Figure EV1. PIS and CEPT1 bare enriched in domains following LE/Ly-mediated ER hitchhiking.

A Representative stills of spinning disk confocal images of \( n = 2 \) of COS7 cells stably expressing GFP-TMD (green), RFP-PIS (orange) (left panels), or RFP-CEPT1 (right panels) (orange) and containing lysosomal marked SiR-lysosome (magenta). Zoom insets show region of interest depicting a lysosomes pulling an ER tubule containing an RFP-PIS or RFP-CEPT1 enriched domain. All images acquired for 120 frames at 1Hz. Image scale bars: 20 \( \mu \)m and zoom insets: 2 \( \mu \)m.

B Percentage of SiR-lysosomes marked vesicles followed by ER containing a PIS or CEPT1 enriched domain. Graph represents \( n = 20 \) vesicles from 2 independent experiments.

C Graph representing the percentage of SiR-lysosome marked vesicles that either or not continued pulling an ER tubule following junction formation, as indicated. Graph represents \( n = 20 \) vesicles from 2 independent experiments.

D Graph shows mean ± s.d. of validation of siRNA treated COS7 cell depletion efficiencies expressed as fraction of the mRNA of Kinectin-1 (siKTN1) relative to siRNA control (siControl) by qPCR. \( n = 3 \) independent experiments. Normalized to control as set at 1. Significance Student two-tailed t-test. *** \( P < 0.001 \).

E Western blot analyses of COS7 cells transfected with sikinecin1 or siControl and stained with rabbit anti-human KTN1. Loading control is stained for actin.
Figure EV2. ER movement related to various static and mobile endosomal populations.

A–G Representative time-lapse spinning disk confocal images of \( n = 3 \) of live COS7 cells stably expressing mCherry-KDEL (green) and transiently expressing GFP-RAB5 (A and B), GFP-RAB11 (C and D), GFP-RAB14 (E and F), or GFP-Rab7 (G) (orange). Zoom insets shows region of interest containing a mobile (A, C, and E) or static (B, D, F, and G) endosome as denoted by the white arrow. First row represents ER (mCherry-KDEL) (green), the second row shows MDA-generated ER movement (magenta), third row shows a merge of the two preceding channels, and the fourth row shows a merge of ER (green) and marked endosomal population (orange). Panels A and B; C and D; and E and F are representatives of mobile and static RAB-positive structures within the same cells. Scale bar: 10 \( \mu \)m, zoom insets 3 \( \mu \)m.

Images related to Movies EV14–EV20. Images were acquired for 90 frames at 1Hz.

H Normalized MDA quantification of total ER movement of COS7 cells not transfected or overexpressing GFP-RAB5, GFP-RAB7, GFP-RAB11, or GFP-RAB14. Graph represents mean ± s.d. of analyses at multiple locations within one cell of \( n = 15 \) cells from 3 independent experiments. Normalized to control set at 1. Significance two-tailed Student t-test. ns = not significant.

I Normalized MDA quantification of total movement of vesicles marked with GFP-RAB5, GFP-RAB7, GFP-RAB11, or GFP-RAB14 in COS7 cells. Graph represents mean ± s.d. of \( n = 15 \) cells from 3 independent experiment. Normalized to control set at 1. Significance two-tailed Student t-test. ns = not significant.

J MDA quantification of total vesicle movement and total ER movement of COS7 cells treated with SiR-Lysosome or transfected with GFP-RAB7. All images acquired for 90 frames at 1Hz. Graph represents mean ± s.d. from \( n = 15 \) (two left bars) and the mean ± s.d. of analyses at multiple locations within one cell of \( n = 15 \) (two right bars) cells from 3 independent experiments. Significance two-tailed Student t-test. ns = not significant.

K Still images of \( n = 3 \) of COS7 cells transfected with RFP-SEC61B (magenta) and GFP-RAB5, GFP-RAB7, GFP-RAB11, or GFP-RAB14 (green). Zoom insets show regions of interest of peripheral ER morphology. Scale bar 5 \( \mu \)m zoom inset 2 \( \mu \)m.
Figure EV2.
Figure EV3. TMEM55B- and starvation-induced endosomal sequestration limits ER movement and peripheral ER junctions.

A Representative time-lapse spinning disk confocal images of n = 3 of live COS7 cells stably expressing GFP-TMD (green) and transiently expressing mScarlet-TMEM55B (magenta) and SiR-lysosome stained LE/Lys (orange). Zoom insets show region of interest containing ER (GFP-TMD). First row represents ER (GFP-TMD) (green), the second row shows MDA-generated ER movement (magenta), third row shows a merge of the two preceding channels. Scale bar: 10 µm, zoom insets 3 µm. Images related to Movie EV21. Images were acquired for 90 frames at 1 Hz.

B MDA quantification of MitoTracker-labeled mitochondria in cells overexpressing GFP-RILP or GFP-TMEM. The scatter plot represents n = 15 cells from 2 independent experiments. All images acquired for 90 frames at 1 Hz.

C MDA quantification of peroxisomes in cells overexpressing GFP-RILP or GFP-TMEM. The scatter plots represent n = 15 cells from 2 independent experiments. All images acquired for 90 frames at 1 Hz.

D MDA quantification of total vesicle movement of COS7 cells stably expressing GFP-TMD (green), the second row shows MDA-generated ER movement (magenta), third row shows a merge of the two preceding channels. Scale bar: 10 µm, zoom insets 3 µm. Images related to Movies EV22 and EV23. Images were acquired for 90 frames at 1 Hz.

E MDA quantification of total vesicle movement of COS7 cells stably expressing GFP-TMD (green) to mark the ER and SiR-lysosome labeled LE/Lys (orange) merged under control or starvation conditions. Zoom insets highlight region of interest containing a mobile endosome as denoted by the white arrow. First row represents ER (GFP-TMD) (green), the second row shows MDA-generated ER movement (magenta), third row shows a merge of the two preceding channels. Scale bar: 10 µm, zoom insets: 3 µm. Images related to Movies EV22 and EV23. Images were acquired for 90 frames at 1 Hz.

F MDA quantification of total vesicle movement of COS7 cells under control or starved conditions overexpressing either GFP-RAB5, GFP-RAB7, GFP-RAB11 or GFP-RAB14. Graph represents mean ± s.d. of analyses at multiple locations within one cell of n = 15 cells per condition from 3 independent experiments. Normalized to 1 to control conditions (fed). All images acquired for 90 frames at 1 Hz. Significance two-tailed Student t-test. **P < 0.001.

G MDA quantification of SiR-lysosome stained LE/Lys of panel (E) normalized to 1 for control cells showing total average endosomal movement. Graph represents n = 12 - 15 cells per condition from 3 independent experiments. Normalized to control conditions (fed). Significance two-tailed Student t-test. *P < 0.05.

H MDA quantification of total local ER movement of COS7 under control or starved conditions overexpressing either GFP-RAB5, GFP-RAB7, GFP-RAB11 or GFP-RAB14. Graph represents mean ± s.d. of analyses at multiple locations within one cell of n = 15 cells per condition from 3 independent experiments. Normalized to control conditions set at 1 (fed). Significance two-tailed Student t-test. ****P < 0.0001, ns = not significant.

I MDA quantification of total local ER movement of COS7 under control or starved conditions marking the ER and overexpressing either GFP-RAB5, GFP-RAB7, GFP-RAB11 or GFP-RAB14. Graph represents mean ± s.d. of analyses at multiple locations within one cell of n = 15 from 3 independent experiments. Normalized to control conditions set at 1 (fed). Significance two-tailed Student t-test.****P < 0.0001, ns = not significant.

J Representative stills of n = 3 of spinning disk confocal images of COS7 cells stably expressing TMD-GFP (green) and SiR-lysosome stained endosomes/lysosomes (orange). COS7 cells under control and starvation conditions. Zoom insets show regions of interest containing ER (TMD-GFP) (green) and junction analysis of skeletonized ER where number of ER junctions representing the quantified average. Scale bars: 15 µm. zoom insets: 3 µm.

K Quantification of number of ER junctions per µm² from (J) as resulted from ER junction analysis. Graph represents mean ± s.d. of analyses at multiple locations within one cell of n = 15 from 3 independent experiments. Significance two-tailed Student t-test. ****P < 0.0001.
Figure EV3.
Figure EV4. ER tethers limit ER movement.

A Graph represents mean ± s.d. validation of siRNA treated depletion efficiencies expressed as fraction of the mRNA of VAPA (siVAPA), VAPB (siVAPB) or MOSPD2 (siMOSPD2) relative to siRNA control (siControl) by qPCR, n = 3 independent experiments.

B Western blot analysis of COS7 cells transfected with siControl, siVAPA, siVAPB or siMOSPD2 and stained with rabbit anti-human VAPA or VAPB. The loading control is stained for actin.

C Western blot analysis of COS7 cells transfected with a combination of siControl, siVAPA, siVAPB and siMOSPD2 and stained with rabbit anti-human VAPA or VAPB. Loading control is stained for actin.

D–I Representative images of n = 3 of time-lapse spinning disk confocal images of COS7 cells stably expressing GFP-TMD (green) and SiR-lysosome stained LE/Lys (orange). Cells shown were transfected with siRNA against VAPA (siVAPA) (D), VAPB (siVAPB) (E), MOSPD2 (siMOSPD2) (F), siVAPA and siVAPB (G), siVAPA and siMOSPD2 (H) or siVAPB and siMOSPD2 (I). Zoom insets show regions of interest containing mobile LE/Lys as denoted by the white arrow. First row represents ER (TMD-GFP) (green), the second row shows MDA-generated ER movement (magenta), third row shows a merge of the two preceding channels and the fourth row shows a merge of ER (green) and SiR-lysosome stained LE/Lys (orange). Scale bar: 5 µm, zoom insets: 3 µm. Images related to Movies EV24–EV29. Images were acquired for 90 frames at 1Hz.

J Still confocal images of n = 3 COS7 cells transfected with RFP-SEC61B and either siControl or a combination of siVAPA, siVAPB and siMOSPD2. Zoom insets show regions of interest depicting ER morphology. Scale bars: 5 µm and zoom insets: 2 µm.

K Normalized MDA quantification of total ER movement of COS7 cells transfected with either siControl, siVAPA, siVAPB, siMOSPD2 or a combination thereof. Graph represents n = 15 cells from 3 independent experiments. Significance two-tailed Student t-test. *P < 0.05, **P < 0.01, ns = not significant.
Figure EV4.
Figure EV5. ER tethers limit peripheral ER junctions.

A–F Representative stills of $n = 3$ of spinning disk confocal images of COS7 cells stably expressing TMD-GFP (green) and SiR-lysosome stained endosomes/lysosomes (orange). Cells shown were transfected with siRNA against VAPA (siVAPA) (A), VAPB (siVAPB) (B), MOSPD2 (siMOSPD2) (C), siVAPA and siVAPB (D), siVAPA and siMOSPD2 (E) or siVAPB and siMOSPD2 (F). Zoom insets show regions of interest containing ER (TMD-GFP) (green) and junction analysis of skeletonized ER with number of ER junctions representing the quantified average. Scale bars: 15 µm. zoom insets: 3 µm.