Rivera-Molina and Toomre, http://www.jcb.org/cgi/content/full/jcb.201212103/DC1

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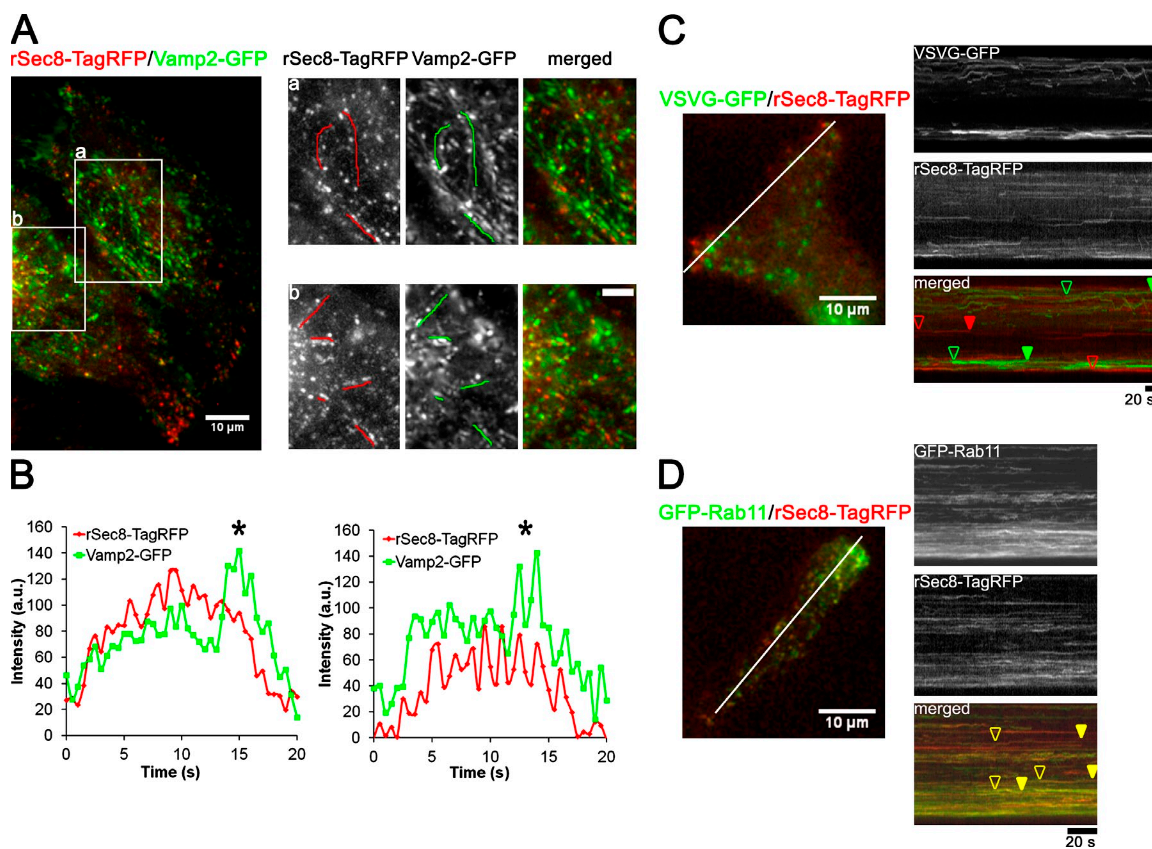


Figure S1. **Localization of rSec8-TagRFP with Vamp2-GFP, VSVG-GFP, or GFP-Rab11 vesicles.** (A) HeLa cells that were transiently knocked down for Sec8 and cotransfected with rSec8-TagRFP and Vamp2-GFP were imaged by TIRFM at a deeper penetration depth (>300 nm) for 3 min at 2 Hz. Bar, 10 µm. A merged maximum projection is shown (left), and individual maximum projections are shown for the regions enclosed in box a and b (right). Red and green lines indicate the curvilinear tracks of puncta that contained rSec8-TagRFP and Vamp2-GFP. Bar, 2 µm. (B) Graphs showing the intensity changes in rSec8-TagRFP and Vamp2-GFP (from Fig. 2 E) signals show a clear rapid brightening (asterisk) and full fusion in the left panel, but fusion is less clear in the right panel (these data are from a single representative experiment of >50 vesicles that was repeated twice). (C) Stable Sec8KD/rSec8-TagRFP HeLa cells were transiently transfected with VSVG-sp-GFP and imaged by TIRFM for 4 min at 2 Hz. Kymographs for the white line are shown for each protein plus the merged image (right). Examples of vesicles containing only VSVG-GFP appearance and disappearance are indicated by open and closed green arrowheads, respectively. Examples of vesicles containing only rSec8-TagRFP are indicated with red arrowheads. (D) Stable Sec8KD/rSec8-TagRFP HeLa cells were transiently transfected with GFP-Rab11 and imaged by TIRFM for 2.5 min at 2 Hz. Kymographs for the white line are shown for each protein plus the merged (right). Examples of vesicles containing both GFP-Rab11and rSec8-TagRFP are indicated with yellow arrowheads.

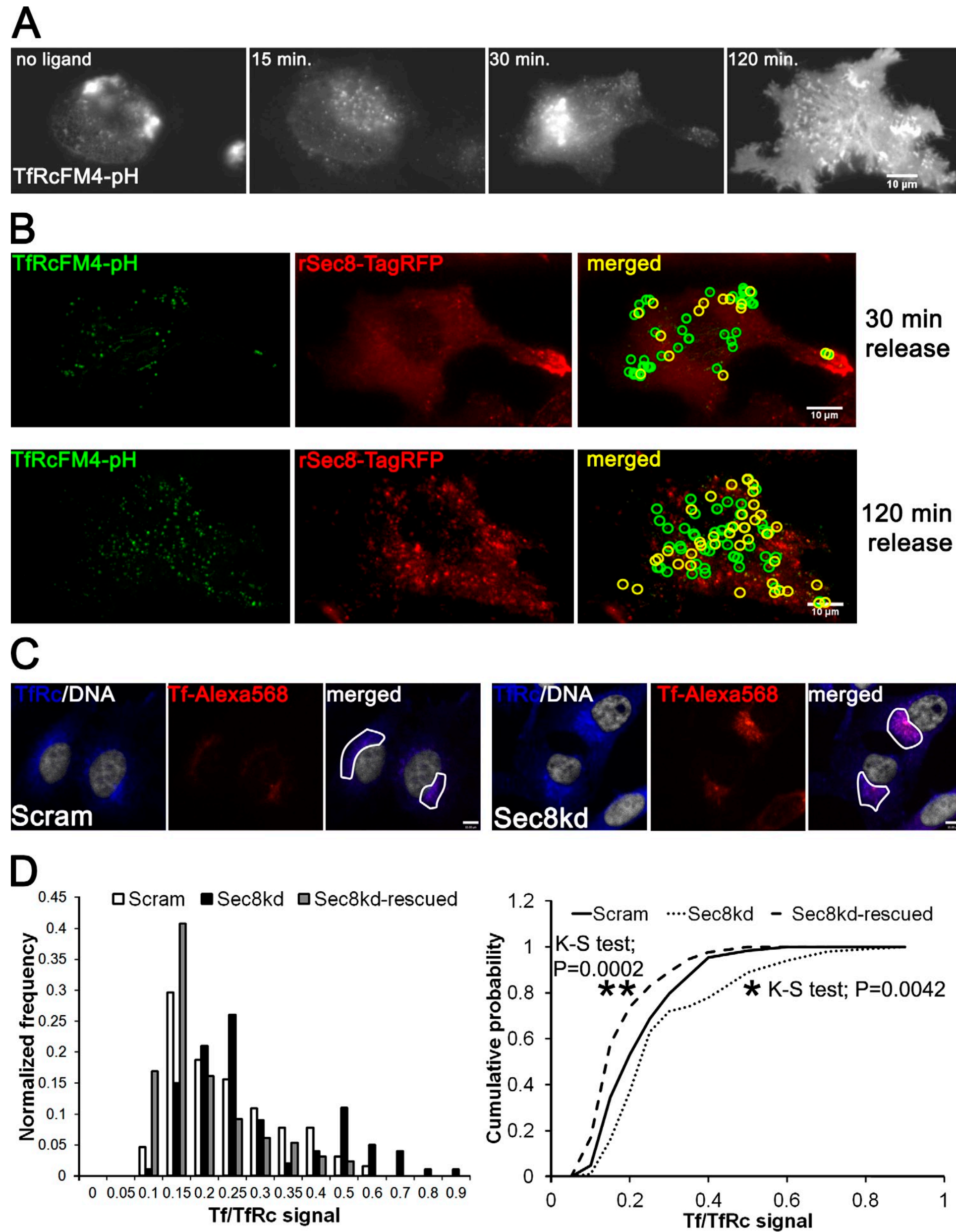


Figure S2. **Kinetic and colocalization of TfRcFM4-pH with rSec8-TagRFP and Tf-Alexa568 recycling defect in Sec8KD cells.** (A) TIRFM images of stable HeLa Sec8KD/rSec8-TagRFP cells transfected with TfRcFM4-pH with no AP21988 ligand or at different time points after addition of 2 µM Ap21988. Images represent different cells showing the release of TFRcFM4-pH from the ER aggregates (no ligand to 15 min) to post-Golgi and PM (30–120 min). Bar, 10 µm. (B) Maximum projection images of TfRcFM4-pH fusion events and rSec8-TagRFP at two different time points (30 and 120 min) to illustrate the increase in colocalization of TfRcFM4-pH fusion events with rSec8-TagRFP after TfRcFM4-pH accumulates at the PM. TfRcFM4-pH fusion events that colocalize with rSec8-TagRFP are shown as yellow circles; noncolocalizing events are in green circles. (C) Stable HeLa cells for Scrambled (Scram), Sec8KD, and Sec8KD/rSec8-TagRFP (Sec8 replaced) were loaded for 1 h with 10 µg/ml Tf-Alexa-568. After washing, the cells were incubated with unlabeled Tf for 10 min at 37°C, fixed, and labeled by immunofluorescence with an antibody against TfRc to identify the perinuclear region. Images were captured and the ratio of Tf-Alexa568 signal was divided by the TfRc-Cy5 in the perinuclear region (white lines). Bars, 10 µm. (D) Analysis of Tf-Alexa568/TfRc-Cy5 signal was performed on 64 Scram, 100 Sec8KD, and 130 Sec8KD-recued cells. A bar histogram (left) and cumulative probability line graph (right) show the increased number of cells with a high level of Tf-Alexa568/TfRc (>0.5) in Sec8KD but not in Scram or Sec8-replaced cells. P-value results for the K-S test between Scram and Sec8KD (*) and Scram and Sec8KD-recued cells (**) are included in the cumulative probability graph to show the significant difference between the samples.

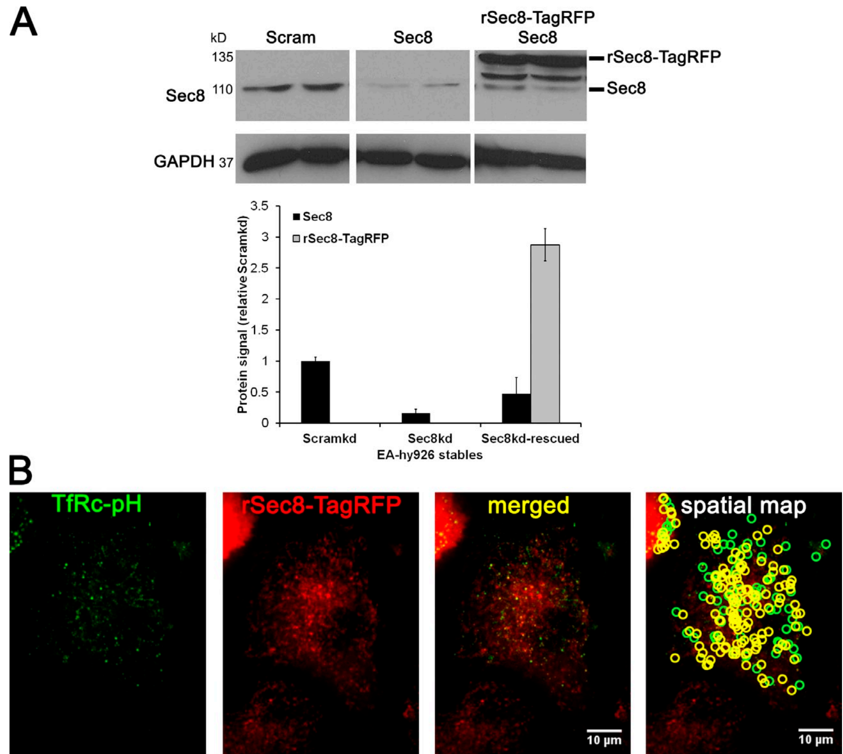
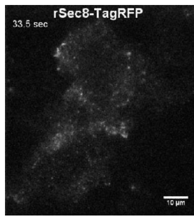
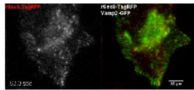


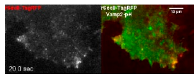
Figure S3. **Validation of Sec8kd and rSec8-TagRFP expression in stable EA hy926 cells and colocalization with TfRc-pH.** (A) EA hy926 cells were infected and selected for Scram and Sec8 shRNA. The Sec8KD cells were later infected with rSec8-TagRFP to generate EA hy926 Sec8KD/rSec8-TagRFP stables. Total protein lysates (6 µg) from the stable cells were run in duplicate on an SDS-PAGE for Western blot analysis against Sec8 and GAPDH. Densitometry analysis of the Sec8 signal was done and normalized relative to the signal in Scram. The Sec8KD was ~80% and the rSec8-TagRFP was overexpressed approximately threefold relative to endogenous Sec8. (B) TIRFM images of stable EA hy926 Sec8KD/rSec8-TagRFP cells transiently transfected with TfRc-pH. Maximum projection images of TfRc-pH fusion events and rSec8-TagRFP to show the colocalization of TfRc-pH fusion events with rSec8-TagRFP. TfRcFM4-pH fusion events that colocalize with rSec8-TagRFP are shown as yellow circles; noncolocalizing events are shown as green circles. A mean of 64.7 ± 5.5% of TfRc-pH fusion events colocalized with rSec8-TagRFP events in EA hy926 cells (*n* = 2 cells; 400 events/cell).



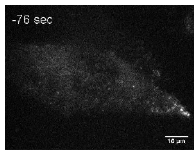
Video 1. **Localization of rSec8-TagRFP after knockdown of endogenous Sec8.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP. Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 30 fps.



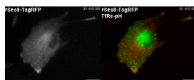
Video 2. **Dual color TIRFM of rSec8-TagRFP and Vamp2-GFP.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP (red) and Vamp2-GFP (green). Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 30 fps. Left shows rSec8-TagRFP signal and right shows the merged signal between rSec8-TagRFP and Vamp2-GFP.



Video 3. **Dual color TIRFM of rSec8-TagRFP and Vamp2-pHluorin.** HeLa cells with a transient Sec8KD were transfected with rSec8-TagRFP (red) and Vamp2-pHluorin (green). Images were acquired on a custom-built objective type TIRF microscope. Frames were taken every 0.5 s for 5 min and are shown at 10 fps. Left shows rSec8-TagRFP signal and right shows the merged between rSec8-TagRFP and Vamp2-pHluorin. Representative red circles were drawn on the rSec8-TagRFP and yellow in the merged to show examples of Vamp2-pHluorin events with rSec8-TagRFP.



Video 4. **TIRFM/FRAP analysis of rSec8-TagRFP in membrane protrusion.** HeLa cells stable for Sec8KD/rSec8TagRFP were imaged on a custom-built objective type TIRF/FRAP microscope. Frames were taken every 1 s for 2 min before photobleaching and 5 min after and are shown at 30 fps.



Video 5. **Dual color TIRFM of rSec8-TagRFP and TfRc-pH during cell migration.** EA hy926 stables for Sec8kd/rSec8-TagRFP (red) transiently transfected with TfRc-pH (green) were imaged on a custom-built objective type TIRF microscope. Images were collected every 30 s for 30 min and are shown at 15 fps. Bars, 10 µm.