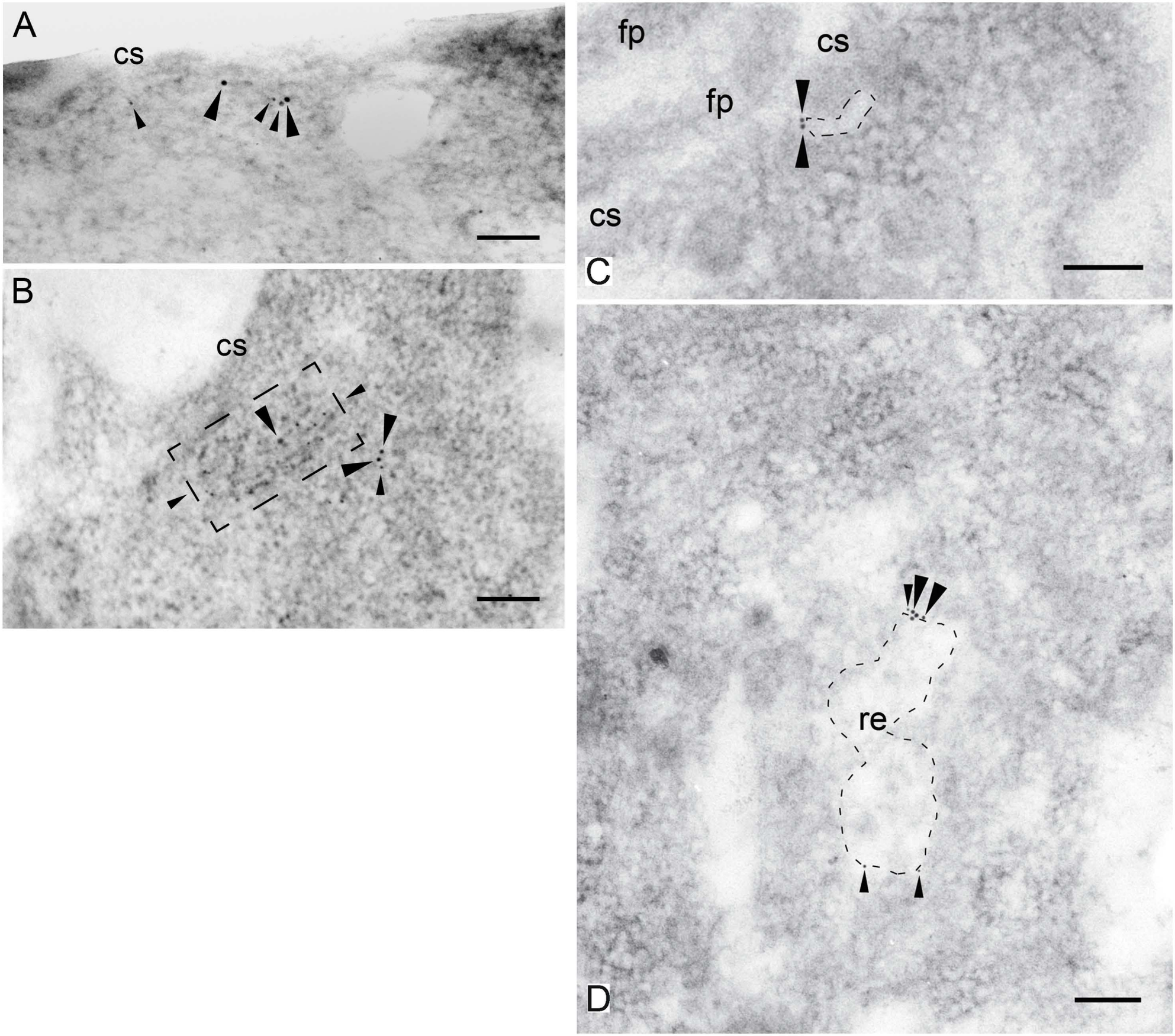


Figure S3

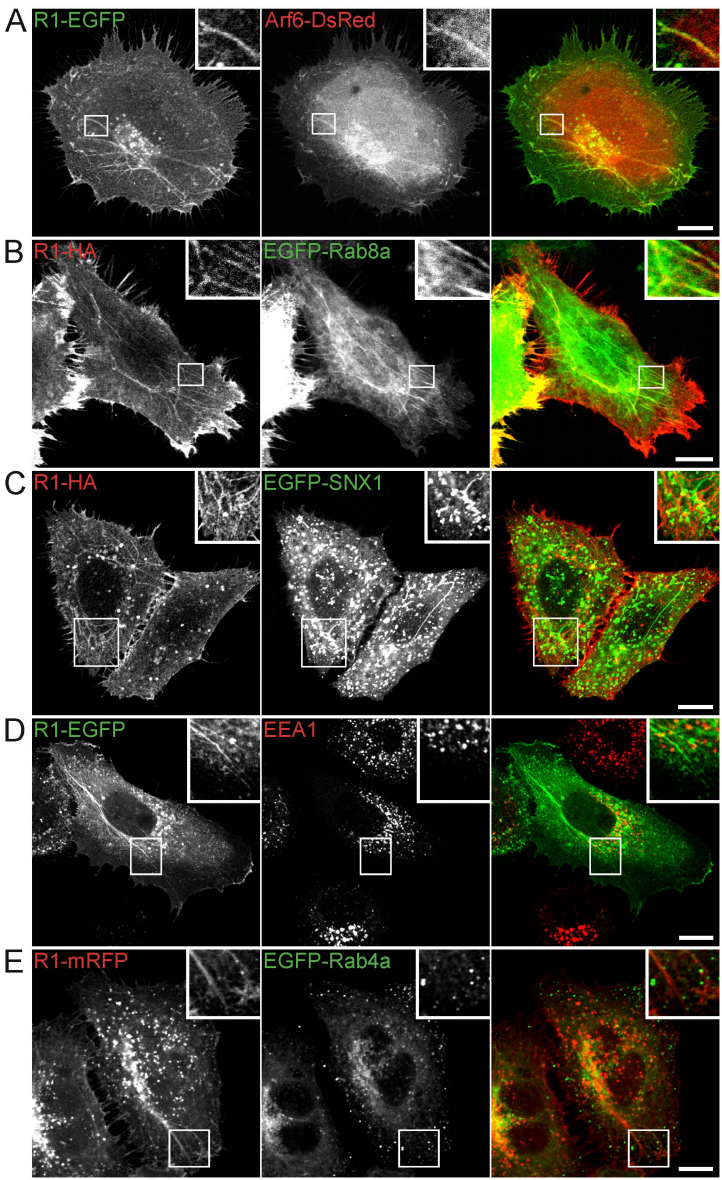
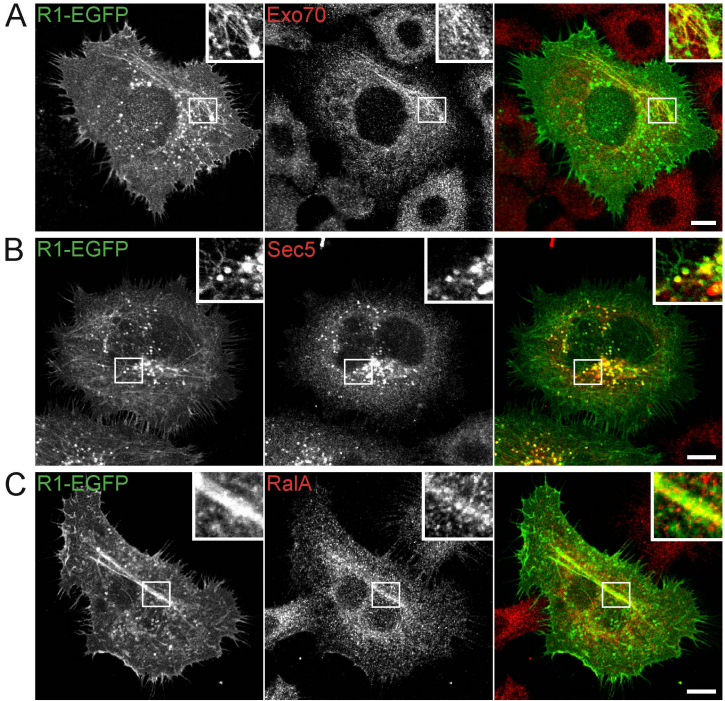

A
R1-EGFP
Arf6-DsRed
B
R1-HA
EGFP-Rab8a
C
R1-HA
EGFP-SNX1
D
R1-EGFP
EEA1
E
R1-mRFP
EGFP-Rab4a

Figure S4


A
R1-EGFP
Exo70
B
R1-EGFP
Sec5
C
R1-EGFP
RalA

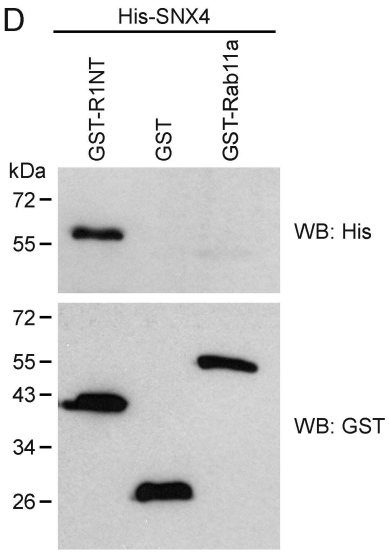

D
His-SNX4
GST-R1NT
GST
GST-Rab11a
kDa
72
55
WB: His
72
55
43
34
26
WB: GST

Figure S5

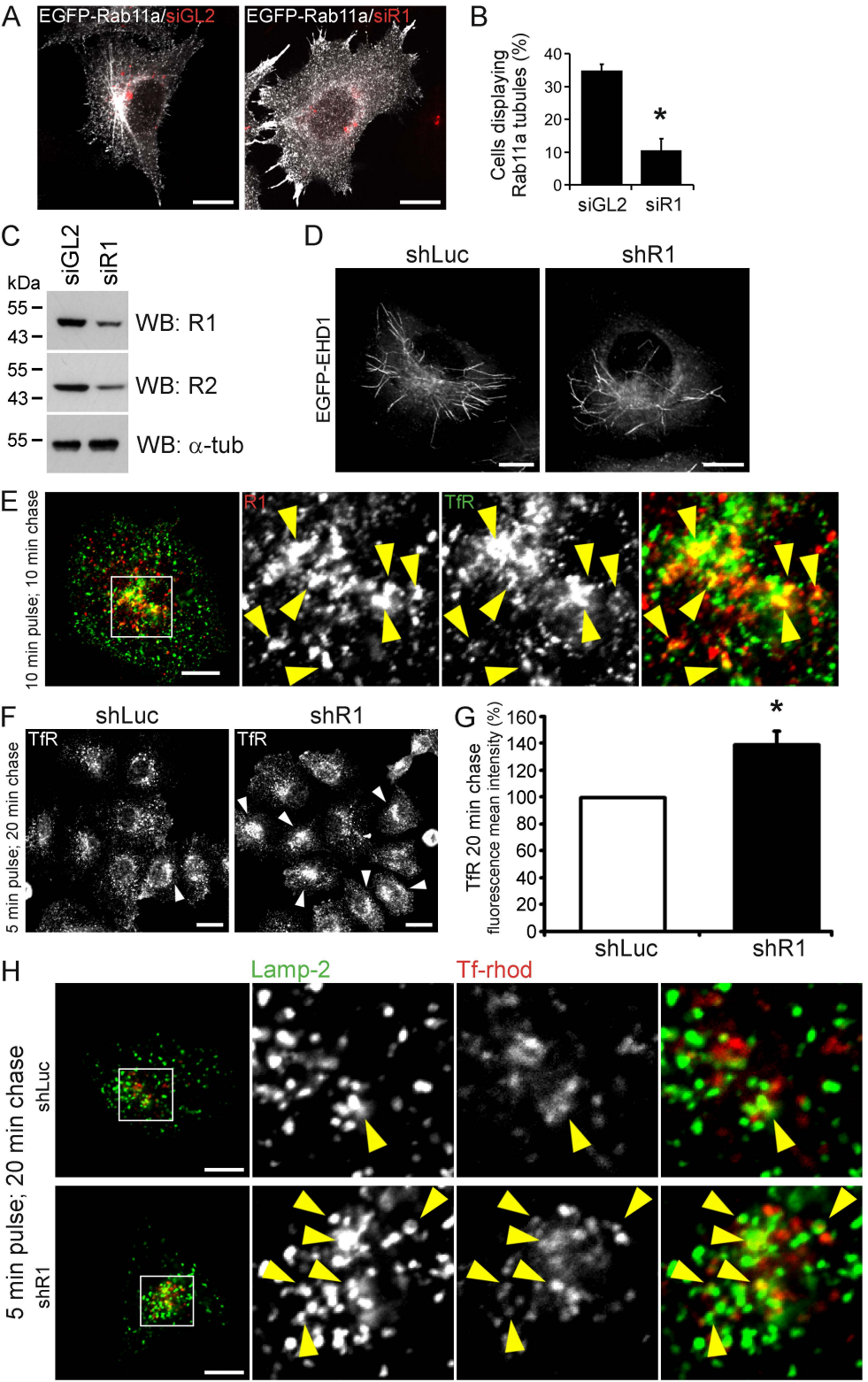
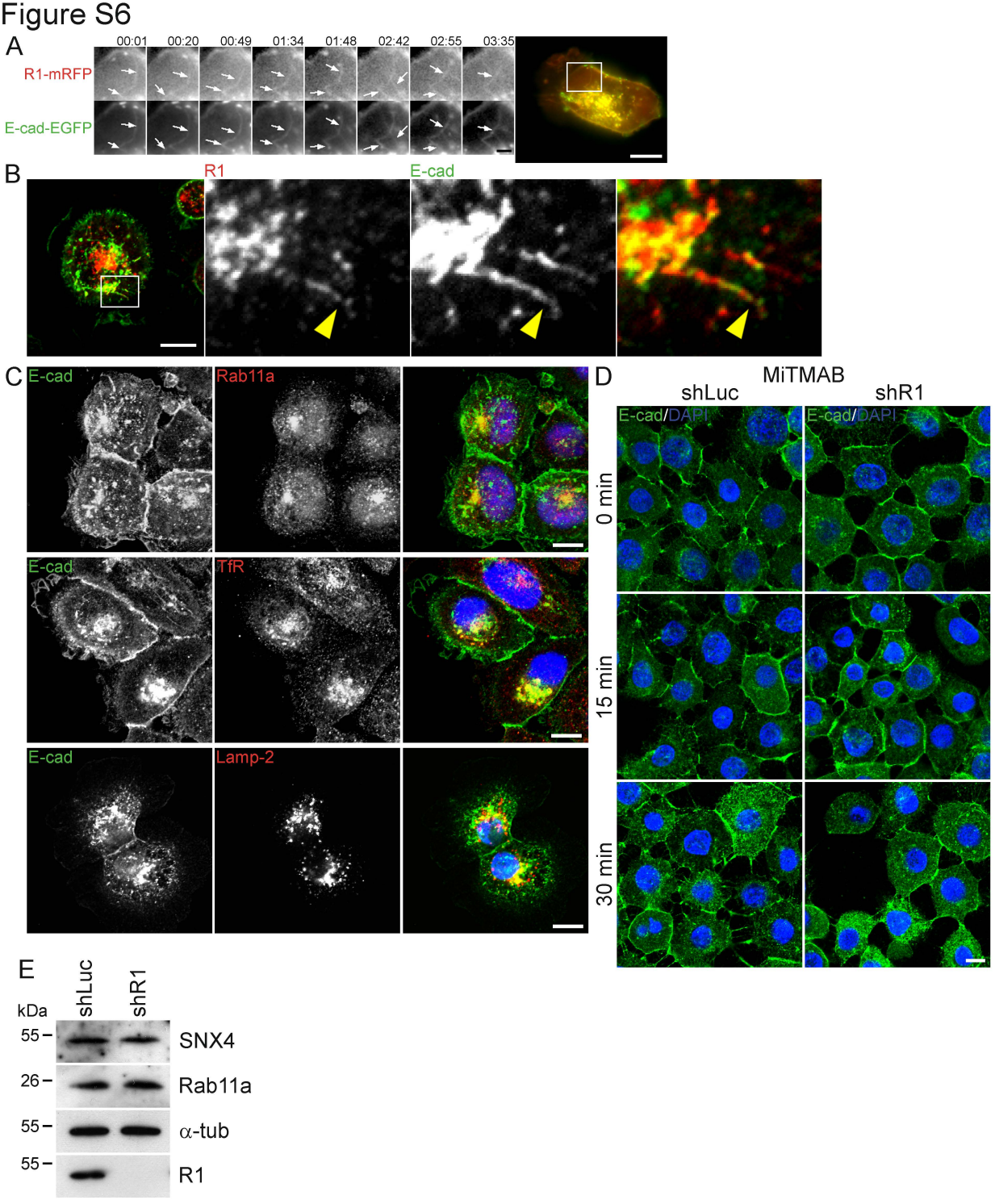

A
EGFP-Rab11a/siGL2
EGFP-Rab11a/siR1
B
Cells displaying Rab11a tubules (%)
40
30
20
10
0
*
siGL2
siR1
C
siGL2
siR1
kDa
55
43
WB: R1
55
43
WB: R2
55
WB: α-tub
D
shLuc
shR1
EGFP-EHD1
E
10 min pulse; 10 min chase
R1
TfR
F
shLuc
shR1
5 min pulse; 20 min chase
TfR
TfR
G
TfR 20 min chase fluorescence mean intensity (%)
160
140
120
100
80
60
40
20
0
*
shLuc
shR1
H
Lamp-2
Tf-rhod
5 min pulse; 20 min chase
shLuc
shR1

Figure S6


A
00:01
00:20
00:49
01:34
01:48
02:42
02:55
03:35
R1-mRFP
E-cad-EGFP
B
R1
E-cad
C
E-cad
Rab11a
E-cad
TfR
E-cad
Lamp-2
D
MiTMAB
shLuc
shR1
E-cad/DAPI
E-cad/DAPI
0 min
15 min
30 min
E
kDa
shLuc
shR1
55
SNX4
26
Rab11a
55
α-tub
55
R1