Supplementary Figure Legends

Figure S1. HaloTag-ESCRT fusion proteins are functional. (A) Quantification of the doubling time for CRISPR/Cas9-modified cell lines as compared to the parental control. Error bars represent mean +/- SEM (n=4 each). No statistically significant differences were found, as calculated using an ANOVA test. (B) Quantification of the relative growth rate of control cells and cells depleted of specified ESCRT subunits. Error bars represent mean +/- SEM (n=4 each). **$p < 0.01$, as calculated using an ANOVA test. (C) Representative immunoblot analyses of control and ESCRT subunit depleted cells (n=3 each) using antibodies directed against Hrs (left), Tsg101 (middle), Vps4B (right), and beta-actin (bottom, load control). (D) Quantification of the relative growth rate (% of mock depleted cells) of control RPE1 cells and cells expressing Vps4B-HaloTag following depletion of Vps4A. Error bars represent mean +/- SEM (n=4 each). No statistically significant difference was found, based on a t-test. (E) Representative cells natively expressing Vps4B-HaloTag were transfected with a GFP-tagged form of Cep55 and imaged live using swept field confocal optics (more than 10 cells imaged; more than 3 biological replicates). Scale bars, 10 μm and 2 μm (zoomed panels). (F) Representative control cells, CRISPR/Cas9-modified cells, and siRNA depleted cells (targeting Hrs or Vps4 isoforms) imaged following fixation and staining using antibodies directed against EEA1 (top) or LAMP1 (bottom) using swept field confocal optics (more than 15 different cells each; more than 3 biological replicates each). Maximum intensity projections of z-stacks are shown. Scale bar, 5 μm. (G) Size distribution of EEA1- (top) and LAMP1-positive (bottom) endosomes in control cells, CRISPR/Cas9-modified cells, and cells depleted of specified ESCRT subunits (based on the analysis of more than 15 different cells each; more than 3 biological replicates each).

Figure S2. The ESCRT complexes function cooperatively. (A) Based on immunoblot analysis using anti-HaloTag antibodies (n=4), the relative expression levels of HaloTag-ESCRT

fusion proteins were quantified. (B) Representative cells natively expressing either HaloTag-Hrs or HaloTag-Vps4B were transfected with a GFP-tagged probe that recognizes endosomal PI3P and imaged using swept field confocal optics (more than 10 cells imaged; more than 3 biological replicates). Scale bars, 5 μm. (C and D) Based on lattice light sheet imaging, the size distributions of endosomes harboring HaloTag-Hrs (C) or HaloTag-Tsg101 (D) were determined (more than 600 endosomes analyzed for each cell line; more than 10 cells imaged per condition in more than 3 biological replicates). (E) Distributions of the residency times of HaloTag-Hrs and HaloTag-Tsg101 on endosomes of three size classifications (more than 600 endosomes analyzed for each cell line; more than 10 cells each in more than 3 biological replicates). (F) Representative trajectories of HaloTag-Hrs positive endosomes under normal growth conditions (two views shown; based on more than 600 endosomes examined). Scale bar, 5 μm. (G and H) Representative HaloTag-Hrs expressing cells imaged live using lattice light sheet microscopy following dye labeling using the JF646–HaloTag ligand (n > 10 cells each; more than 3 biological replicates each). Projected z-stacks are shown for each timepoint. Arrows (G) highlight ESCRT-positive endosomes that come into transient or sustained contact with another endosome. A second timeseries further demonstrating ESCRT-positive endosome fusion (H). Scale bars, 5 μm; inset bar, 2 μm (G); and 2 μm (H). (I) The frequency of HaloTag-Hrs positive endosome contacts were determined based on particle tracking and plotted (more than 600 endosomes analyzed for each cell line; more than 10 cells each in more than 3 biological replicates).

Figure S3. ESCRT dynamics at MVEs. (A and B) Representative trajectories of HaloTag-Hrs positive endosomes following depletion of either Tsg101 (A) or Vps4 isoforms (B). Two views are shown (based on more than 600 endosomes examined). Scale bar, 5 μm. (C) The frequency of HaloTag-Hrs positive endosome contacts were determined following depletion of

Tsg101 or Vps4 isoforms based on particle tracking and plotted (more than 600 endosomes analyzed for each cell line; more than 10 cells each in more than 3 biological replicates). (D and E) Quantification of the average duration of HaloTag-Hrs (D) or HaloTag-Tsg101 (E) on endosomes in control and Vps4-depleted cells. Error bars represent mean +/- SEM (more than 400 endosomes analyzed for each cell line; more than 10 cells imaged per condition in more than 3 biological replicates). ***$p < 0.001$, as calculated using a Student's t test. (F) Representative cells natively expressing either HaloTag-Tsg101 (left) or HaloTag-Vps4B (right) were imaged using swept field confocal optics after growth factor deprivation (more than 10 cells imaged; more than 3 biological replicates). Scale bar, 10 μm. (G) Cells expressing HaloTag-ESCRT fusion proteins were imaged using lattice light sheet microscopy, and representative endosomes exhibiting oscillations in ESCRT complex accumulation are shown. Graphical representations of fluorescence intensities at individual endosomes over time are shown (right), and color coordinated arrows highlight peak fluorescence intensities achieved at each of them. Scale bar, 2 μm. (H) Representative MVEs found in RPE1 cells grown in the presence (right) or absence (left) of 30 ng/mL EGF were imaged following high pressure freezing and freeze substitution using electron tomography. Reconstructed models are shown. Scale bars, 100 nm. (I) Representative HaloTag-Hrs expressing cell imaged live using STED microscopy following dye labeling using the SiR–HaloTag ligand and incubation with Alexa Fluor 594-EGF. A dotted line highlights where linescan analysis was performed to measure degree relative distributions of Hrs and EGF, and individual fluorescence channels are also shown below. Scale bar, 500 nm.

Supplementary Movie Legends

Movie S1. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing HaloTag-Hrs as determined using lattice light sheet microscopy. Playback rate, 24x.

Movie S2. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing HaloTag-Tsg101 as determined using lattice light sheet microscopy. Playback rate, 24x.

Movie S3. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing Vps4B-HaloTag as determined using lattice light sheet microscopy. Playback rate, 24x.

Movie S4. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing HaloTag-Hrs following stimulation by EGF (30 ng/mL) as determined using lattice light sheet microscopy. Playback rate, 48x.

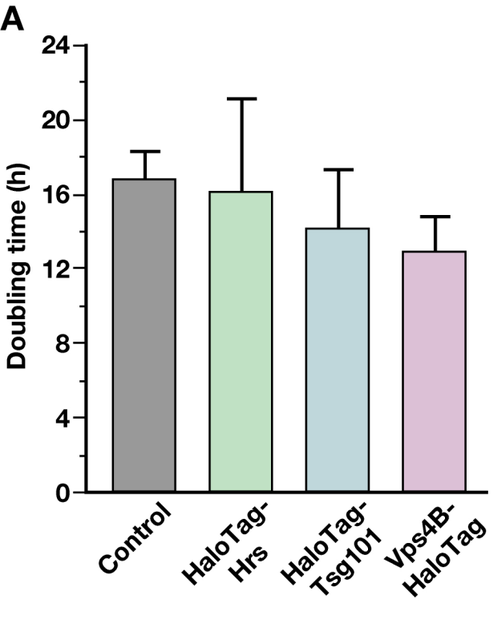
Movie S5. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing HaloTag-Tsg101 following stimulation by EGF (30 ng/mL) as determined using lattice light sheet microscopy. Playback rate, 48x.

Movie S6. Representative movie showing endosome dynamics in CRISPR/Cas9-modified cells expressing Vps4B-HaloTag following stimulation by EGF (30 ng/mL) as determined using lattice light sheet microscopy. Playback rate, 48x.

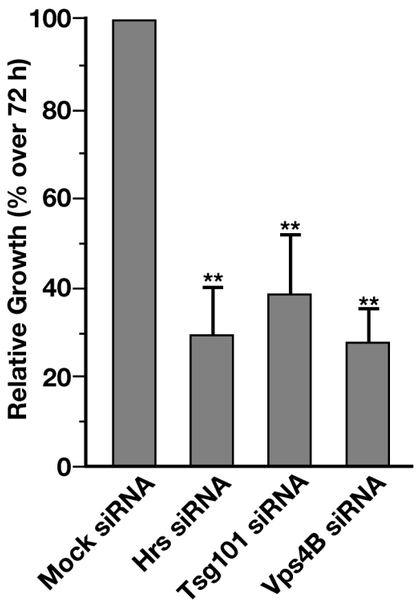
Movie S7. Representative electron tomogram of a MVE in RPE1 cells. High pressure frozen, control RPE1 cells were processed for electron tomography following growth on sapphire discs. Bar, 100 nm.

Movie S8. Representative electron tomogram of a MVE in RPE1 cells following EGF stimulation. High pressure frozen RPE1 cells were processed for electron tomography following treatment with 30 ng/mL EGF. Bar, 100 nm.

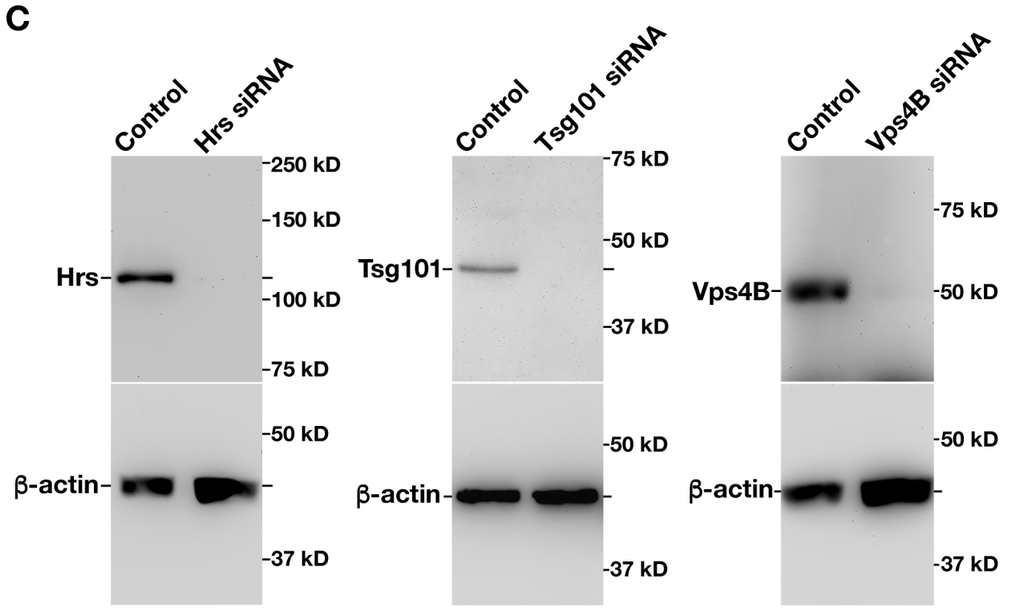
A


Doubling time (h)
0
4
8
12
16
20
24
Control
HaloTag-Hrs
HaloTag-Tsg101
Vps4B-HaloTag

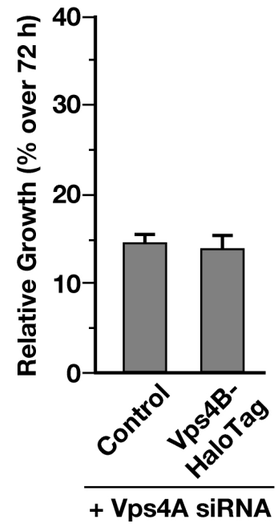
B


Relative Growth (% over 72 h)
0
20
40
60
80
100
**
**
**
Mock siRNA
Hrs siRNA
Tsg101 siRNA
Vps4B siRNA

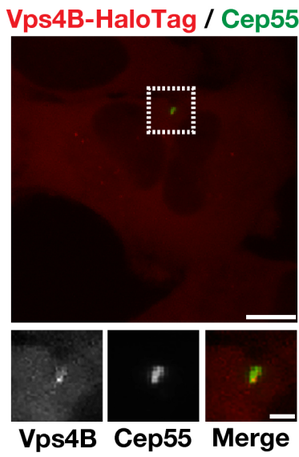
C


Control
Hrs siRNA
Hrs
β-actin
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
Control
Tsg101 siRNA
Tsg101
β-actin
75 kD
50 kD
37 kD
Control
Vps4B siRNA
Vps4B
β-actin
75 kD
50 kD
50 kD
37 kD

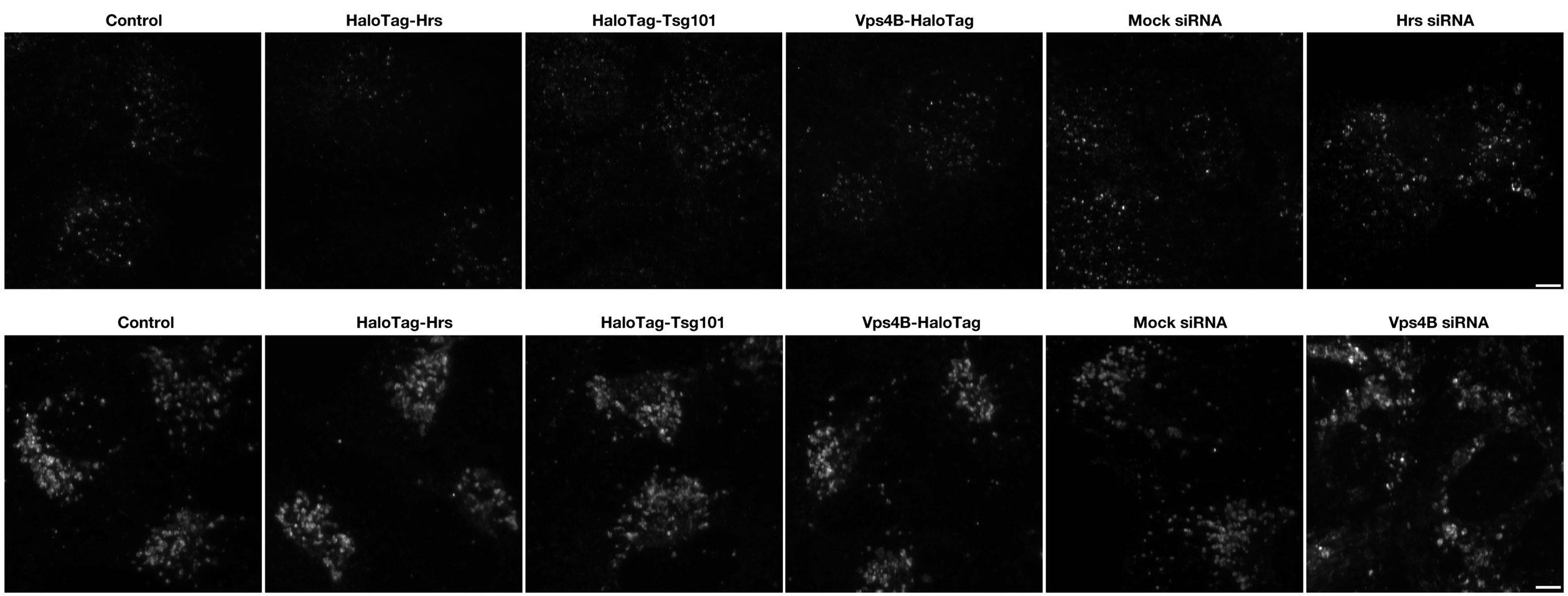
D


Relative Growth (% over 72 h)
0
10
20
30
40
Control
Vps4B-HaloTag
+ Vps4A siRNA

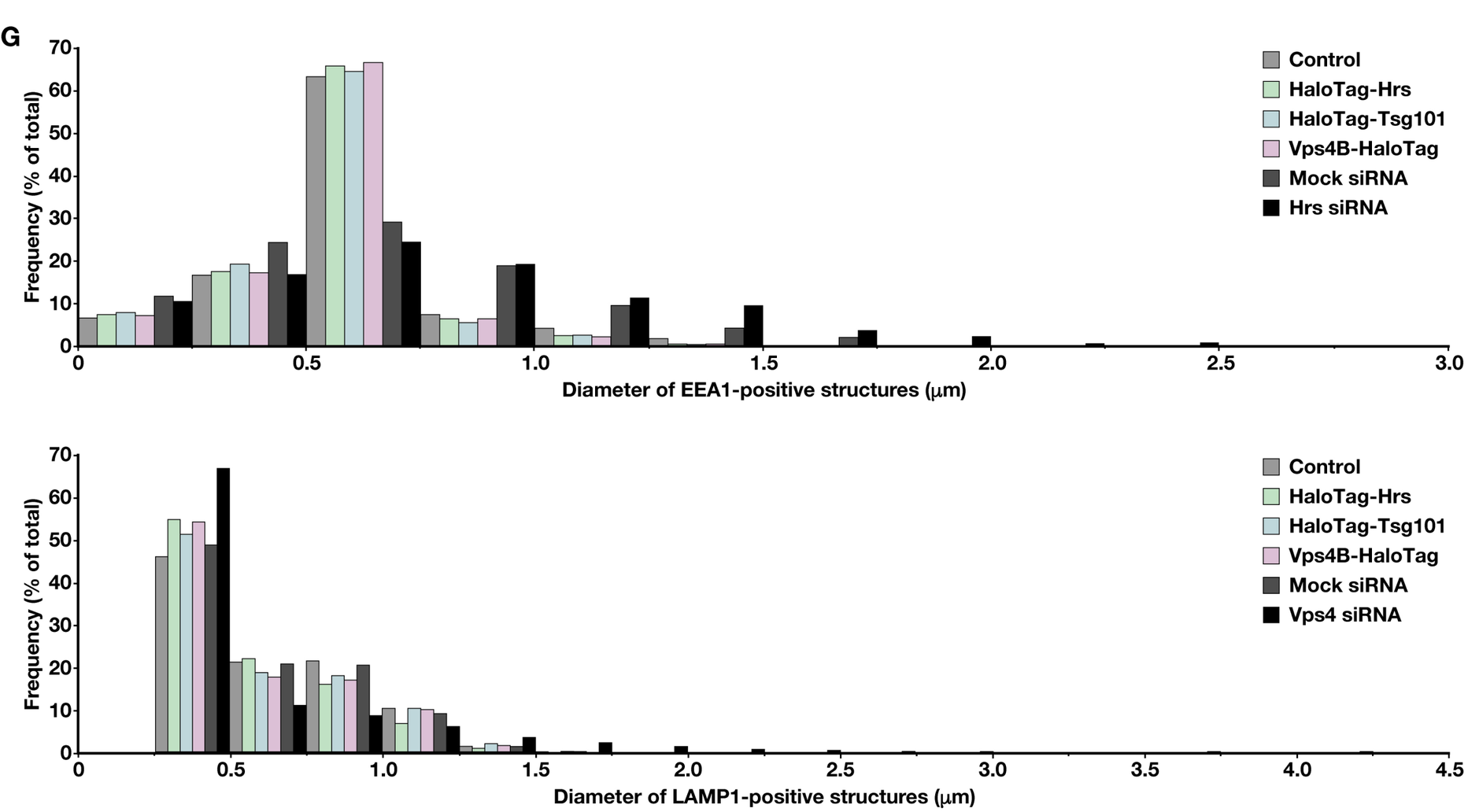
E


Vps4B-HaloTag / Cep55
Vps4B
Cep55
Merge

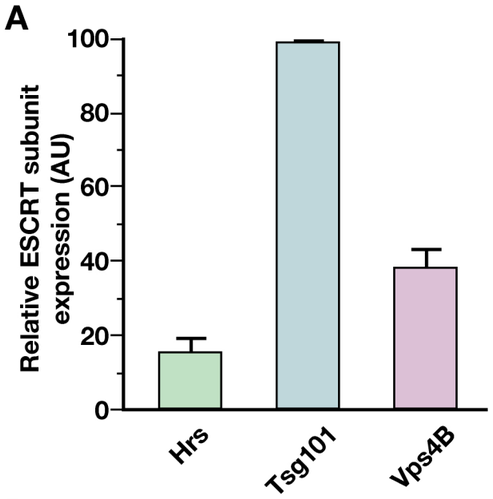
F


Control
HaloTag-Hrs
HaloTag-Tsg101
Vps4B-HaloTag
Mock siRNA
Hrs siRNA
EEA1
Control
HaloTag-Hrs
HaloTag-Tsg101
Vps4B-HaloTag
Mock siRNA
Vps4B siRNA
LAMP1

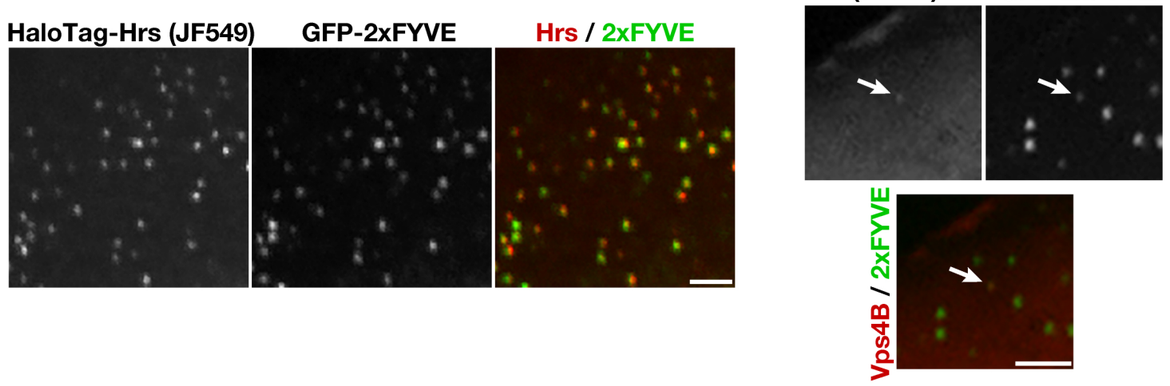
G


Frequency (% of total)
0
10
20
30
40
50
60
70
0
0.5
1.0
1.5
2.0
2.5
3.0
Diameter of EEA1-positive structures (μm)
Control
HaloTag-Hrs
HaloTag-Tsg101
Vps4B-HaloTag
Mock siRNA
Hrs siRNA
Frequency (% of total)
0
10
20
30
40
50
60
70
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Diameter of LAMP1-positive structures (μm)
Control
HaloTag-Hrs
HaloTag-Tsg101
Vps4B-HaloTag
Mock siRNA
Vps4 siRNA

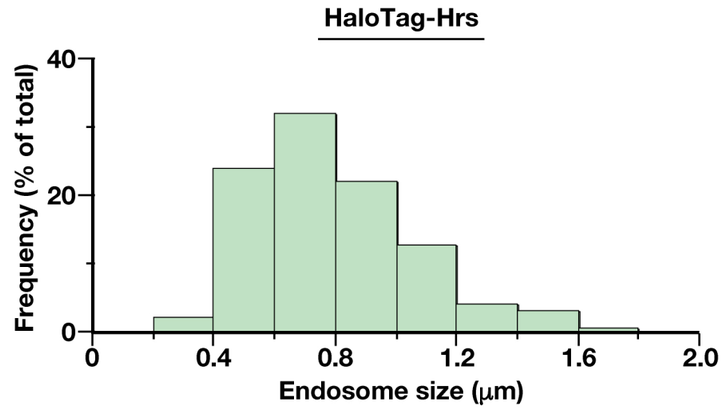
A


Relative ESCRT subunit expression (AU)
100
80
60
40
20
0
Hrs
Tsg101
Vps4B

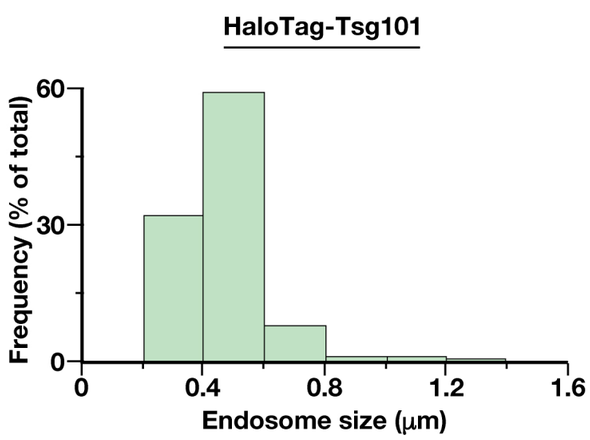
B


HaloTag-Hrs (JF549)
GFP-2xFYVE
Hrs / 2xFYVE
Vps4B-HaloTag (JF549)
GFP-2xFYVE
Vps4B / 2xFYVE

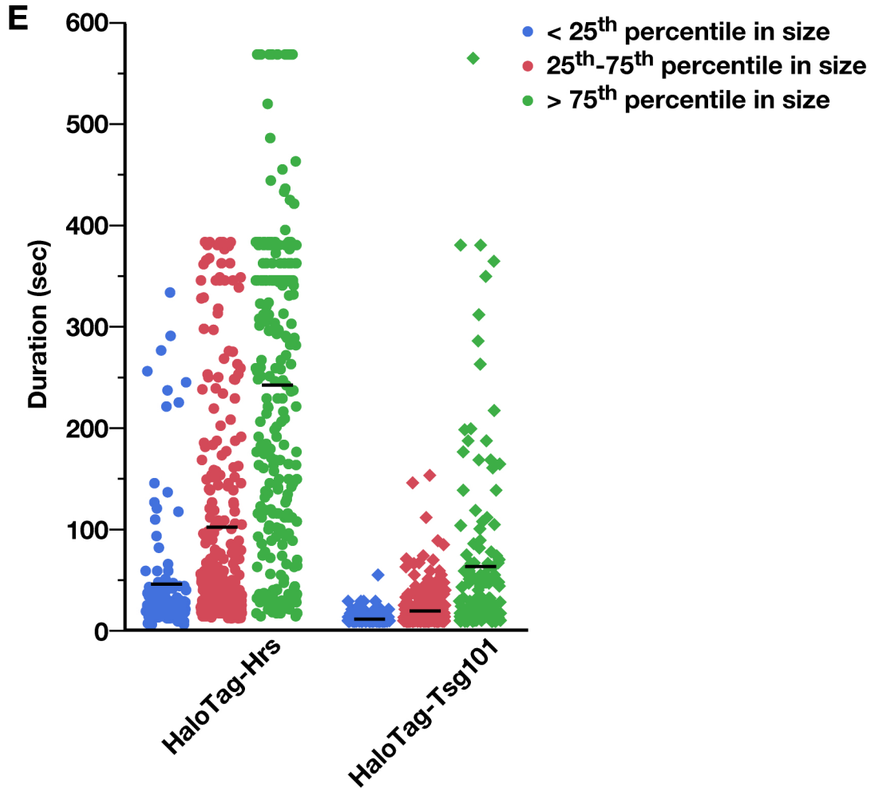
C


HaloTag-Hrs
Frequency (% of total)
40
20
0
0
0.4
0.8
1.2
1.6
2.0
Endosome size (μm)

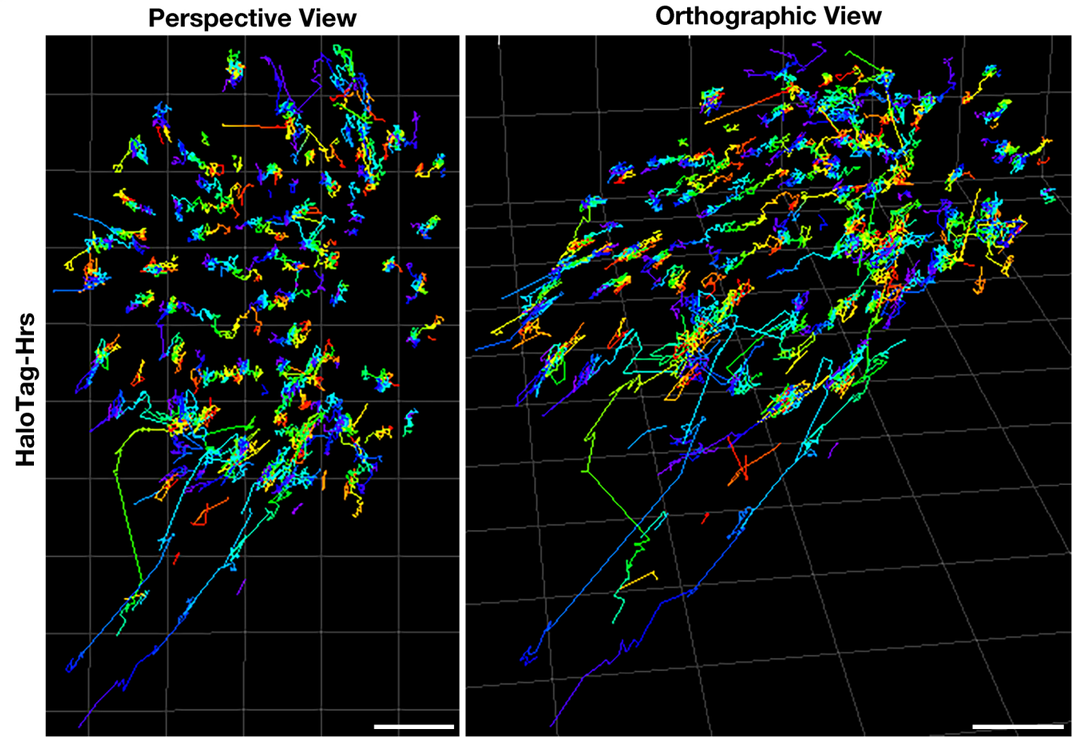
D


HaloTag-Tsg101
Frequency (% of total)
60
30
0
0
0.4
0.8
1.2
1.6
Endosome size (μm)

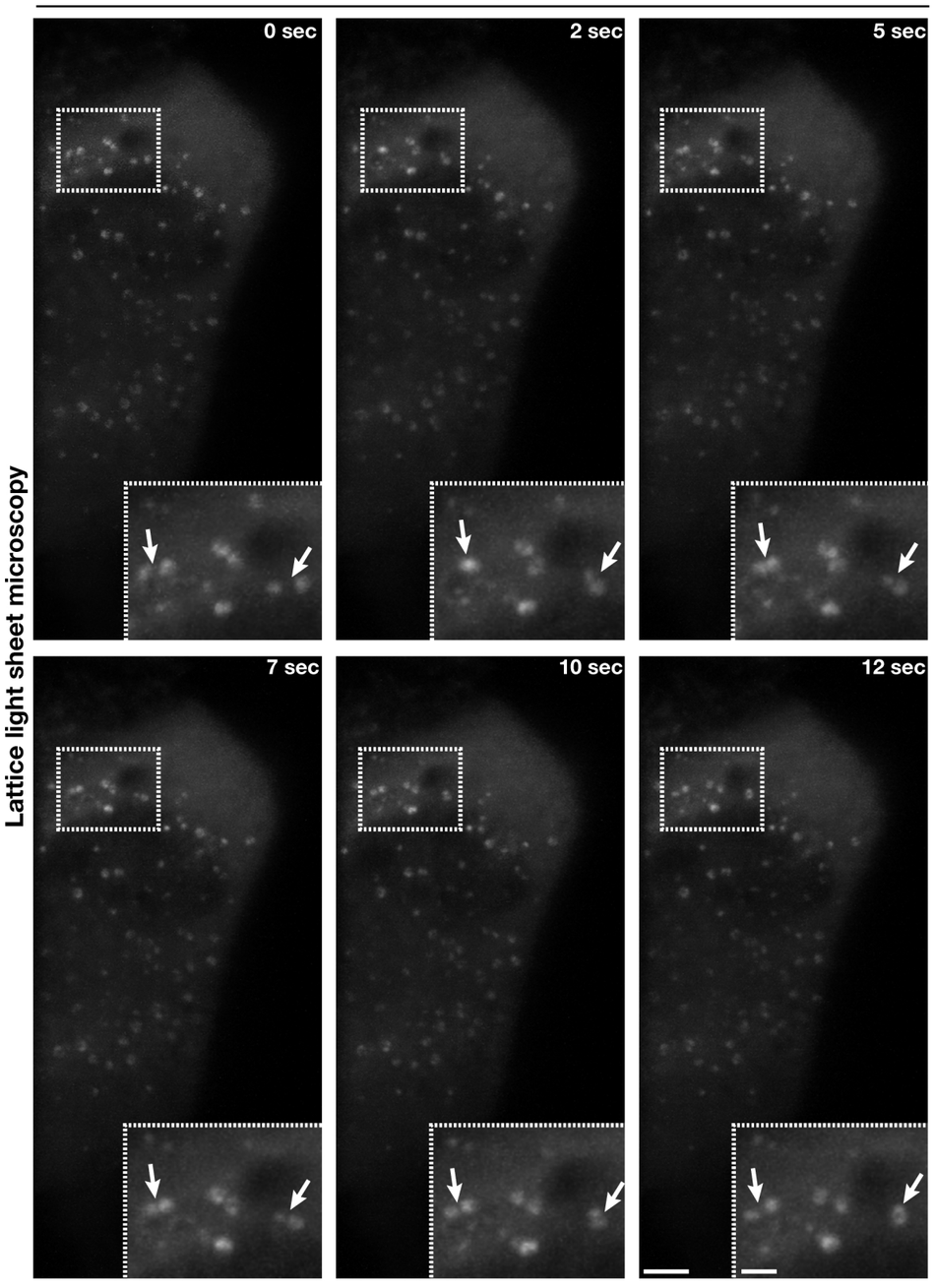
E


< 25th percentile in size
25th-75th percentile in size
> 75th percentile in size
Duration (sec)
600
500
400
300
200
100
0
HaloTag-Hrs
HaloTag-Tsg101

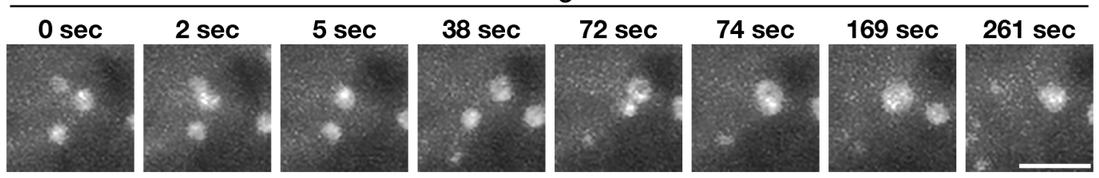
F


Perspective View
Orthographic View
HaloTag-Hrs

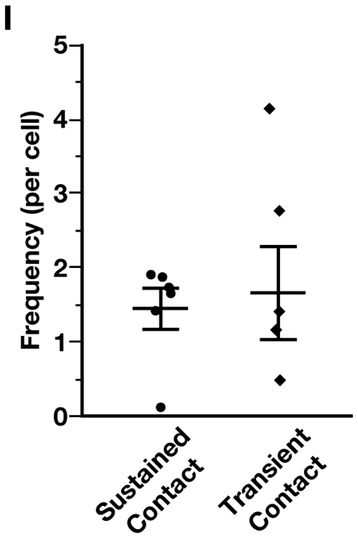
G


HaloTag-Hrs
0 sec
2 sec
5 sec
7 sec
10 sec
12 sec
Lattice light sheet microscopy

H


HaloTag-Hrs
0 sec
2 sec
5 sec
38 sec
72 sec
74 sec
169 sec
261 sec

I


Frequency (per cell)
5
4
3
2
1
0
Sustained Contact
Transient Contact

A
Tsg101 siRNA
HaloTag-Hrs
B
Vps4 siRNA
HaloTag-Hrs
C
Frequency (per cell)
0
1
2
3
4
5
6
7
8
9
Tsg101 siRNA
Vps4 siRNA
Tsg101 siRNA
Vps4 siRNA
Sustained
Transient
D
HaloTag-Hrs
***
Duration of endosome association (sec)
0
20
40
60
80
100
120
140
160
180
Control
Vps4 siRNA
E
HaloTag-Tsg101
***
Duration of endosome association (sec)
0
30
60
90
120
150
Control
Vps4 siRNA
F
HaloTag-Tsg101
Vps4B-HaloTag
Growth Factor Deprived
G
0 sec
15 sec
44 sec
59 sec
74 sec
117 sec
147 sec
176 sec
HaloTag-Hrs
0 sec
15 sec
44 sec
59 sec
74 sec
117 sec
130 sec
159 sec
HaloTag-Tsg101
0 sec
15 sec
44 sec
59 sec
74 sec
117 sec
130 sec
159 sec
Vps4B-HaloTag
Intensity (AU)
0
20
40
60
80
0
60
120
180
Time (sec)
H
RPE1 (steady state)
RPE1 + EGF stimulation
I
STED microscopy
1452 sec
HaloTag-Hrs / EGF
EGF
Hrs