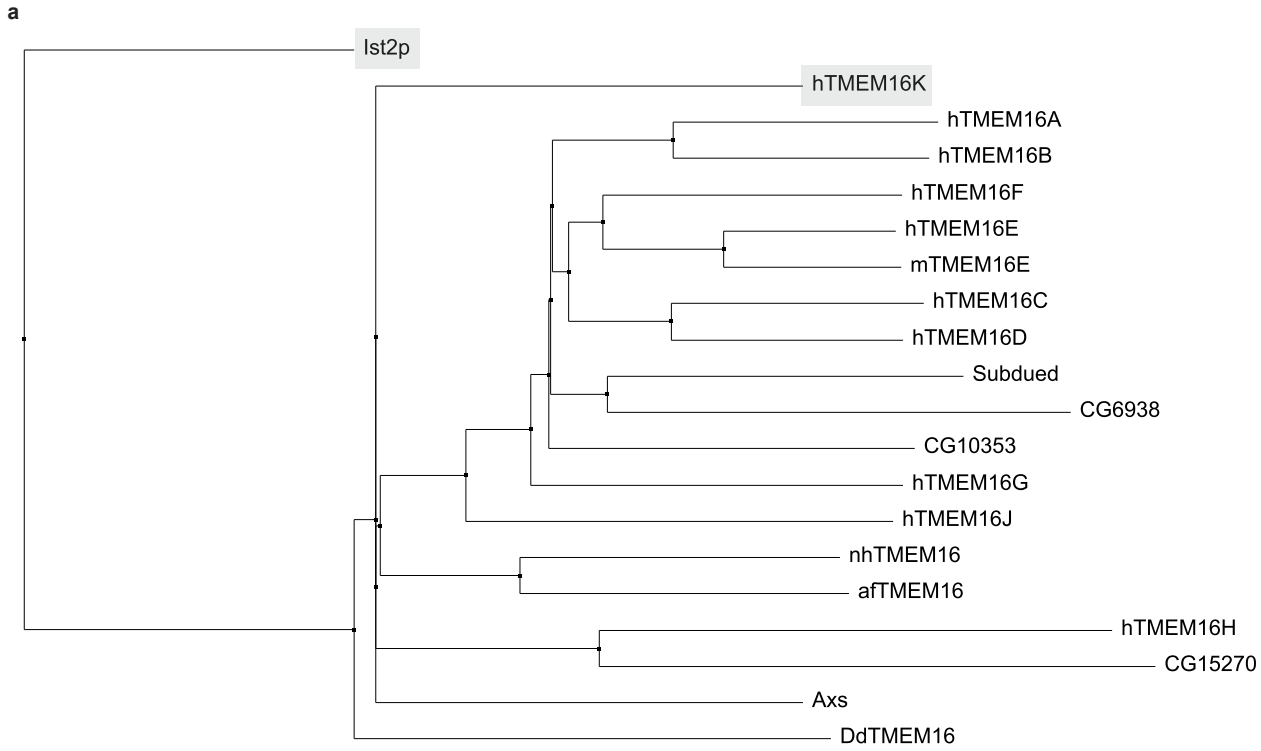
TMEM16K is an interorganelle regulator of endosomal sorting

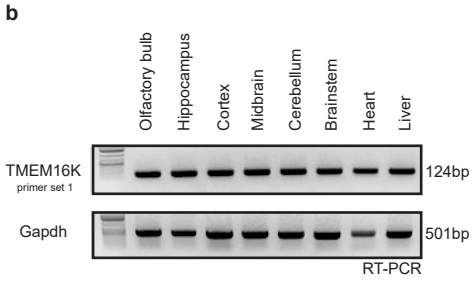
Maja Petkovic, Juan Oses-Prieto, Alma Burlingame, Lily Yeh Jan, Yuh Nung Jan

Supplementary Information

Content:

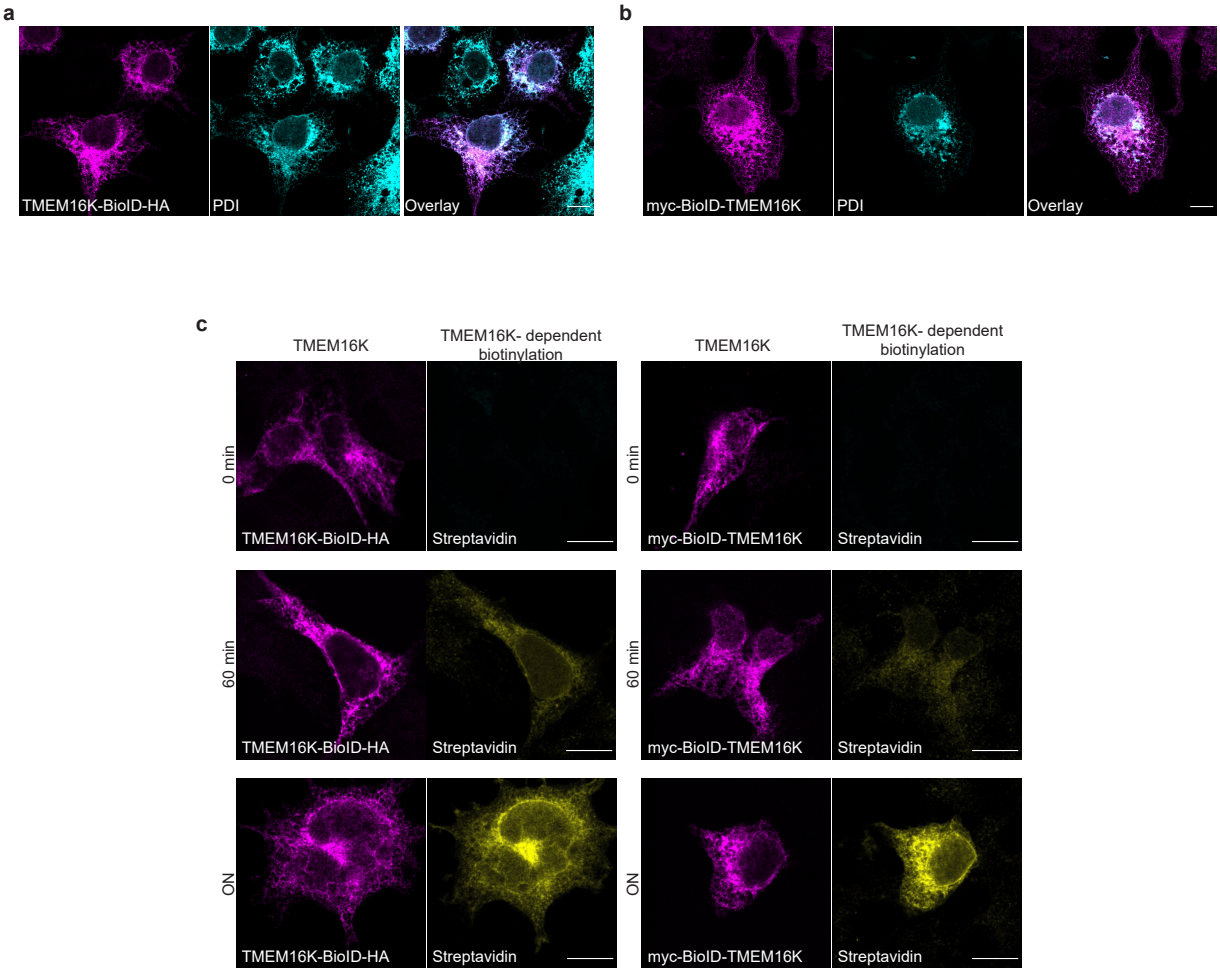
Supplementary Figure 1





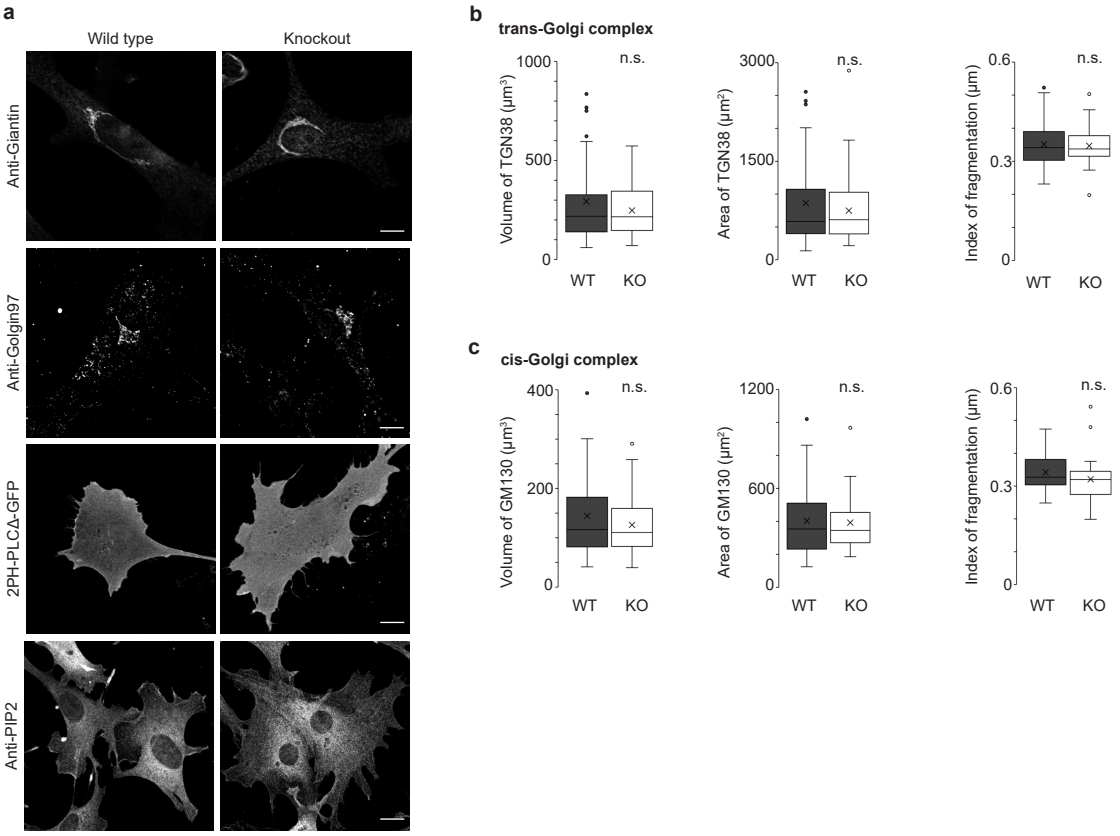
Supplementary Figure 1.a. Phylogenetic tree of TMEM16 family of proteins represented with only homolog in yeast *Saccharomyces cereviseae* (Ist2p), 10 homologs in humans (TMEM16A-H), 5 homologs in Drosophila (Axs, Subdued, CG6938, CG10353, CG15270), only homolog in fungi *Aspergillus fumigatus* (afTMEM16), only homolog in fungi *Nectria haematococca* (nhTMEM16) and only homolog in amoebozoa *Dictyostelium discoideum* (DdTMEM16). Representation was constructed in Jalview based on Neigbourhood Joining algorithm generated from the MUSCLE alignment of the sequences. **b.** RT-PCR from C57BL/6J mouse tissue shows TMEM16K is broadly expressed. RNA was extracted, reverse transcribed and equal amounts of each tissue sample were loaded on agarose gel and revealed with ethidium bromide. Housekeeping gene Gapdh was shown here as a control. DNA Ladder used was GeneRuler 1 kb Plus DNA Ladder from ThermoScientific.

Supplementary Figure 2



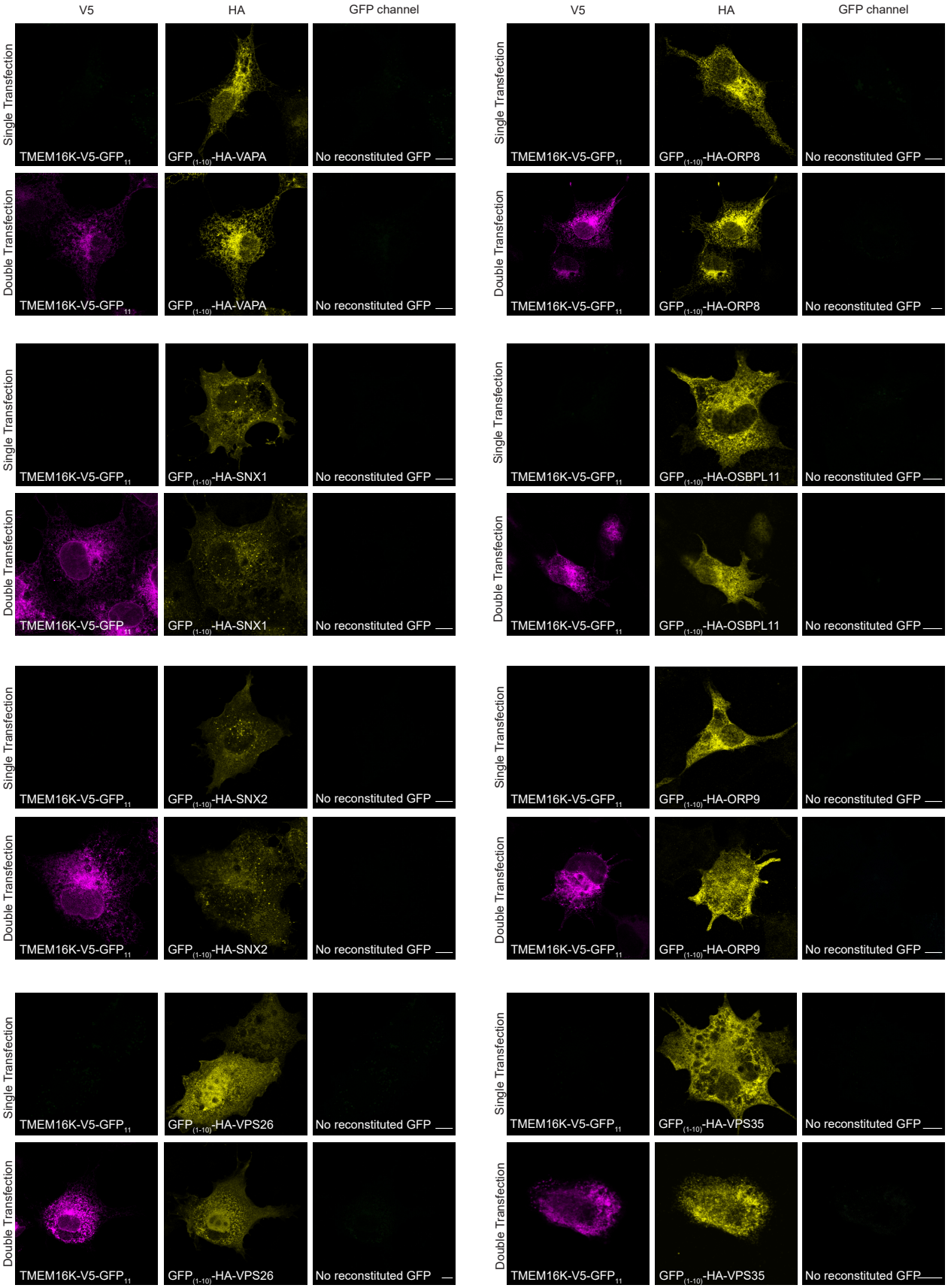
Supplementary Figure 2. a. Immunocytochemistry of TMEM16K tagged C-terminally with proximity biotinylation ezyme revealed with anti-HA antibody (magenta) and ER- marker PDI (cyan). Scale bar 10 µm. **b.** Immunocytochemistry of TMEM16K N-terminally tagged to proximity biotinylation enzyme revealed with anti-myc antibody (magenta) and ER-marker marker PDI (cyan). Scale bar 10 µm. **c.** Immunocytochemistry evaluating retention of biotinylation activity of the BioID tagged to TMEM16K. HEK293 cells were transfected with TMEM16K tagged with biotinylation enzyme constructs (magenta) and 24 hours post-transfection were exposed to no biotin, 60 min or overnight (ON) biotin at 50 µM final concentration. Distribution of TMEM16K-mediated proximity biotinylation was revealed with fluorescently conjugated Streptavidin (yellow). Scale bar 10 µm. Images are represented using pseudocolors suitable for color-blind palette.

Supplementary Figure 3



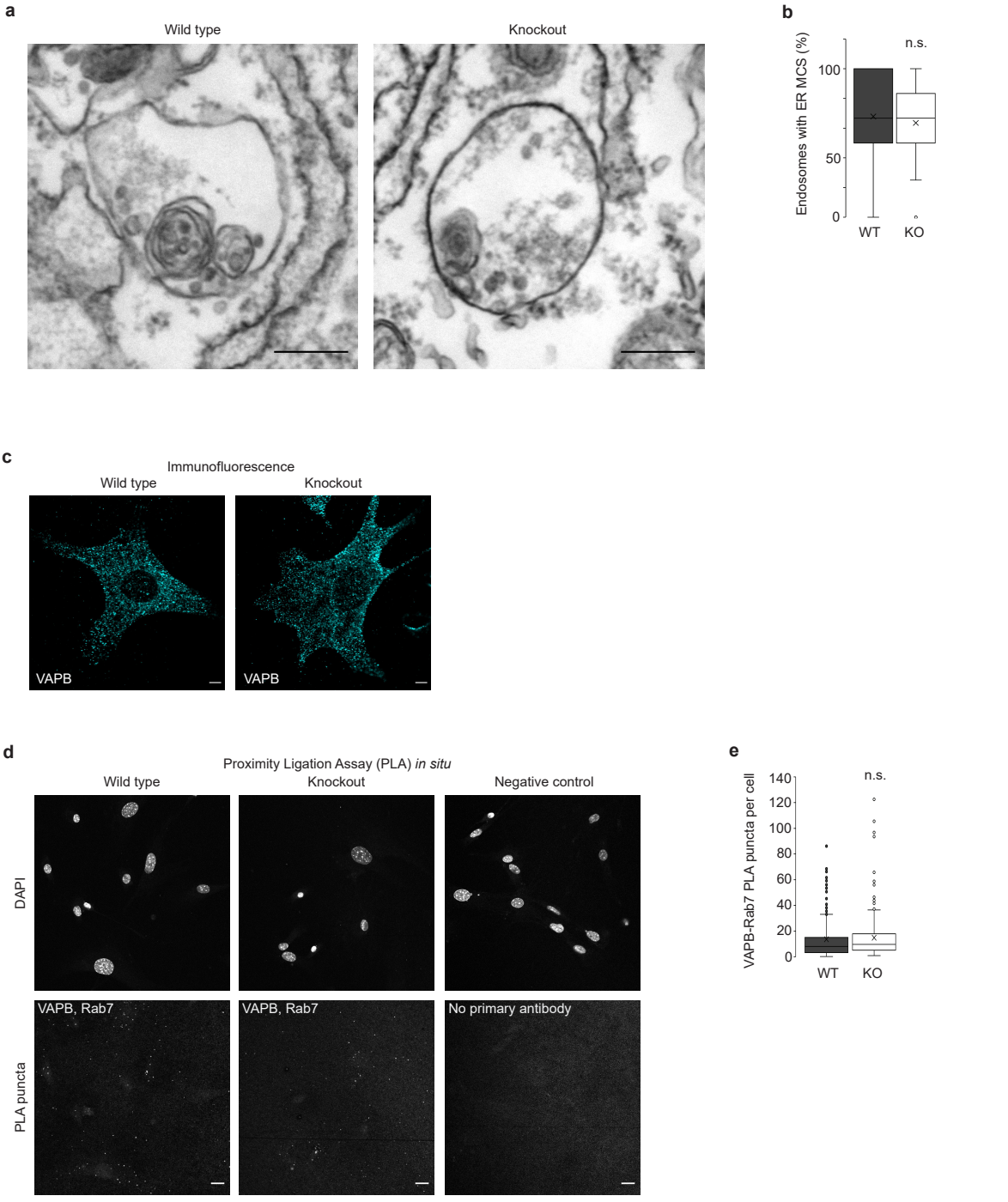
Supplementary Figure 3.a. Cellular markers evaluated in WT or TMEM16K KO cells with immunocytochemistry and confocal microscopy. Giantin and Golgin97 are Golgi markers. 2PH-PLCΔ-GFP and anti-PIP2 antibody are markers of PtdIns(4,5)P_2. Representative images from 2 independent experiments. Scale bar 10 µm. **b.** Evaluation of 3D trans-Golgi complex morphology from 3D reconstructions of endogenous immunolabeling of the trans-Golgi marker TGN38 in the WT (n=99) and TMEM16K KO (n=82) cells. Data obtained from 3 independent experiments Two-tailed Student t-test. Quantification of the volume (p-value=0.76 n.s.), area (p-value=0.89 n.s.) and index of fragmentation, expressed as the TGN38 volume divided by its area for each cell (p-value=0.13 n.s.). **c.** Evaluation of 3D cis-Golgi complex morphology from 3D reconstructions of endogenous immunolabeling of the cis-Golgi marker GM130 in the WT (n=40) and TMEM16K KO (n=36). Data obtained from 3 independent experiments. Two-tailed Student t-test. Quantification of the volume (p-value=0.32 n.s.), area (p-value=0.81 n.s.) and index of fragmentation, expressed as volume of GM130 divided by its area for each cell (p-value=0.17 n.s.).

Supplementary Figure 4



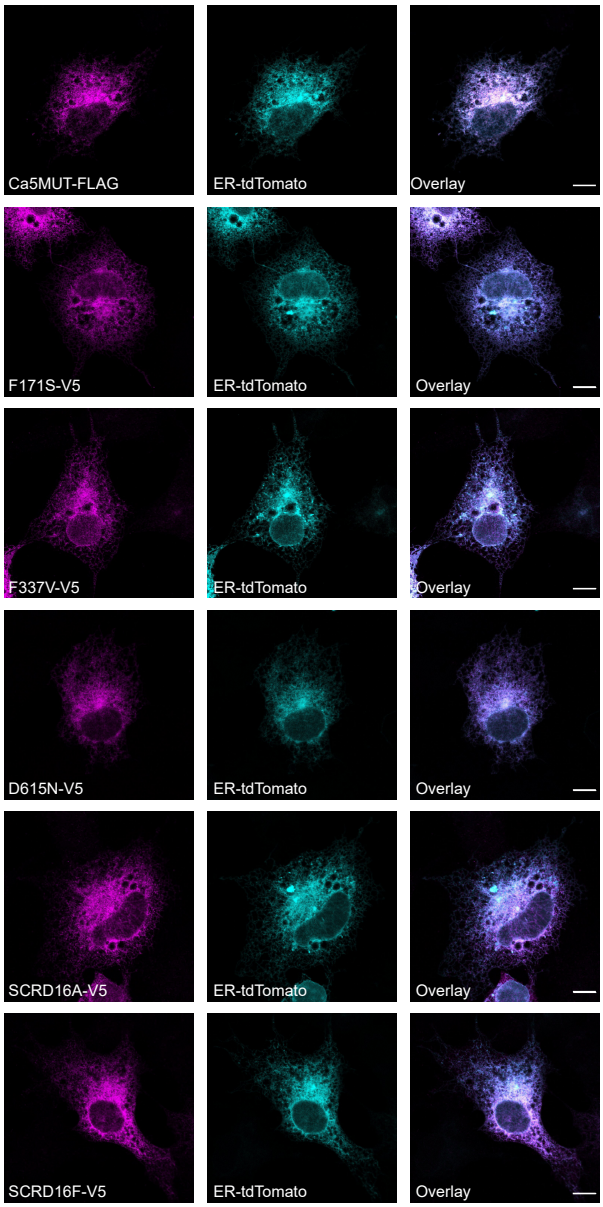
Supplementary Figure 4. Split-GFP reconstitution assay. Immunocytochemistry of single and double transfected COS7 cells with TMEM16K-V5-GFP11 (magenta) and tested proteins tagged with GFP(1-10) (yellow). Images are represented using pseudocolors suitable for color-blind palette. Representative images from 3 independent experiments. Scale bar 10 μm.

Supplementary Figure 5



Supplementary Figure 5. a. Electron micrographs showing membrane contact sites between ER and endosomes in the WT and TMEM16K KO primary fibroblasts. Scale bar, 200 nm. **b.** The percentage of endosomes with an ER contact site defined as proximity under 30 nm was quantified. Data are from two independent experiments. (n=68 electron micrographs of WT, 71 micrographs of TMEM16K KO). Two-tailed Student t-test, p-value=0.40 n.s. **c.** Immunofluorescence of WT and TMEM16K KO mouse primary fibroblast to evaluate the VAPB antibody. Scale bar, 5 µm. **d.** Proximity ligation assay (PLA) *in situ* evaluating the extent of ER- endosome contact sites in WT and TMEM16K KO primary fibroblasts. Cells were fixed, permeabilized, labelled with mouse anti-VAPB and rabbit anti-Rab7 antibodies, or with omitted primary antibodies as indicated to confirm the specificity of the observed signal, and subjected to proximity ligation assay *in situ* (PLA). Nuclei were detected with DAPI. Scale bar, 20 µm. **e.** Number of PLA puncta and nuclei from 3 independent experiments was measured, and quantified as PLA puncta per cell. Two-tailed Student t-test, p-value=0.43 n.s. (3 biological replicates, n=231 non-overlapping fields of view with WT cells, 252 with TMEM16K KO cells). Source data are provided as a Source Data file.

Supplementary Figure 6



Supplementary Figure 6. Immunocytochemistry to evaluate subcellular localization of TMEM16K mutants to endoplasmic reticulum. COS7 were co-transfected with ER-tdTomato labeling ER (cyan) and TMEM16K mutant constructs tagged with FLAG or V5 tag, which was revealed with anti-FLAG or anti-V5 antibody, respectively (magenta). Scale bar 10 μm. Images are represented using pseudocolors suitable for color-blind palette.

Supplementary Table 2. List of all primers and constructs generated for this study.

TMEM16K genotyping and RT-PCR primers

Primer name	Primer sequence
Ano10_257998_F	CACTCCCTCATCCCATTCTTG
Ano10_257998_R	AGACGGCCACCTTACCACAG
Ano10_257998_F and CAS_R1_Term	TCGTGGTATCGTTATGCGCC
TMEM16K primer set 1.1	CATGGCCATCATTGGACTGCCC
TMEM16K primer set 1.2	GCACAGCCACGCTTCCACAC
TMEM16K primer set 2.1	GCCATGCGGGCCTTCACCTA
TMEM16K primer set 2.2	GCCATGCGGGCCTTCACCTA
Gadph 1.1	TGGCCCCTCTGGAAAGCTGTG
Gadph 1.2	AGTTGGGATAGGGCCTCTCTTGC
β-actin 1.1	ATGAGCTGCGTGTGGCCCCTG
β-actin 1.2	GACGCAGGATGGCGTGAGGG

Classical subcloning

List of constructs	Backbone	RE to cut backbone	Source of insert	Primers to amplify insert	RE to cut the insert
myc-BioID-TMEM16K	pcDNA3.1-myc-BioID (Addgene)	NotI, KpnI	pcDNA3.1-TMEM16K-FLAG (Genscript)	F:gcggcggccgctaagagtgactttatcaacgctggatacttgt R:cccggtacctaggtagcttccttcccatcttcctggt	NotI, KpnI
TMEM16K-BioID-HA	BioID-HA (Addgene)	AgeI, BamHI	pcDNA3.1-TMEM16K-FLAG (Genscript)	F:gcgaccggtatgagagtgactttatcaacgctggatacttgt R:cccggatcccaaggtagcttccttcccatcttcctggt	AgeI, BamHI
mcherry-CAAX	CIBN-CAAX (Pietro de Camilli)	NheI, AgeI	mcherry cDNA	F:ccgctagcatggtgagcaagggcgaggaggata R: cgaccggtccgcttcgagatctcttgtacagctc	NheI, AgeI
N16F-GFP	pEGFP-N1 (Clontech)	NheI, EcoRI	TMEM16F-GFP (Tien et al. 2009)	F:ggcgctagcatgcagatgatgactaggaaggtcctgctgaac R:gcggaattctcctgataagatccaagggctgct	NheI, EcoRI
N16K-GFP	pEGFP-N1 (Clontech)	NheI, HindIII	pcDNA3.1-TMEM16K-FLAG (Genscript)	F:gcggctagcaccgccaccatgagagtgacttttat R:ggcaagctttgtctccccaaatagctacgaatgc	NheI, HindIII
N16A-GFP	pEGFP-N1 (Clontech)	NheI, EcoRI	TMEM16A-GFP (Tien et al. 2009)	F:gcggctagcccggggttgtggatggggag R:ggcgaattcggccaaccttctcaccaaagta	NheI, EcoRI
pet15b-His-N16F-GFP	pet15b (Novagene, Millipore)	NdeI, BlpI	N16F-GFP	F:caagcttccatatgaccgtcagatccgctagcatgc R:gatccggtgctcagcgtccatgccgagagtgatcccg	NdeI, BlpI

pet15b-His-N16K-GFP	pet15b (Novagene, Millipore)	NdeI, BlpI	N16K-GFP	F:caagcttccatatgaacaccgccaccatgagagtgact R:gatccggtgctcagctcgtccatgccgagagtgatc	NdeI, BlpI
pet15b-His-N16A-GFP	pet15b (Novagene, Millipore)	NdeI, BlpI	N16A-GFP	F:caagcttccatatggttgtggatggggagcgcgag R:gatccggtgctcagcgtccatgccgagagtgatcccg	NdeI, BlpI

Gibson assembly

List of constructs	Backbone	Primers to amplify backbone	Source of insert	Primers to amplify insert
TMEM16K-V5	pCDNA3.1-TMEM16K-FLAG (Genescript)	Backbone is amplified in 2 segments splitting the AmpR gene (increases the probability that picked colony is a positive hit) and adding V5 tag with primers Backbone part 1: AmpF: gtaagttggccgcaggttatcactcatgg R1:gtccaagcccagcaaagggttaggaataggcttaccggtagcttccttcccatcttcctggtac Backbone part 2: F2:cctattcctaacccttgctgggcttggactcaacctagcgctgatcagcctcgactgt AmpR: ccatgagtgataacactgcggccaacttac	V5 tag was added with primers.	
TMEM16K-V5-GFP11	pCDNA3.1-TMEM16K-FLAG (Genescript)	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: AmpF: gtaagttggccgcaggttatcactcatgg R1: caccgcctccgccaccggtagcttccttcccatcttcctggtac Backbone part 2: F2:gctgctgggattacatagcgctgatcagcctcgactgt AmpR: ccatgagtgataacactgcggccaacttac	Synthesized by Integrated DNA Technologies ggtggcggaggcggtggaggtaagcctattcctaacccttgctgggcttggactcaaccggtggaggaggctcaggtggcggaggatcaggcggtggtggatcacgtgaccacatggtccttcatgagtatgtaaatgctgctgggattacatag	
GFP$_{(1-10)}$-HA-TMEM16K	pcDNA3.1-GFP$_{(1-10)}$ (Addgene)	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: AmpF: gtaagttggccgcaggttatcactcatgg R1:gggtaacctccacctccgcctccttttttcatttggatctttgctcaggactgtttgtg Backbone part 2: F2: gcttaggcggccttaagtttaaaccgc AmpR: ccatgagtgataacactgcggccaacttac	pcDNA3.1-TMEM16K-FLAG (Genscript)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggatacgcaagagtgactttatcaacgctggatacttgt R:gcggtttaaacttaaggccgcctaagctcaggtagcttccttcccatcttcctg
GFP$_{(1-10)}$-HA-Rab7			Rab7-tdTomato (UCSF Nikon Imaging Center)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggatacgcaacctctaggaagaaagtgttgctgaaggtt R:gcggtttaaacttaaggccgcctaagctcagcaactgcagctttctgccg
GFP$_{(1-10)}$-HA-VAPA			pEGFP-N1-VAPA (Addgene)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggatacgcagcgaaacacgagcaaatcctggtcc R: gcggtttaaacttaaggccgcctaagcctacgagatgaatttccctagaaagaatccaat
GFP$_{(1-10)}$-HA-SNX1			SNX1 cDNA (Ewan Reid)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggatacgcagatccggagtcggaaggggc R:gcggtttaaacttaaggccgcctaagctcaggagatggcctttgcctcag

$GFP_{(1-10)}$-HA-SNX2			SNX2 cDNA (Marcel Verges)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggat tacgcagcggccgagagggaacc R: gcggtttaaacttaaggccgcctaagcctaggcaatggctttggcttca ggtag
$GFP_{(1-10)}$-HA-VPS26			VPS26A cDNA (Transomics)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggat tacgcaatgagttttcttggaggctttttggtcca R:gcggtttaaacttaaggccgcctaagctcacatttcaggctgttcggc agatgc
$GFP_{(1-10)}$-HA-ORP8			ORP8 cDNA (Francesca Giordano)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggat tacgcagagggaggtttggcagatggagaac R:gcggtttaaacttaaggccgcctaagcctacttgaacatgaagtttat tatgacttgaag
$GFP_{(1-10)}$-HA-OSBPL11			OSBPL11 cDNA (Transomics)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggat tacgcacaggggggtgaaccagtgtcc R:gcggtttaaacttaaggccgcctaagctcactctgctggttgtgttgtt ggaattatttt
$GFP_{(1-10)}$-HA-VPS35			VPS35 cDNA (Marcel Verges)	F:gaaaaaggaggcggaggtggaggttacccttacgatgtaccggat tacgcacctacaacacagcagtcccctcag R:gcggtttaaacttaaggccgcctaagcttaaaggatgagaccttcat aaattggccc
$GFP_{(1-10)}$-HA-ORP9	$GFP_{(1-10)}$-HA-TMEM16K	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: AmpF: gtaagttggccgcaggttatcactcatgg R1-ORP9: gcctcctcctccgccgcctgcgtaatccggtacatcgtaagggtaacc Backbone part 2: F2-ORP9:gctgccaagcattaggcttaggcggccttaagtttaaaccgc AmpR: ccatgagtgataacactgcggccaacttac	ORP9 cDNA (Transomics)	F:ggcggcggaggaggaggcatggtagaatcaattaaacactgcatt gtgttgc R:cttaaggccgcctaagcctaatgcttggcagcaccaagacg
mClover3-TMEM16K	pSBtet-RB (Addgene)	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: AmpF:gtaagttggccgagtgttatcactcatgg R1:gcccttctcaccatggtggcctcagaggcctttcg Backbone part 2: F2:gaaggagctacctgaggctgtcaggccaagcttc AmpR:ccatgagtgatacactgcggccacttac	pKanCMV-mClover3-mRuby3 (Addgene) pcDNA3.1-TMEM16K-FLAG (Genscript)	Insert 1 (mClover3): F1:ctctgaggccaccatggtgagcaagggcgaggagc R1:gataaagtcactctcattcttcgcctttgctcaccatcgg Insert 2 (TMEM16K): 2F:gagcaaaggcgaagaaatagagtgactttatcaacgctggatctt gtgagag 2R:cttggcctgacaggctcaggtagcttccttcccatctcctg
TMEM16K-V5-mNeonGreen	TMEM16K-V5-GFP11	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: AmpF:gtaagttggccgagtgttatcactcatgg NG-R1: GCCCTTGCTCACCATTGAtccaccaccgccTGAtcc Backbone part 2: AmpR:ccatgagtgatacactgcggccacttac NG-F2: GACGAGCTGTACAAGtagcgctgatcagcctcgactgt	mNeonGreen-mRuby2-FRET-10 (UCSF Nikon Imaging Center Library)	Insert (mNeonGreen) NG-F1: CAggcggtggtggaTCAATGGTGAGCAAGGGCGAGG AG NG-R2: ggctgatcagcgctaCTTGTACAGCTCGTCCATGCCCA TC
SCRD16A-V5	TMEM16K-V5	Backbone is amplified in 2 segments splitting the AmpR gene.	Synthesized by Integrated DNA Technologies	

		Backbone part 1: R1:gccgtaaacttcatccatgatctcaatcacaacggcataaacaatgctgg AmpF: gtaagttggccgcaggttatcactcatgg Backbone part 2: F2: gccttcctgctcaagttcctgaattgcttcgcctcactcttctac AmpR: ccatgagtgataacactgcggccaacttac	gtgattgagatcatggatgaagtttacggctgcattgccaggtggctcaccaagattgaggtcccaaa gacagagaagagctttgaggagaggctaaccttcaaggccttcctgctcaagttcctgaattgcttc	
SCRD-16F-V5	TMEM16-V5	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: R1:ctcgtagatcgtgttcatgatctcaatcacaacggcataaacaatgctgg AmpF: gtaagttggccgcaggttatcactcatg Backbone part 2: F2: gatgttcttgttccag ttcctgaattgcttcgcctcactcttctac AmpR: ccatgagtgataacactgcggccaacttac	Synthesized by Integrated DNA Technologies gtgattgagatcatgaacacgatctacgagaaggtggccatcatgatcaccaacttcgagctccca aggacccagacggattatgagaacagcctgaccatgaagatgttcttgttccagttcctgaattgcttc	
SCRD16A-V5-GFP11	TMEM16K-V5-GFP11	Backbone is amplified in 2 segments splitting the AmpR gene. Backbone part 1: R1: CTCTCACAAGTATCCAGCGTTGATAAAGTCACTCTCAT AmpF: gtaagttggccgcaggttatcactcatg Backbone part 2: F2: CCAGGAAGATGGGAAGGAAGCTACC AmpR: ccatgagtgataacactgcggccaacttac	SCRD16A-V5	All respective inserts were amplified with following primers K-F: ATGAGAGTGACTTTATCAACGCTGGATACTTGTG AGAG K-R: GGTAGCTTCCTTCCCATCTTCCTGG
SCRD16F-V5-GFP11			SCRD16F-V5	
F171S-V5-GFP11			F171S-V5	
F337V-V5-GFP11			F337-V5	
D615N-V5-GFP11			D615N-V5	
deltaN16K-V5-GFP11	TMEM16K-V5-GFP11	Backbone is amplified in 2 segments splitting the AmpR gene, and removing the N-terminal domain of TMEM16K Backbone part 1: AmpF: gtaagttggccgcaggttatcactcatgg dNtR: GTCATGCAGCGGGAACACcatgccagcttgggtctccctatag Backbone part 2: dNtF:gacccaagctggcatgGTGTTCCCGCTGCATGACACTGA AmpR: ccatgagtgataacactgcggccaacttac		

Site-directed mutagenesis

List of constructs	Backbone	Site-directed mutagenesis primers	Comments
Ca5MUT-FLAG (TMEM16K-E448Q/D497N/E500Q/E529Q/D533N-FLAG)	pCDNA3.1.-TMEM16K-FLAG (Genscript)	448-F: CTGAACCAAGTCGTAcAATCTCTTCTTCCTT 448-R: AAGGAAGAAGAGATTgTACGACTTGGTTCAG 497-500-F: CTGGGAACCTTTGATaATTACCTGcAGTTGTTCCTGCAGT 497-500-R: ACTGCAGGAACAACTgCAGGTAATtATCAAAGGTTCCCAG 529-533-F: GTTAAATAACTTCACTcAAGTCAACTCAaATGCCTTGAAAATGTG 529-533-R: CACATTTTCAAGGCATtTGAGTTGACTTgAGTGAAGTTATTTAAC	All 5 mutations were introduced in one multiplex-site-directed mutagenesis reaction.
F171S-V5	TMEM16K-V5	F171S.F: GGCATCGTGACCCAAGTGTcCCCGCTGCAT F171S.R: ATGCAGCGGGgACACTTGGGTCACGATGCC	
F337-V5	TMEM16K-V5	F337V.F: TATGTCATGATGATCTACgTTGACATGGAGGACTGGG F337V.R: CCCAGTCCTCCATGTCAAcGTAGATCATCATGACATA	
D615N-V5	TMEM16K-V5	D615N.F: TCGCATTTGCCATCCCTaATAAACCACGGCACATC D615N.R: GATGTGCCGTGGTTTATtAGGGATGGCAAATGCGA	
$GFP_{(1-10)}$-HA-Rab7 T22N	GFP(1-10)-HA-Rab7	T22N-R: CTGGTTCATGAGTGAgtTCTTCCCGACTCCAGA T22N-F: CTGGAGTCGGGAAGAacTCACTCATGAACCAGT	
$GFP_{(1-10)}$-HA-Rab7 Q67L	GFP(1-10)-HA-Rab7	Q67L-R: GAGACTGGAACCGTTCCaGTCCTGCTGTGTCCC Q67L-F: GGGACACAGCAGGACtGGAACGGTTCCAGTCT	