SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Figure 1). Expression and localization of E-Syts

(A-D) Immunofluorescence images showing localization of the three E-Syts in HeLa cells expressing Myc-tagged (A-C) or FLAG-tagged (D) constructs. The N- or C-terminal position of the tag is indicated in the labeling of each figure. Note the localization of E-Syt1 throughout the ER, the predominant cortical localization of E-Syt2 and the nearly exclusively cortical localization of E-Syt3. (E) Immunoblots using commercial antibodies directed against the three E-Syts showing that E-Syts constructs tagged with EGFP at either the N- or C-terminus have the expected molecular weights (MW) when expressed in HeLa cells. Expected MW are as follows. EGFP: 27 kDa; native E-Syt1: 121 kDa; native E-Syt2 and E-Syt3: 100 kDa. Note the overexpression of the tagged E-Syts relative to the endogenous proteins. Endogenous E-Syt3, which is known to be expressed at low levels in most tissues, is not detectable. (F) Confocal images of HeLa cells co-expressing untagged E-Syt2 or E-Syt3 and mRFP-Sec61β. Note the recruitment of mRFP-Sec61β to the cell periphery. (G-J) Representative confocal images of HeLa cells expressing mRFP-Sec61β and N-terminal EGFP-tagged E-Syts (G-I) or Syt1 (J). Note that EGFP-Syt1, in contrast to Syt1-EGFP (compare with Figure 2A) forms intracellular aggregates, while E-Syts-EGFP have the same subcellular localization as EGFP-E-Syts (compare with Figure 1B). (K) Representative confocal images of HeLa cells co-expressing E-Syt2 or E-Syt3 independently tagged at the N-and C-terminus with two different

fluorescent proteins as indicated. Note the precise overlap of the two fluorescence patterns for each of the two E-Syts. Scale bar, 10 μm.

Figure S2 (related to Figures 2 and 3). E-Syts are inserted into the ER membrane via hydrophobic hairpin sequences

(A) Schematic representation of a fluorescence protease protection (FPP) assay. Low concentration of digitonin allows permeabilization of the plasma membrane without disrupting the ER membrane. Application of proteinase K selectively cleaves cytosolically exposed mRFP of mRFP-Sec61β without affecting luminally exposed EGFP of VAP-A-EGFP. (B - F and H - I) Confocal images showing representative unfixed HeLa cells before digitonin application (left), 50 seconds after digitonin application (middle) and 50 seconds after proteinase K application (right). (B) Confocal images of cells co-expressing VAP-A-EGFP (top panel) and mRFP- Sec61β (bottom panel)(see also Supplemental movie 1). (C - D) Confocal images of cells expressing N-terminal (C) or C-terminal (D) EGFP fusions of E-Syt proteins (see also Supplemental movie 2). (E - F) Confocal images of cells co-expressing N-terminal (E) or C-terminal (F) EGFP fusion of E-Syt2 (top) and the luminal ER marker ER-mRFP (bottom panels). (G and H) Schematic representation of WT E-Syt2 and a mutant E-Syt2 harboring an internal EGFP (top) and confocal images of the mutant construct in cells upon the FPP assay (bottom). (I) Schematic representation of E-Syt2 fused to mCherry at its N-terminus and to EGFP at its C-terminus (top) and confocal images of the construct in cells upon the FPP assay. (J) Hydrophilicity plots of the E-Syts and

of the tricalbins (Tcb) according to the Goldman-Engelman-Steitz scale with a window of seven residues. The portions of the plots colored in red indicate the putative transmembrane regions (sequences in Figure 3A). (K) Illustration of expected protected fragments (PF) obtained after protease K (PK) digestion of dog pancreas rough microsomes (RMs) containing integrated Myc-Syt1 (1,2) or Myc-E-Syt3 (3). Because the Myc epitope is at the N-terminus of the constructs, a protected fragment (PF) that can be immunoprecipitated with anti-Myc antibodies should be detected if translocation of the N-terminus occurs. As the expected protected fragment of Syt1 is predicted to undergo glycosylation, its motility should be different in control conditions (1) and when glycosylation is inhibited (2). If the E-Syt3's N-terminus is not translocated, an immunoprecipitable Myc-positive protected fragment should not be detectable (3). This is the case, as shown in Figure 3E. (L) Tight association of Myc-Syt1 and Myc-E-Syt3 with microsomal membranes assayed by Na_2CO_3 extraction. After translation in the presence or absence of rough microsomes and $^{[35}S$methionine, samples were brought to 0.1 M Na_2CO_3, incubated for 30 minutes on ice and centrifuged to separate soluble or extracted proteins from insoluble or membrane-integrated ones. Equal aliquots of the total sample (T), pellet (P) and supernatant (S) fractions were loaded for SDS-PAGE followed by auoradiography. The lower autoradiogram shows that a cytosolic protein (soluble form of cytochrome b5 reductase) is mainly recovered in the supernatant, regardless of the presence of rough microsomes (RM). The numbers below the lanes indicate the percentages of each protein recovered in the P and S

fractions. (M) Representative immunoblots of membranes prepared from HeLa cells that were subjected to different extraction methods. (S) soluble material; (P) extraction-resistant pellet. Samples were analyzed by SDS-PAGE and immunoblotted for the proteins indicated. E-Syt1 was recovered in the pellets of the Na_2CO_3 and NaCl extraction, like the integral membrane proteins calnexin and VAP-A while BiP, a soluble protein of the ER lumen is recovered in the supernatant. E-Syt1, however, like calnexin, are extracted by Triton X-100. HB: control homogenate buffer; Triton: Triton X-100.

Figure S3 (Related to Figure 4). Recruitment of E-Syt2/3 to the plasma membrane and its dependence on PI(4,5)P_2 in the plasma membrane.
(A - B) Representative images of HeLa cells co-expressing E-Syt2-EGFP or EGFP-E-Syt3 deletion constructs and the ER marker mRFP-Sec61β. Schematic representation of E-Syts constructs examined and of their localizations is shown on the top. Note the cER localization of the E-Syt2 construct lacking only the SMP domain (A) and the ER localization of the E-Syt3 construct lacking the C2C domain (B). Scale bar, 10 μm. (C) Cartoon depicting the optogenetic system used to acutely dephosphorylate plasma membrane PI(4,5)P_2 in the experiments shown in E and Figures 4C-4J. Upon blue light illumination, cytosolic CRY2-5-ptase$_{OCRL}$ heterodimerizes with membrane anchored CIBN (CIBN-CAAX) leading to selective loss of plasma membrane PI(4,5)P_2 (see (Idevall-Hagren et al., 2012) for details). (D) Loss of cortical ER, as assessed by fluorescence in the TIRF microscopy field, in HeLa cells expressing ER-mRFP and E-Syt2-EGFP together

with two components of blue light-dependent PI(4,5)P_2 depletion system following blue light illumination (mean±SEM, n=3). (Left) Time-course of mRFP fluorescence showing loss of the cortical ER, which had been exaggerated by the overexpression of E-Syt2, upon blue light illumination. (Right) Bar-graph showing the corresponding loss of cortically localized E-Syt2-EGFP from the same cells (mean±SEM, n=3, * P<0.001).

Figure S4 (Related to figure 5). Calcium dependence of E-Syt1 cortical localization

(A) Electrostatic surface potential of selected C2 domains of the E-Syts highlighting predicted Ca^{2+} binding sites (red) and basic surface patches (blue), based on the known structure of the C2B domain of Syt1 (pdb: 1TJX) which is shown at left. Images were created in Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.). Electrostatic potentials were calculated by APBS (Baker et al., 2001), with positive surfaces colored blue, and negative surfaces in red (-5 to +5). (B) Confocal micrographs of the ventral region of a COS-7 cell expressing EGFP-E-Syt1 before and after treatment with Thapsigargin (2 uM). (C) Confocal micrographs of the ventral region of a COS-7 cell co-expressing ER-oxGFP and untagged E-Syt1 before and after UV photolysis of caged Ca^{2+}. Below are kymographs showing the transient accumulation of ER-oxGFP upon Ca^{2+} increases in control cells (bottom) or cells overexpressing E-Syt1 (top).

Figure S5 (Related to figure 6). Reciprocal interactions of the three E-Syts

(A-B) HeLa cells were co-transfected with EGFP-tagged and Myc-tagged (A) or untagged (B) E-Syts, as indicated. Twenty-four hours after transfection, anti-GFP immunoprecipitation was performed, and immunoprecipitates were blotted with anti-GFP and anti-Myc (A) or anti E-Syts (B) antibodies to detect the co-immunoprecipitated bands corresponding to E-Syts homomers (A) or heteromers (B) (arrows). 2.5% of the total cell lysate was loaded in the input lane.

Figure S6 (Related to figure 7). E-Syt triple knock-down (TKD) impacts on ER-PM contacts formation

(A) HeLa cells were transfected with E-Syt1, E-Syt2, E-Syt3 or control (Ctrl) siRNA oligos. E-Syt1 protein levels were evaluated by western blot. (B-C) HeLa cells were transfected with control siRNAs (Ctrl) or siRNAs against all E-Syts (TKD). E-Syts mRNA expression was measured by Real-time qPCR (B) and protein levels of E-Syt1 and E-Syt2 as detected by western blot are shown (C). Real-time qPCR reactions in (B) were performed in triplicate and averaged. *Y*-axis: $2^{-\Delta Ct}$ value represents differences between the mean Ct (cycle threshold) values of tested gene (E-Syt1, E-Syt2 and E-Syt3) and those of reference gene (S26) (reduction of E-Syts mRNA levels: 90% (E-Syt1 and E-Syt2) and 85% (E-Syt3), *P<0.001 compared to control). (D) TIRF microscopy images of oxGFP fluorescence from Ctrl or TKD cells expressing ER-OxGFP with or without untagged E-Syts rescue constructs (E-Syt1/2). (E) Quantification of the ER-oxGFP signal visible in cells of D, expressed as number of ER-oxGFP -positive

puncta after setting an arbitrary threshold (mean±SEM, n=8 for each, * P<0.001 compared to Ctrl, # P<0.005 compared to TKD+E-Syt1/2). (F) Time-course of the normalized YFP fluorescence of Ctrl and TKD cells transfected with SP-YFP-STIM1 and stimulated with TG (2 µM). (G) TIRF microscopy images of mRFP and GFP fluorescence from Ctrl and TKD cells expressing mRFP-STIM1 and GFP-Orai1, before and 10 min after TG treatment (2 µM, 10 min). (H) Box and whisker plot showing the percentage of plasma membrane surface occupied by mRFP-STIM1- and GFP-Orai1-positive puncta after TG treatment (2 µM, 10 min) from TIRF images of Ctrl and TKD cells (* P<0.001). (I) Quantifications of the number of STIM1 and Orai1-positive puncta/ area ($10\mu m^2$) from cells treated as in G. (mean±SEM, n=16 for each, * P<0.001). (J) Schematic drawing illustrating the role of C2 domains in tethering cytosolic proteins or membranous organelles to the $PI(4,5)P_2$-rich plasma membrane. Examples shown include 1) synaptotagmin, an intrinsic membrane protein of secretory vesicles, 2) the ER-anchored E-Syts that comprise C2 and SMP domains and 3) protein kinase C, which in addition to the C2 domain contains a C1 domain and a protein kinase module.

Supplemental Movie 1. FPP assay (movie related to Figure 3 and Figure S2B).

Spinning disc confocal microscopy imaging of HeLa cells expressing VAPA-EGFP (left) and mRFP-Sec61β (right). 20 μM digitonin was applied at 1.5 min followed by Proteinase K (approximately 1.5 units/ml) application at 3 min. The rapid loss of the fluorescent signal for mRFP-Sec61β is observed after Proteinase K application, while the fluorescent signal of VAPA-EGFP is not affected. This is consistent with the luminally exposed EGFP and the cytosolically exposed mRFP of these tagged proteins (see cartoon in Figure S2A).

Supplemental Movie 2. FPP assay (movie related to Figure 3 and Figure S2C).

Spinning disc confocal microscopy imaging of HeLa cells expressing EGFP-E-Syt2 (left) and mRFP-Sec61β (right). 20 μM digitonin was applied at 1.5 min followed by Proteinase K (approximately 1.5 units/ml) application at 3 min. The fluorescent signal of both EGFP-E-Syt2 (left) and mRFP-Sec61β is rapidly lost after Proteinase K application. This result confirms that the N-terminus of EGFP-E-Syt2 is exposed to the cytosolic space.

Supplemental Movie 3. Redistribution of E-Syt3 and $PH_{PLC\delta1}$ upon $PI(4,5)P_2$ dephosphorylation at the plasma membrane (movie related to Figure 4C)

Spinning disc confocal microscopy imaging of a COS-7 cell expressing E-Syt3-mCherry (left) and iRFP-$PH_{PLC\delta1}$ (right). Blue light illumination was applied after

40s from the start of the recording. E-Syt3 and iRFP-PH $_{PLC\delta1}$ were initially concentrated at the plasma membrane and upon illumination E-Syt3-mCherry redistributes to the reticulum of the ER and iRFP-PH-$_{PLC\delta1}$ to the cytosol.

Supplemental Movie 4. Modulation of E-Syt2 cortical localization upon plasma membrane PI(4,5)P_2 dephosphorylation (movie related to Figure 4H)

TIRF microscopy imaging of four COS-7 cells expressing mCherry-E-Syt2. Blue light illumination was applied after 200s from the start of the recording. mCherry-E-Syt2 was initially concentrated at the plasma membrane and upon illumination mCherry fluorescence disappears from the cell cortex and gradually re-appears after termination of illumination.

Supplemental Movie 5. Cortical recruitment of EGFP-E-Syt1 upon thapsigargin-induced depletion of intracellular Ca^{2+} stores (movie related to Figure 5A).

TIRF microscopy imaging of a group of HeLa cells expressing EGFP-E-Syt1. The cells were treated with 2 μM thapsigargin (TG) that resulted in a rapid (within 10 s) and transient recruitment of EGFP-E-Syt1 to the cortical region of the cell. The movie has been inverted to show EGFP-fluorescence in black.

Supplemental Movie 6. Cortical recruitment of E-Syt1-mCherry upon photolysis of caged Ca^{2+} (movie related to Figure 5F).

Spinning disc confocal microscopy imaging of the ventral-most region of a COS-7

cell expressing E-Syt1-mCherry. The cell was preloaded with caged Ca^{2+} (NP-EGTA) which was released by a 3 s 405-nm light-pulse (UV) to cause an acute increase in cytosolic Ca^{2+}. This results in rapid and transient recruitment of E-Syt1-mCherry to the ventral (cortical) region of the cell. The movie has been inverted to show mCherry-fluorescence in black.

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies and chemicals

Primary antibodies used in this study were: polyclonal anti-GFP (Abcam), monoclonal anti-FLAG M2 antibody (Sigma-Aldrich), polyclonal anti-FLAG (Sigma-Aldrich), anti-E-Syt1 (HPA016858, Sigma-Aldrich), anti-E-Syt2 (HPA002132, Sigma-Aldrich), anti-E-Syt3 (HPA039200, Sigma-Aldrich), anti-Calnexin (Spa860, Enzo Life Sciences), anti-VAP-A (HPA009174, Sigma-Aldrich), anti-VAP-B (HPA013144, Sigma-Aldrich), anti-BiP/GRP78 (610978, BD Transduction Laboratories), anti-PDI (GeneTex), anti-Myc 9B11 or 9E10 (Alexa Fluor 488 conjugate is #2279 from Cell Signaling Technology), anti-actin (69100, MPBiosciences), monoclonal anti-α-tubulin (clone B-5-1-2, Sigma-Aldrich). Oxo-M and Atropine (Sigma-Aldrich).

Biochemical analysis

For immunoprecipitation of GFP-tagged E-Syts, cell lysates were incubated with Chromotek GFP-trap agarose beads (Allele Biotech), and solubilized bead-bound material was processed for SDS-PAGE and immunoblotting. Tandem affinity purification of EGFP-TEV-3xFLAG-E-Syt2 and the cell-free translation/translocation assay are described below.

Plasmids

The following reagents were kind gifts: mRFP-Sec61β from T. Rapoport (Harvard University) (Shibata et al., 2008). mRFP-STIM1 from B. Baird (Cornell University)

(Calloway et al., 2009). GFP-Orai1 and HRP-STIM1 from R. Lewis (Stanford University) (Mullins et al., 2009; Wu et al., 2006). ssHRP-KDEL from T. Schikorski (St. Jude Children's Research Hospital) (Schikorski et al., 2007). M1 muscarinic acetylcholine receptor (M1R) from B. Hille (University of Washington) (Suh et al., 2004). ER-mRFP and ER-oxGFP (comprising an ER-retention sequence fused to a version of GFP optimized for expression in the ER lumen) from EL. Snapp (Albert Einstein College of Medicine). pEX-SP-YFP-STIM1 (23-685) was obtained from Addgene (Plasmid 18857). iRFP-$PH_{PLC\delta1}$, EGFP-$PH_{PLC\delta1,}$ CIBN-CAAX, CRY2-5-$ptase_{OCRL}$, CRY2-DEAD 5-$ptase_{OCRL}$ were previously described (Idevall-Hagren et al., 2012). Pm-mRFP (mRFP with N-terminal plasma membrane localization module from Lyn kinase).

cDNA clones:

cDNA clones of human E-Syt1, E-Syt2, E-Syt3 and Syt1 were obtained from: Open Biosystems (E-Syt1: clone 2958094) or Origene (E-Syt2: RC224839, E-Syt3-myc-Flag: RC218538, Syt1: SC116605). Two coding changes were identified in the E-Syt2 cDNA from Origene. We mutagenized the corresponding nucleotides by site-directed mutagenesis to match it to the E-Syt2 cDNA sequence in the GenBank (NM_020728.1). The E-Syt2 protein encoded by this clone, which had been used in previous studies (Jean et al., 2010), had a slightly slower mobility in SDS-PAGE than the endogenous protein as revealed by western blotting. Inspection of other E-Syt sequences revealed that several of them started with a methionine that aligned with a second methionine in human

NM_020728.1. A construct using this other methionine as the N-terminus (defined below as E-Syt2S) aligned more closely with endogenous E-Syt2 in SDS-PAGE. In spite of this difference both constructs had the same subcellular localization and both constructs were used in this study. An additional E-Syt2 construct (Min et al., 2007), which differed in the N-terminal sequence, also had the same subcellular distribution, but was not used for the experiments reported in this study. The generation of all the tagged constructs, including those encoding E-Syt2S is described below.

To mutate the two coding changes in the E-Syt2 cDNA from Origene, we used the following primers (targeted nucleotide is showed in lower case):

ESyt2_QC1_Fw,

GAGACGAGCAGCACCAGTGTtCCCTGGGGAATCTGAAGGTC

ESyt2_QC1_Rw,

GACCTTCAGATTCCCCAGGGaACACTGGTGCTGCTCGTCTC

ESyt2_QC2_Fw,

GTGAGCCAGCGCTTCCAGCTCaGTAACTCGGGTCCAAACAG

ESyt2_QC2_Rw,

CTGTTTGGACCCGAGTTACtGAGCTGGAAGCGCTGGCTCAC

Cloning of E-Syt1-EGFP, E-Syt2-EGFP, E-Syt3-EGFP, E-Syt2-mCherry, E-Syt3-mCherry, Syt1-EGFP:

cDNAs of E-Syt1, E-Syt2, E-Syt3 and Syt1 were amplified by PCR. Primers used were:

5’ SalI-E-Syt1_Fw, ACCG*GTCGAC*atggagcgatctccaggaga

3’ SacII-E-Syt1 No Stop_Rw, ACCG*CCGCGG*ggagctgcccttgtccttgt

5' EcoRI-ESyt2b_Fw, CAAGCTTC*GAATTC*atgacgccaccgtcccgggc

3' BamHI-ESyt2b NoStop_Rw, CGACCGGT*GGATCC*tttgtcatcgcctgaggcctcg

5’ XhoI-E-Syt3_Fw, CCG*CTCGAG*atgcgagcagaggagccct

3’ SalI-E-Syt3 No Stop_Rw, ACCG*CCGCGG*ggagctgcccttgtccttgt

5' EcoRI-g-Syt1,

GCGGCCGCGAATTCgATGGTGAGCGAGAGTCACCATGAGG

3' BamHI_Syt1 No Stop,

CGACCGGTGGATCCTTCTTCTTGACGGCCAGCATGGC

PCR products were ligated between SalI and SacI for E-Syt1, EcoRI and BamHI for E-Syt2 and Syt1 and XhoI and SalI for E-Syt3 in the pEGFP-N1 vector (Clontech) to generate E-Syt1-EGFP, E-Syt2-EGFP, E-Syt3-EGFP and Syt1-EGFP and in the pmCherry-N1 (Clontech) to generate E-Syt2-mCherry and E-Syt3-mCherry.

Cloning of EGFP-E-Syt1, EGFP-E-Syt2, EGFP-E-Syt3, mCherry-E-Syt1, mCherry-E-Syt2:

cDNAs of E-Syt1, E-Syt2 and E-Syt3 were amplified by PCR. In all PCR reactions, Herculase II fusion DNA polymerase (Agilent) was used for E-Syt1 and E-Syt3 and Pfu UltraII DNA polymerase (Agilent) for E-Syt2. Primers used were (coding sequence shown in lowercase):

5’ SalI-E-Syt1_Fw, ACCG*GTCGAC*atggagcgatctccaggaga

3’ SacII-E-Syt1 Stop_Rw, ACCG*CCGCGG*ctaggagctgcccttgtcct

5' EcoRI-g-ESyt2b_Fw, CAAGCTTC*GAATTC*gatgacgccaccgtcccgggc

3' BamHI-ESyt2 Stop_Rw, GATCCGGT*GGATCC*ctatgtcatcgcctgaggcctc

5’ XhoI-E-Syt3_Fw, CCG*CTCGAG*CTatgcgagcagaggagccct

3’ SalI-E-Syt3 Stop_Rw, ACCG*GTCGAC*tcagcttctgggctgtccat

PCR products were ligated between SalI and SacI for E-Syt1, EcoRI and BamHI for E-Syt2 and XhoI and SalI for E-Syt3 in the pEGFP-C1 vector (Clonetech) to generate EGFP-E-Syt1, EGFP-E-Syt2 and EGFP-E-Syt3 and in the pmCherry-C1 (Clonetech) to generate mCherry-E-Syt1 and mCherry-E-Syt2.

Cloning of mCherry-E-Syt2-EGFP

E-Syt2-EGFP was digested with BglII and MfeI, and the fragment was ligated between BglII and MfeI in the mCherry-C1.

Cloning of Myc-E-Syt1, Myc-E-Syt2, Myc-E-Syt3 and Myc-Syt1

cDNA encoding Myc epitope was synthesized in the pIDTSMART-Kan vector backbone (Integrated DNA Technologies). Sequence used was,

GCTAGCGCTACCGGTCGCCACCATGCAGAAGCTGATCTCAGAGGAGGACC

TGTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTA

CCGCGGGCCCGGGATCCACCGGATCTAGA

To generate pMyc-C1, the vector was digested with NheI and BamHI and the fragment was ligated into pEGFP-C1 to replace EGFP with a Myc tag.

E-Syt1, E-Syt2 and E-Syt3 cDNA were digested from EGFP-E-Syt1, EGFP-E-Syt2 and EGFP-E-Syt3 (see above) and ligated between SalI and SacI for E-

Syt1, EcoRI and BamHI for E-Syt2 and XhoI and SalI for E-Syt3 in the pMyc-C1 vector to generate Myc-E-Syt1, Myc-E-Syt2 and Myc-E-Syt3.

cDNA of Syt1 was amplified by PCR. Primers used were:

5' EcoRI-g-Syt1,

GCGGCCGCGAATTCgATGGTGAGCGAGAGTCACCATGAGG

3' BamHI_Syt1 No Stop,

CGACCGGTGGATCCTTCTTCTTGACGGCCAGCATGGC

The PCR product was ligated between EcoRI and BamHI in the pMyc-C1 vector to generate Myc-Syt1.

Cloning of pGEM-4Z-Myc-E-Syt3 and pGEM-4Z-Myc-Syt1

Myc-E-Syt3 and Myc-Syt1 were digested with AgeI and XbaI, and fragments were ligated in the pGEM-4Z (promega) using XmaI and XbaI to generate pGEM-4Z-Myc-E-Syt3 and pGEM-4Z-Myc-Syt1 (used for Cell-free transcription and translation-translocation assay: see below).

Cloning of 3xFLAG-E-Syt1, 3xFLAG-E-Syt2

cDNAs of E-Syt1 and E-Syt2 were amplified by PCR. Primers used were:

5’ NotI-E-Syt1_Fw, ATAAAGAAT*GCGGCCGC*atggagcgatctccaggaga

3’ XbaI-E-Syt1_Rw, CG*TCTAGA*ctaggagtcgcccttgtcct

5' EcoRI-g-ESyt2b_Fw, CAAGCTTCGAATTCgatgacgccaccgtcccgggc

3' BamHI-ESyt2 Stop_Rw, GATCCGGTGGATCCctatgtcatcgcctgaggcctc

PCR products were ligated between NotI and XbaI for E-Syt1, EcoRI and BamHI for E-Syt2 in the p3XFLAG-CMV-10 (Sigma-Aldrich) to generate 3xFLAG-E-Syt1 and 3xFLAG-E-Syt2.

Cloning of Internal EGFP fusion for E-Syt2

Predicted transmembrane region of E-Syt2 was amplified using the following primers:

5' AgeI_ESyt2b_H, CTAGCGCTACCGGTGCCACCatgacgccaccgtcccgggc

3' AgeI_ESyt2b_H,

TGGTGGCGACCGGTgccctgaaaataaagattctcccacaccaggcgagcagcgcgagcgcg

PCR products were ligated at AgeI of E-Syt2 TMΔ construct described below to generate TM-EGFP-E-Syt2.

Cloning of EGFP-TEV-3xFLAG-E-Syt2

cDNA encoding TEV protease cleavage site and 3xFLAG was synthesized in the pIDTSMART-Kan vector backbone (Integrated DNA Technologies) to generate TEV-3xFLAG. Sequence used was,

GATCTtccaccggcgggggccgctctagagagaatctttattttcagggcgagctcgggtccgccaccatgg actacaaagaccatgacggtgattataaagatcatgacatcgattacaaggatgacgatgacaagcttgcggc cgcGAATTC

To generate EGFP-TEV-E-Syt2, TEV-3xFLAG was digested and the cDNA fragment was ligated between BglII and EcoRI in the EGFP-E-Syt2.

Constructs encoding the short isoform of E-Syt2 (E-Syt2S) were generated using the following primers:

For E-Syt2S-EGFP

5' EcoRI-g-ESyt2b_short, CAAGCTTCGAATTCgatgagcggcgcccggggcgagggcc

3' BamHI-ESyt2 Stop, GATCCGGTGGATCCctatgtcatcgcctgaggcctc

For EGFP-E-Syt2S and Myc-E-Syt2S

5' EcoRI-g-ESyt2b_short, CAAGCTTCGAATTCgatgagcggcgcccggggcgagggcc

3' BamHI-ESyt2b NoStop, CGACCGGTGGATCCtttgtcatcgcctgaggcctcg

PCR products were ligated as described for N- or C- terminal fusion of E-Syt2 (see above).

E-Syt2 deletion and mutation analysis

For deletion analysis of E-Syt2 (except for E-Syt2 SMPΔ), primers flanking target regions were created and individual cDNAs were amplified by PCR from EGFP-E-Syt2 using following primers:

E-Syt2 C2CΔ

5' EcoRI-g-ESyt2b, CAAGCTTCGAATTCgatgacgccaccgtcccgggc

3' BamHI-ESyt2b C2Cdel NoStop,

CGACCGGTGGATCCttGTTTCTCTGCGAGCTGTGCC

E-Syt2 C2ABC

5' EcoRI-g-ESyt2_TM-SMPdel, CAAGCTTCGAATTCgagtgaagttcaaatagctcag

3' BamHI-ESyt2 Stop, GATCCGGTGGATCCctatgtcatcgcctgaggcctc

E-Syt2 C2C

5' EcoRI_g-ESyt2C2C_L,

CAAGCTTCGAATTCgAGGCTGAGGCAGCTGGAAAACGGG

3' BamHI-ESyt2 Stop, GATCCGGTGGATCCctatgtcatcgcctgaggcctc

E-Syt2 TMΔ (See above for generation of TM-EGFP-E-Syt2)

5' EcoRI-g-ESyt2_TMdel, CAAGCTTCGAATTCgcgccgcagccgcggcctcaa

3' BamHI-ESyt2 Stop, GATCCGGTGGATCCctatgtcatcgcctgaggcctc

These cDNAs were inserted between EcoRI and BamHI in pEGFP-N1 (E-Syt2 C2CΔ) or pEGFP-C1 (E-Syt2 C2ABC, E-Syt2 C2C and E-Syt2 TMΔ).

E-Syt2 SMPΔ was generated by site-directed mutagenesis using the following primers in E-Syt2-EGFP:

SMP deletion QC_F, ctgggttcattttccagacactgaaagtgaagttcaaatagctcagttgc

SMP deletion QC_R, gcaactgagctatttgaacttcactttcagtgtctggaaaatgaacccag

Basic Patch of E-Syt2 C2C domain was mutated using the following primers in E-Syt2-EGFP to generate E-Syt2 AAA:

C2C_ESyt2_QC_AA>AAA_Fw,

CGCATGTATTTATTACCAGACgccgccgccTCAGGAgccgccgccACACACGTGTCAAAG

C2C_ESyt2_QC_AA>AAA_Rw,

CTTTGACACGTGTGTggcggcggcTCCTGAggcggcggcGTCTGGTAATAAATACATGCG

Generation of EGFP-E-Syt3 C2C deletion mutant

C2C domain of EGFP-E-Syt3 was deleted using site-directed mutagenesis (QuickChange II-XL, Stratagene) to generate EGFP-E-Syt3_C2CΔ mutant. The following primers were used:

E-Syt3_C2CΔFw 5' ctcaggcgacggcagctgattgacttatcaaaagaag 3'

E-Syt3_C2CΔRw 5' cttcttttgataagtcaatcagctgccgtcgcctgag 3'

Generation of Ca^{2+}-binding deficient E-Syt1

7 aspartic acid residues predicted to confer Ca^{2+}-binding properties to E-Syt1 was mutated to alanine using site-directed mutagenesis (QuickChange II-XL, Stratagene) to generate mCherry-E-Syt1-mut. The following primers were used (targeted nucleotides are shown in uppercase:

E-Syt1_C2C_D663A_S, gacctgattgccaaagCccgtttcttgggggg

E-Syt1_C2C_D663A_AS, ccccccaagaaacggGctttggcaatcaggtc

E-Syt1_C2C_D675A_S, ggtgaagggcaagtcagCcccctatgtcaaactaa

E-Syt1_C2C_D675A_AS, ttagtttgacatagggGctgacttgcccttcacc

E-Syt1_C2C_D722-729A_S,

ctagaggttgaagtctttgCcaaggCcttggCcaaggCtgCttttctgggcaggtgtaaa

E-Syt1_C2C_D722-729A_AS,

tttacacctgcccagaaaaGcaGccttgGccaagGccttgGcaaagacttcaacctctag

Cloning of untagged E-Syts, including RNAi-resistant E-Syt1

Untagged E-Syt1 and E-Syt3 were generated from the respective N-terminal EGFP-fusion construct by excision of EGFP using Age1/BsrG1 followed by Mungbean nuclease treatment and blunt-end ligation. RNAi resistant E-Syt1 was generated by introducing 6 silent point mutations in the region targeted by the RNAi oligos using site-directed mutagenesis and the following primers (targeted nucleotides are shown in uppercase:

E-Syt1_siRNA-res_S,

ctgggccaggtgaaactgactctgtggtaTtaTTCCgaGgagcgaaagctggtcagcattgttc

E-Syt1_siRNA-res_AS,

gaacaatgctgaccagctttcgctcCtcGGAAtaAtaccacagagtcagtttcacctggcccag

To generate untagged E-Syt2 and E-Syt2S, EGFP-E-Syt2 and EGFP-E-Syt2S were digested with EcoRI and MfeI, and digested fragments (E-Syt2 and E-Syt2S) were ligated between EcoRI and MfeI in pEGFP-N1.

Cloning of VAP-A-GFP:

The human VAP-A coding sequence was amplified by PCR using the following primers containing XhoI and HindIII restriction sites:

5' XhoI-VAP-A_Fw CCG*CTCGAG*atggcgtccgcctcaggg

3' HindIII-VAP-A _Rw CCC*AAGCTT* caagatgaatttccctagaaagaatc

The PCR product was cloned in XhoI/HindIII-digested pEGFP-N1 vector (Clontech) to express VAP-A with an EGFP-tag at the C-terminus.

Cell culture and transfection

HeLa, COS-7 and SH-SY5Y cells were cultured in DMEM containing 10% FBS at 37°C and 5% CO_2. Transfection of plasmids and RNAi oligos was carried out with Lipofectamine 2000 and RNAIi MAX (Life Technologies).

siRNAs

HeLa cells were transfected with control or E-Syts siRNA oligos by using Lipofectamine RNAi MAX (Life technologies), and cultured for 72 hours prior to analysis. Double-stranded siRNAs were derived from the following references:

E-Syt1 (J-010652-06-0010 from Dharmacon)

E-Syt2 (human ESYT2 HSC.RNAI.N020728.12.5 and 12.1 from IDT; 12.1,

targeting UTR, was used for rescue experiments)

E-Syt3 (human ESYT3 HSC.RNAI.N031913.12.6 from IDT)

Control (NC1 negative control duplex from IDT)

Real time qPCR analysis

Total RNA was extracted from HeLa cells transfected with control or E-Syts siRNAs using RNeasy MiniKit (Qiagen). cDNA was synthesized from 2 μg of RNA with the iScriptTM cDNA synthesis kit (Bio-Rad). Resulting DNA was subjected to real-time qPCR with SYBR green fast kit (Applied Biosystems) according to manufacturers instructions. Quantification results were expressed in terms of the cycle threshold (Ct). All real-time qPCR reactions were run in triplicate, and the Ct values were averaged from three independent samples. Data were normalized to the internal control, the reference gene S26. Differences between the mean Ct values of each gene and those of the reference gene were calculated as $\Delta Ct = Ct^{gene} - Ct^{reference}$ and represented as $2^{-\Delta Ct}$. (Figure S5B).

List of the intron-spanning primers used:

Human S26_Fw: CCGTGCCTCCAAGATGACAA;

Human S26_Rw: GCAATGACGAATTTCTTAATGGCCT;

Human E-Syt1_Fw: CATGTCAAGAGAGCGTGAGC;

Human E-Syt1_Rw: ACCCTGGGAAAGGTCTGTCT;

Human E-Syt2_Fw: ACCCAAATCCTGTTGTCCAG;

Human E-Syt2_Rw: TTTTCCTCCCACACAGGTTC;

Human E-Syt3_Fw: GGTGGATGAGTGGTTTGTCC

Human E-Syt3_Rw: ACGAGAATGGCAGTGGAAAG

Fluorescence microscopy

Fixed cells: Transfected cells were fixed with 4% PFA/PBS at room temperature, washed in PBS, mounted (ProLong Gold antifade reagent, Invitrogen) and imaged under a spinning disc confocal (SDC) microscope. Images from a mid-focal plane are shown.

Live cells: Before imaging, cells were transferred to imaging buffer containing, in mM: 125 NaCl, 5 KCl, 1.3 $CaCl_2$, 1.2 $MgCl_2$ and 3 D-glucose with pH adjusted to 7.4 with NaOH.

For imaging experiments, cells were plated on 35mm glass bottom dishes at low density (MatTek Corp, Ashland, MA). Live cell imaging and immunofluorescence were carried out one day after transfection. Spinning disc confocal (SDC) microscopy was performed using the Improvision UltraVIEW VoX system (Perkin-Elmer) built around a Nikon Ti-E inverted microscope, equipped with PlanApo objectives (60X1.45-NA) and controlled by Volocity (Improvision) software. Total internal reflection fluorescence (TIRF) microscope was performed on a setup built around a Nikon TiE microscope equipped with 60X1.49-NA and 100X1.49-NA objectives. Excitation light was provided by 488-nm (for GFP and blue-light activation), 561-nm (mCherry and mRFP) and 640-nm (iRFP) DPSS lasers coupled to the TIRF illuminator through an optical fiber. The output from the lasers was controlled by an acousto-optic tunable filter and fluorescence was detected with an EM-CCD camera (Andor DU-887). Acquisition was controlled by

Andor iQ software. Images were typically sampled at 0.25 Hz with exposure times in the 100-500 ms range. SDC microscopy was carried out at room temperature (20-25°C) and TIRF microscopy at 37°C.

Optogenetic depletion of PI(4,5)P_2 in the plasma membrane

Optogenetics:

Blue light-induced 5-dephosphorylation of plasma membrane PI(4,5)P_2 was performed as previously described (Idevall-Hagren et al., 2012; Kennedy et al., 2010). Briefly, the 5-phosphatase domain of human OCRL (residues 234-539) was ligated to the C-terminus of the PHR-domain of Arabidopsis thaliana cryptochrome 2 (CRY2) to generate CRY2-5-ptase$_{OCRL}$ and Arabidopsis thaliana CIBN was fused to a C-terminal CAAX-box motif for plasma membrane anchoring (CIBN-CAAX). When expressed in cells, blue light (488 nm) illumination induces immediate binding of CRY2 to CIBN, thus bringing the 5-ptase to the plasma membrane. SDC microscopy was performed as described above. 488-nm laser light was used to induce dimerization between CRY2 and CIBN and to image GFP-fusion proteins and 561-nm and 640-nm laser lines were used to image mCherry/mRFP and iRFP (Filonov et al., 2011), respectively. Images were typically sampled at 0.2 Hz with exposure times in the 100-500 ms range. TIRF microscopy was performed as described above. Note that due to the spectral overlap of GFP with the absorption spectrum of CRY2 (Liu et al., 2008), GFP-tagged proteins could only be imaged when dimerization was promoted. For TIRF imaging, dimerization was induced by 50x100 ms 488 nm

light pulses delivered through the evanescent field. For SDC imaging, dimerization was induced by 40x200 ms 488 nm light pulses delivered so that the whole cell was illuminated.

Quantification:

Changes in plasma membrane fluorescence over time (TIRF miscoscopy) were analyzed off-line using Image J (NIH) or Fiji (http://fiji.sc/wiki/index.php/Fiji) by manually drawing regions to cover as large area of the cell foot-print as possible. Quantification of fluorescence changes were performed using Excel (Microsoft) and all data are presented as mean ± SEM. In Figure S6D/E, the number of fluorescent spots was quantified after setting an arbitrary threshold identical for the three conditions.

Calcium imaging

HeLa cells were loaded with 5μM Fura-2-AM with Pluronic F127 (invitrogen) in Ca^{2+}-containing buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 2 mM $CaCl_2$ [pH 7.4]) for 1 hour at room temperature. Fura-2 fluorescence was measured by illuminating the cells with an alternating 340/380 nm light every 5 second. Fluorescence intensity was measured at 510 nm. Changes in intracellular Ca^{2+} concentration are presented as the change in the ratio of fluorescence intensity for excitation at 340 and 380 nm (F340/F380). To monitor store-operated calcium entry, cells were washed twice and incubated with Ca^{2+}-free buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 3 mM EGTA, [pH 7.4]) before imaging. Thapsigargin was

added in Ca^{2+}-free buffer, and Ca^{2+} buffer was added back after Ca^{2+} store depletion. Cells were imaged with Axio Observer Z1 (Carl Zeiss) equipped with ORCA-Flash4.0 (Hamamatsu photonics), and fluorescence intensities were measured with the Zen 2012 software (Carl Zeiss).

Antibody accessibility assay and immunofluorecence

HeLa cells or SH-SY5Y cells co-transfected with N-terminus Myc-tagged E-Syts and mRFP-Sec61β (or HeLa cells transfected with N-terminus FLAG-tagged E-Syts alone) were washed twice with ice-cold PBS and either directly incubated at 4°C with anti-Myc antibodies for one hour (non-permeabilized conditions) or fixed with 4% PFA at room temperature for 20 minutes, washed with PBS and incubated with PBS containing 0.1% Triton X-100 at room temperature for 10 minutes prior to anti-Myc antibody (or anti-FLAG antibody) incubation for 45 min (permeabilized conditions). Cells without prior permeabilization were washed with PBS and fixed with PBS containing 4% PFA at room temperature for 20 minutes. Both non-permeabilized and permeabilized cells were then incubated with Alexa Fluor 488 (or 594) -conjugated anti-mouse (or anti-rabbit for FLAG antibody) secondary antibody in 0.5% BSA/PBS for one hour at room temperature. After 4x washing with PBS, cells were examined under a SDC microscope. Images from a mid-focal plane are shown.

Digitonin assay

Transfected HeLa cells were washed three times with KHM buffer (110mM potassium acetate, 20mM HEPES, 2mM $MgCl_2$, pH7.3), and KHM buffer containing 20µM digitonin was applied with anti-Myc antibody-conjugated with Alexa Fluor 488. Cells were incubated at room temperature for 5 min, washed twice with KHM buffer and fixed with KHM buffer containing 4% PFA at room temperature for 20 minutes. After 4x washing with PBS, cells were examined under a SDC microscope.

Fluorescent Protease Protection (FPP) assay

FPP assay was carried out as previously described (Lorenz et al., 2006). Briefly, transfected HeLa cells were washed three times with KHM buffer, and images were taken every 10 seconds under a SDC microscope. After 100 seconds of baseline acquisition, cells were permeabilized by KHM buffer containing 20µM digitonin. 100 seconds after permeabilization, KHM buffer containing Proteinase K (Sigma-Aldrich, P4850), (400 fold dilution, approximately 1.5 units/ml) was applied to cleave cytosolically exposed portions of proteins.

Electron Microscopy analysis

Conventional EM:

HeLa cells grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, postfixed in 2% OsO_4+1% $K_4Fe(CN)_6$ in 0.1M cacodylate buffer at 4°C for 1h, dehydrated in ethanol and embedded in Epon while on the coverslips as described in (Giordano et al., 2009). Ultrathin sections were

counterstained with 2% uranyl acetate and lead citrate before observation under a Philips CM10 microscope equipped with a Morada 2kx2k CCD camera (Olympus).

HRP detection:

HeLa cells expressing HRP-KDEL or HRP-STIM1 were fixed on coverslips with 1.3% glutaraldehyde in 0.1M cacodylate buffer, washed in 0.1M ammonium phosphate [pH 7.4] buffer for 10 min and HRP was visualized with 0.5mg/ml DAB and 0.005% H_2O_2 in 0.1M Ammonium Phosphate [pH 7.4] buffer. Development of HRP (DAB dark reaction product) took between 5 min to 20 min and was stopped by extensive washes with cold water. Cells were postfixed in 2% OsO_4+1% $K_4Fe(CN)_6$ in 0.1M cacodylate buffer at 4°C for 1h, washed in cold water and then contrasted in 0.5% uranyl magnesium acetate for 2hrs at 4°C, dehydrated in ethanol and embedded in epon as for conventional EM. Ultrathin sections were counterstained with 2% uranyl acetate before observation.

Immunogold labeling:

HeLa cells were fixed with a mixture of 2%PFA and 0.125% glutaraldehyde in 0.1M phosphate buffer [pH 7.4], and processed for ultracryomicrotomy as described previously (Slot and Geuze, 2007). Ultrathin cryosections were single- or double-immunogold-labeled with antibodies and protein A coupled to 5 or 10 or 15 nm gold, as indicated in the legends to the figures. Sections of resin-embedded cells and immunogold-labeled cryosections were observed under a Philips CM10 microscope equipped with a Morada 2kx2k CCD camera

(Olympus).

Quantifications:

For the quantification of cortical ER in HRP-stained Epon sections, the total length of plasma membrane and the length of the multiple HRP-positive cER segments were measured by iTEM software on acquired micrographs of HeLa cells for each of 30 uninterrupted cell profiles showing the ER-PM contact phenotype.

For the quantification of E-Syts immunogold labeling on ultrathin cryosections, 600 gold particles were counted in randomly selected cell profiles and assigned to either non-cortical or cortical ER, in each of three experiments. All data are presented as mean +/- SD.

Biochemical analysis of E-Syts

Immunoprecipitation of E-Syts:

HeLa cells co-expressing Myc-tagged or untagged E-Syts and EGFP-tagged E-Syts or EGFP alone were washed in cold PBS and lysed on ice in lysis buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail (Roche)]. Cell lysates were then centrifuged at 21000 g for 20 min at 4°C. For immunoprecipitation of EGFP-tagged E-Syts or EGFP alone (control), supernatants were incubated with Chromotek GFP-trap agarose beads (Allele Biotech) for 1h at 4°C under rotation. Subsequently beads were washed in 0.1M phosphate buffer. After extensive washes in cold lysis buffer,

immunoprecipitated proteins bound to the beads were incubated in sample buffer (containing 2% SDS) and then boiled for 2 min. Immunoprecipitates were loaded and separated in 8% SDS–PAGE gel and immunoblotting was carried out as described in (Giordano et al., 2009). The antibodies used here were: polyclonal anti-GFP (Abcam) or polyclonal antibodies anti-Myc (Millipore), anti-E-Syt1, anti-E-Syt2, anti-E-Syt3 (all from Sigma-Aldrich), to detect co-immunoprecipitated bands.

Tandem affinity purification of E-Syt2:

Stable cell lines expressing EGFP-3xFLAG-E-Syt2 were generated using G418 selection media. Cells were cultured on a 15 cm dish, and 10 plates were used for each immunoprecipitation. Expression of tagged constructs was confirmed using anti-GFP antibody, anti-FLAG antibody and anti-E-Syt2 antibody. Cells were washed in cold PBS and lysed on ice in digitonin lysis buffer [1% digitonin, 20 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM EDTA, glycerol 10%, and protease inhibitor cocktail (Roche)]. Cell lysates were then centrifuged at 21000 g for 20 min at 4°C. For immunoprecipitation, supernatants were incubated with Chromotek GFP-trap agarose beads (Allele Biotech) for 30min at 4°C under rotation. After rigorous wash with the lysis buffer, proteins trapped in beads were eluted with TEV protease O/N at 4°C under rotation in elution buffer [20 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM EDTA, glycerol 10%, digitonin 1%, 1mM DTT, 0.3U/μl AcTEV (Invitrogen)]. After brief centrifuge, supernatants were collected and used for second immunoprecipitation with an anti-FLAG M2 affinity gel (Sigma Aldrich) for 3 hours at 4°C. Affinity gel was collected with brief centrifuge,

and proteins were eluted using FLAG peptides in TBS. After standard Methanol/Chloroform precipitation, samples were incubated in sample buffer containing 7% SDS at 60°C for 20min followed by incubation at 70°C for 10 min. Immunoprecipitates were loaded and separated in 8% SDS–PAGE gel (see above), and the gel was stained using colloidal blue staining kit (Invitrogen). Gels were excised, and Mass spectrometry was carried out to identify enriched-proteins according to a standard procedure.

Cell-free transcription and translation-translocation assay;

In vitro transcription of Syt1, E-Syt3 and soluble cytochrome b5 reductase (Pietrini et al., 1992) under the SP6 promoter, translation in the rabbit reticulocyte lysate (from Green Hectares) co-translational translocation reactions with dog pancreas rough microsomes (RMs – a kind gift of Dr. R.S. Hegde) with or without the NYT tripeptide, digestions with proteinase K (PK), immunoprecipitation with anti-Myc antibodies (clone 9E10, Sigma) were performed as described previously (Brambillasca et al., 2005) . Carbonate extractions were performed by addition of an equal volume of 0.2M Na_2CO_3 to the *in vitro* translated samples, which were then incubated for 30 min on ice before centrifugation at 150000 g for 45 min (Beckman TLA ultracentrifuge). Samples were analyzed by SDS-PAGE, followed by exposure to film or to a phosphorimager screen (Storm, GE Healthcare).

Subcellular fractionation and extraction assay:

HeLa cells were homogenized with HB buffer (0.25M sucrose, 1mM EDTA, 20mM Tris pH7.4) using 25G syringe. The homogenate was centrifuged at 1000

g for 15 minutes to isolate postnuclear supernatant (PNS). Equal amount of PNS was divided into 4 ultracentrifuge tubes and centrifuged at 170,000 g for 1 hour. Supernatant was discarded, and microsomal pellets were resuspended with either HB buffer alone, HB buffer containing 1M NaCl, HB buffer containing 2% Triton-X100 or Carbonate buffer (0.1M pH 11.3) and incubated on ice for 1 hour. Samples were again centrifuged at 170,000 g for 1 hour. Pellets were lysed with HB buffer containing 2% Triton-X100, and both the pellet and supernatant were analyzed with 8% SDS-PAGE followed by immunoblotting. Immunoblotting was carried out as described above.

Sequence analysis of E-Syts and Tricalbins

Transmembrane regions were identified by hydropathy plots using the Goldman Engelman Steitz scale and with the TMPred prediction algorithm (http://www.ch.embnet.org/software/TMPRED_form.html). The identified transmembrane regions were then aligned by ClustalW, using the Blosum Series matrix with the default parameters of MacVector 6.0 software. Homology modeling of C2 domains of the extended synaptotagmins was carried out using the HHPRED web server (http://toolkit.tuebingen.mpg.de/hhpred#), and MODELLER.

Structural alignment of C2 domains

The best sequences for alignment were automatically selected by the HHPRED web server.

Sequences used for homology modeling were the following:

E-Syt1 C2C domain: Q9BSJ8 630-668

E-Syt1 C2E domain: Q9BSJ8 964-1102

E-Syt2 C2C domain: A0FGR8 771-912

E-Syt3 C2C domain: A0FGR9 741-880

Statistical analysis

Image J (NIH) was used for generation of kymographs and quantification of fluorescent signals. Briefly, after background subtraction, fluorescent intensity was normalized to an average value of the initial 10 time points (F/F0). Graphical presentations were made using Excel (Microsoft). Comparisons between pre- and post-stimulation values (Figure 4G-J, 5B, 5G, 6D-6H, 7C, 7H, S3D, S6F) were made using Student's paired t-test.

Image J and Volocity 3D Image Analysis software were used for measuring of the area and the number of fluorescent signals in Figure S6H and S6I, respectively. iTEM software was used for quantification on EM sections. Box and whisker plots were made using Excel. The ends of the box represent the upper and lower quartiles (values above or below which the lower 25% of the data are contained, respectively). A vertical line inside the box marks the median. Statistical analysis in all other quantifications, including those of electron microscopy data, was made using Student's t-test for independent samples.

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Supplemental Figure

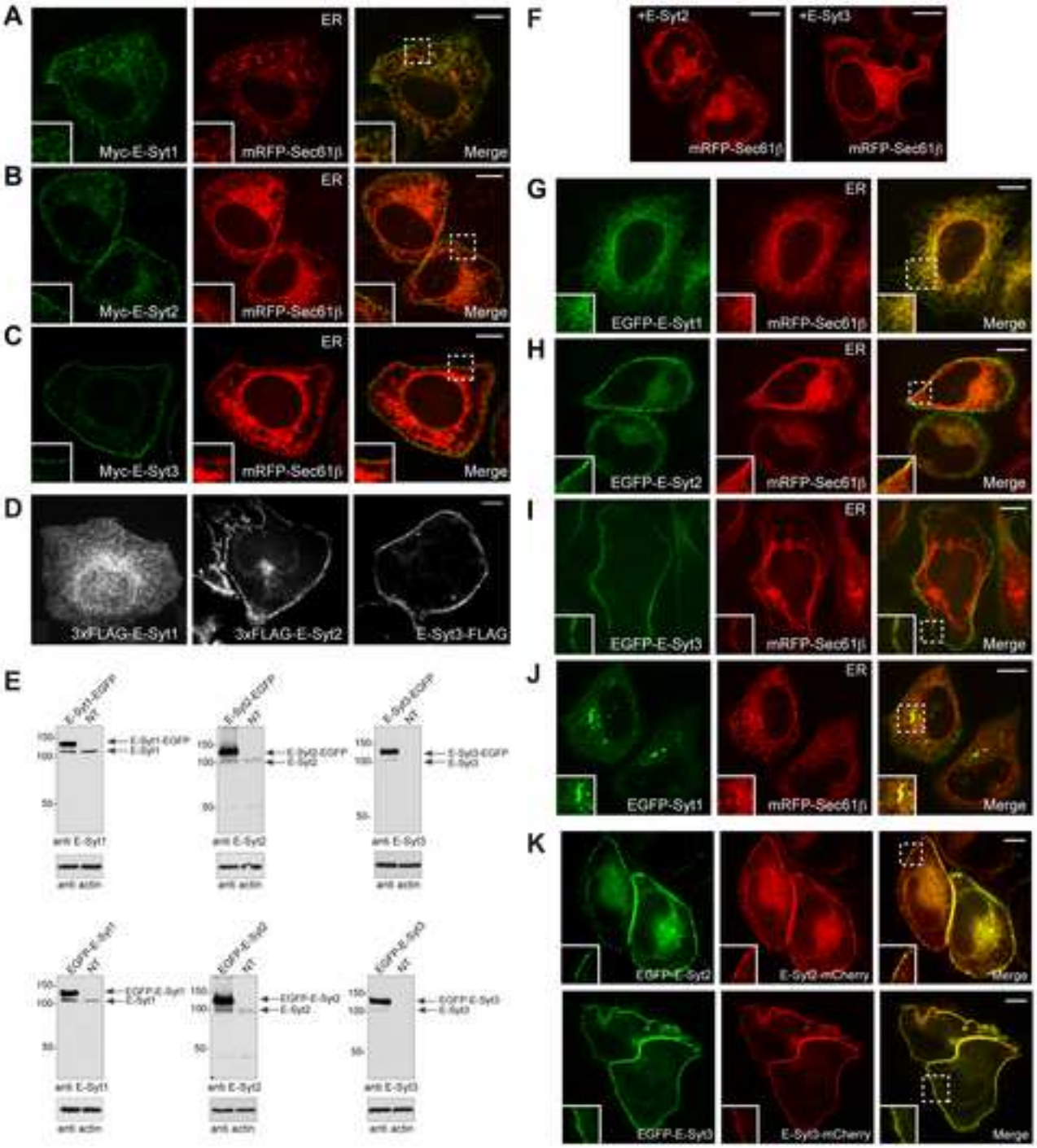


Figure S1

Supplemental Figure

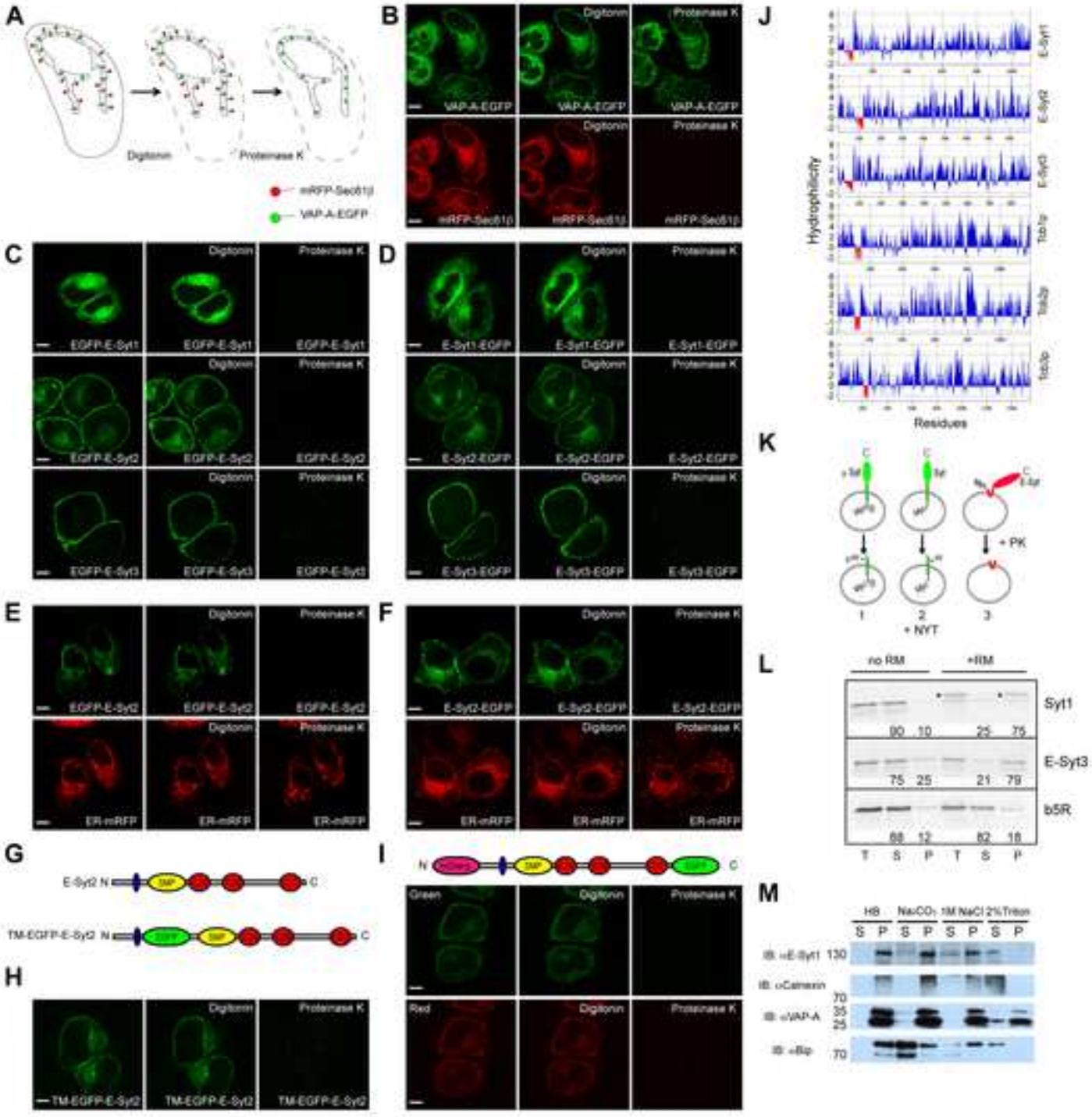
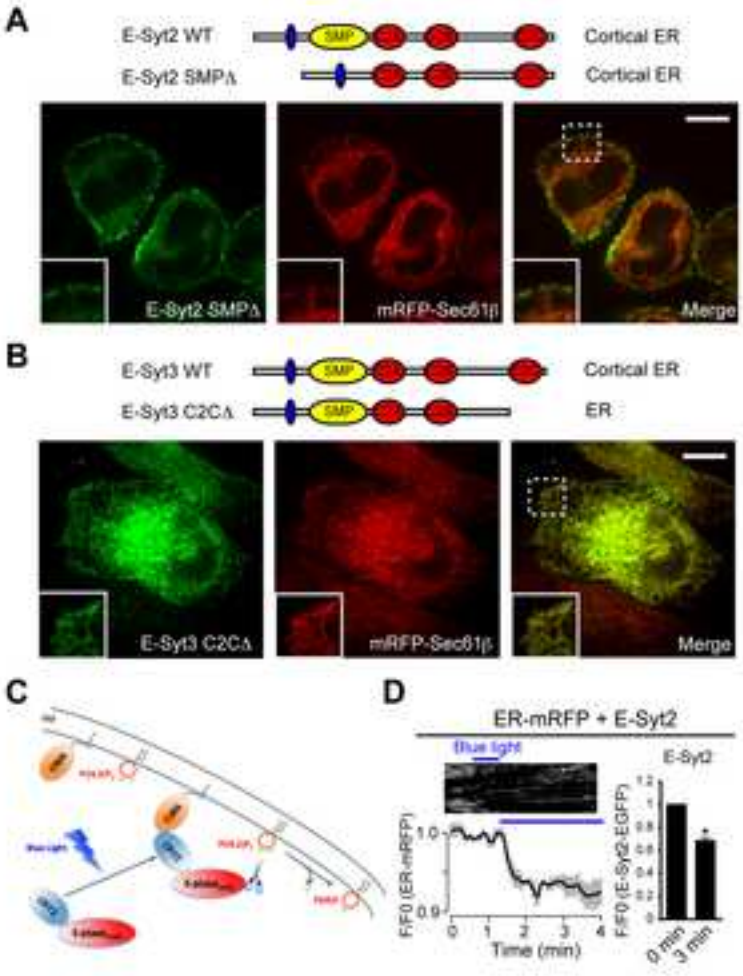


Figure S2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Merge
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SMP
Cortical ER
E-Syt3 C2CΔ
SMP
ER
E-Syt3 C2CΔ
mRFP-Sec61β
Merge
C
D
ER-mRFP + E-Syt2
Blue light
F/F0 (ER-mRFP)
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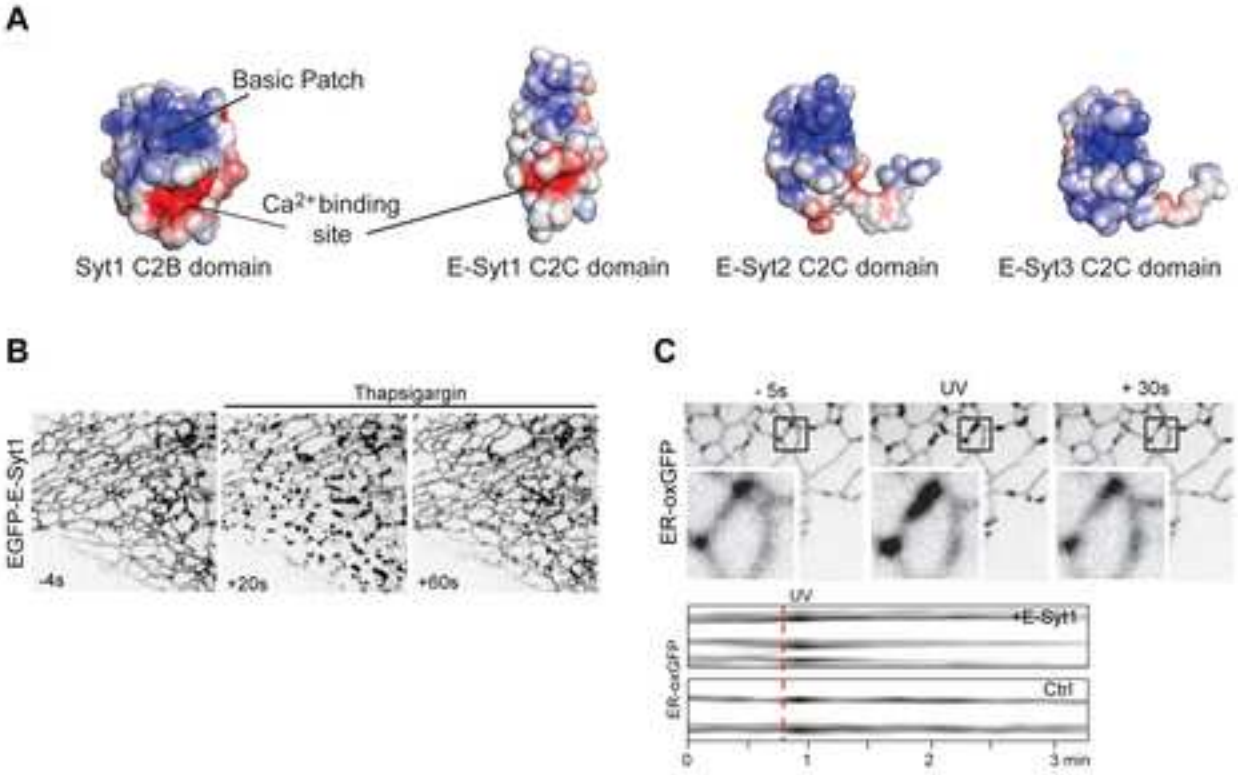
Figure S3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EGFP-E-Syt1
-4s
+20s
+60s
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Figure S4

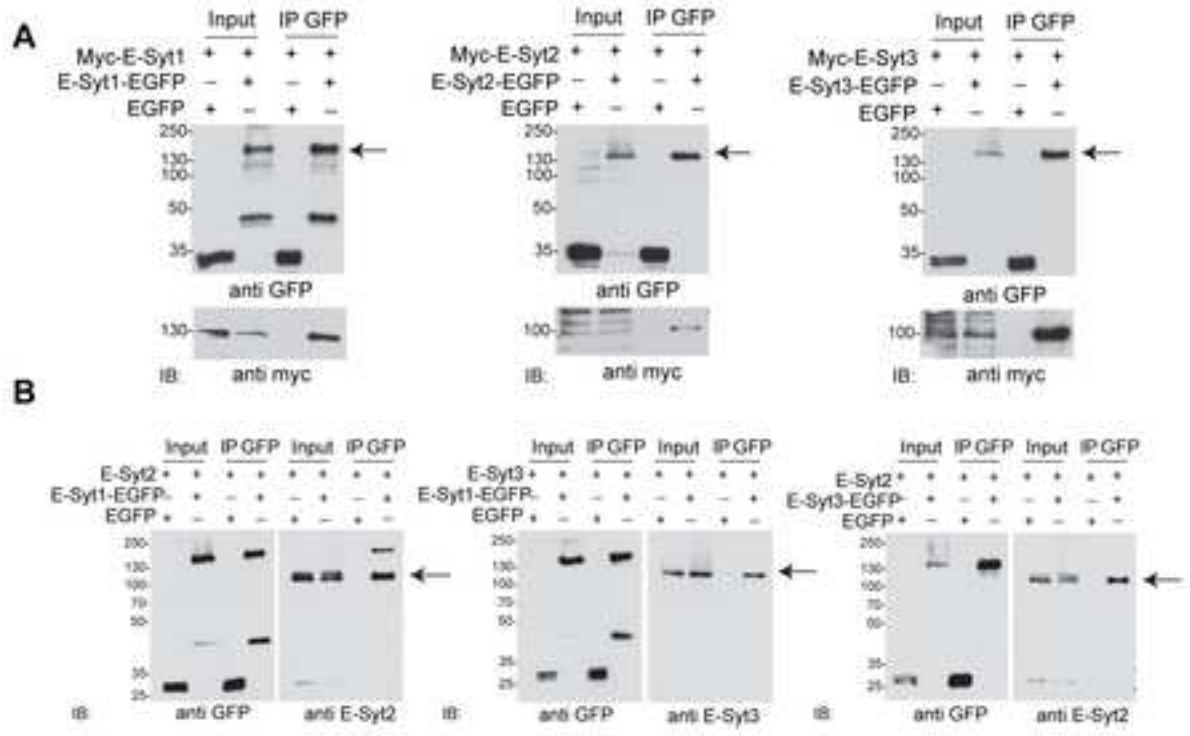
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Figure S5

Supplemental Figure

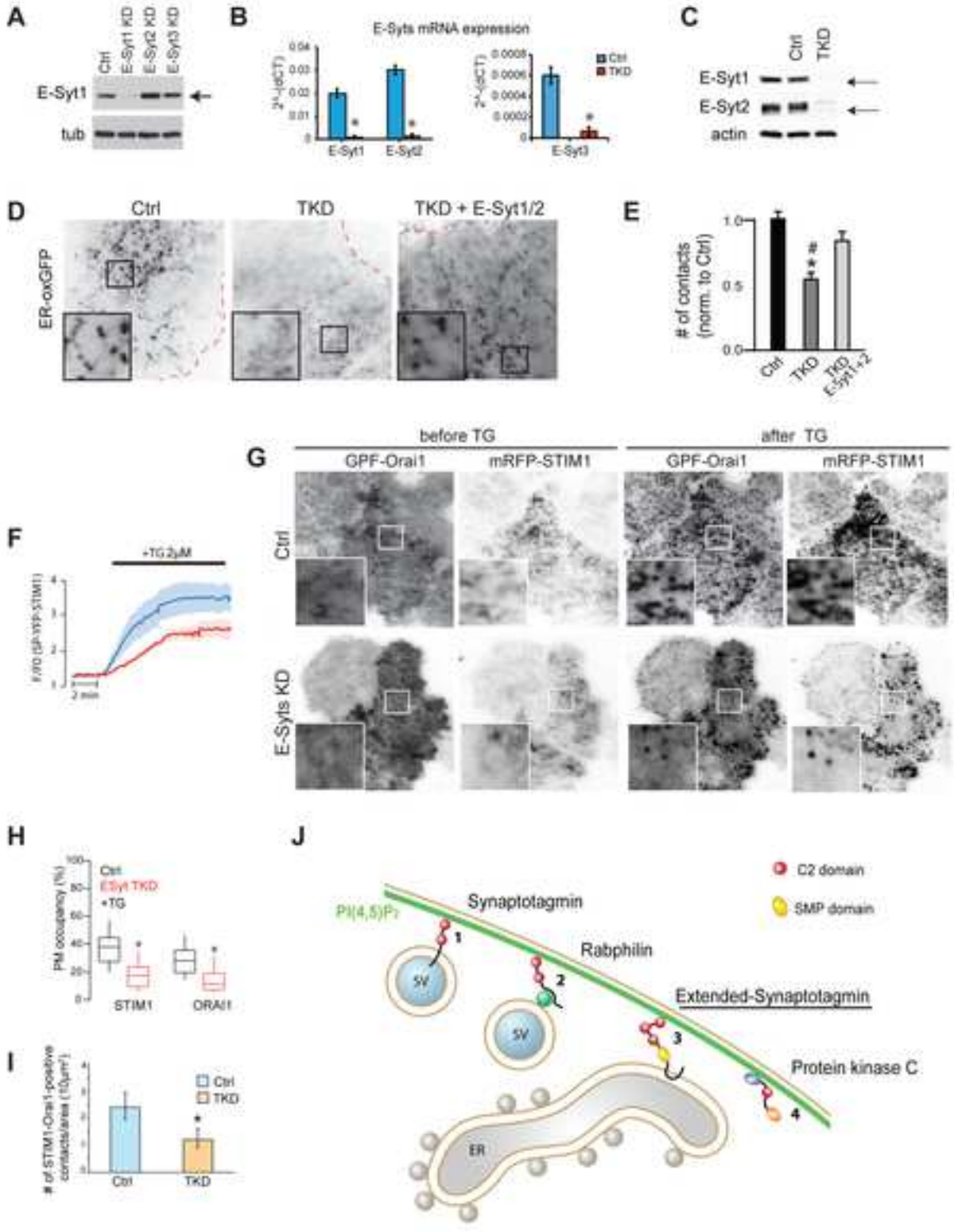

Figure S6