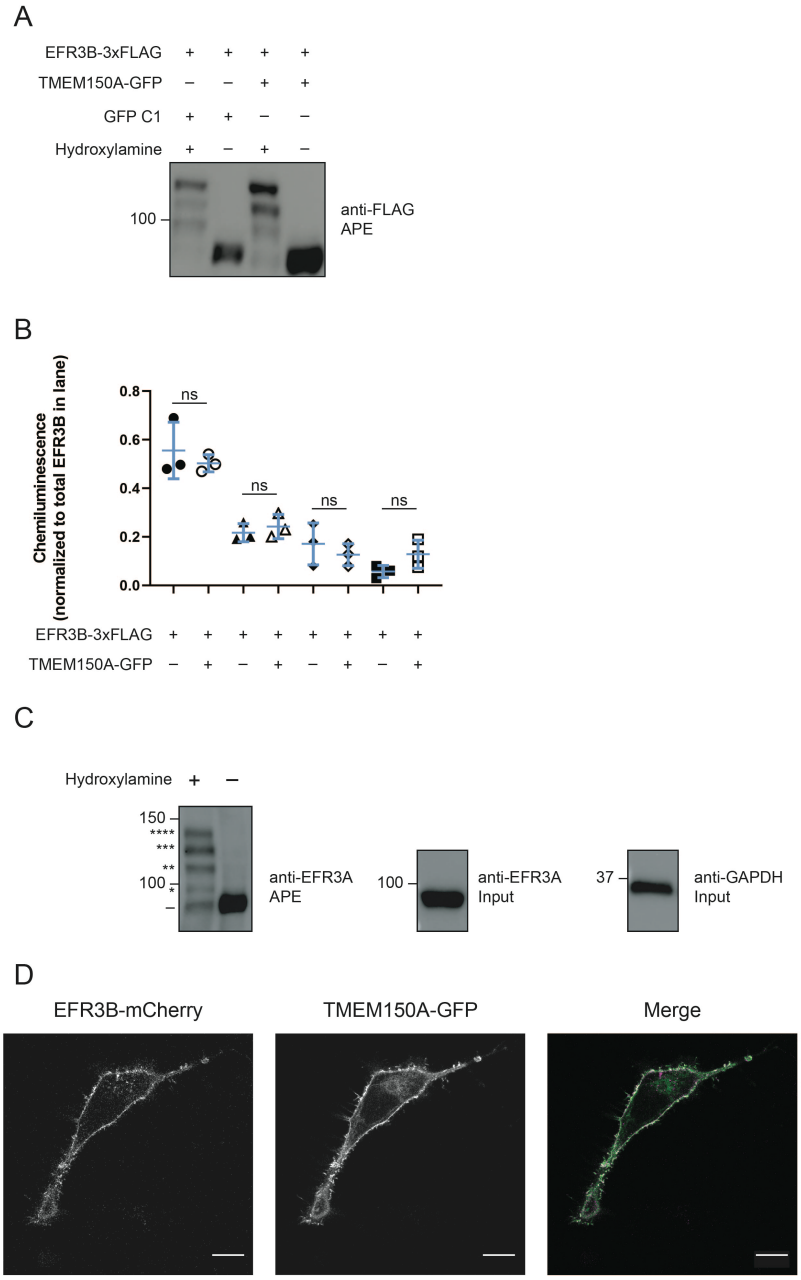

Fig. S1. Overexpression of TMEM150A does not alter the palmitoylation state of EFR3B. (A) Representative Western blot of acyl-PEG exchange (APE) experiments in which EFR3B was co-expressed with TMEM150A-GFP or with a GFP-C1 empty vector. (B) Quantification of three biological replicates of the experiment shown in (A), showing that co-expression of TMEM150A with EFR3B has no effect on its palmitoylation. (C) Endogenous EFR3A exists as a mixture of quadruply, triply, and doubly palmitoylated forms. Western blot for endogenous EFR3A after an APE assay, showing that endogenous EFR3A is heavily palmitoylated. The number of asterisks to indicates the number of palmitoyl groups on EFR3A, and – denotes the non-palmitoylated form. (D) Representative image of a cell overexpressing EFR3B-mCherry (left) and TMEM150A-GFP (middle) and a merged image (right). Scale bars, 15 µm. Statistical significance was assessed using Student's t-test. n=3; ns, not significant.

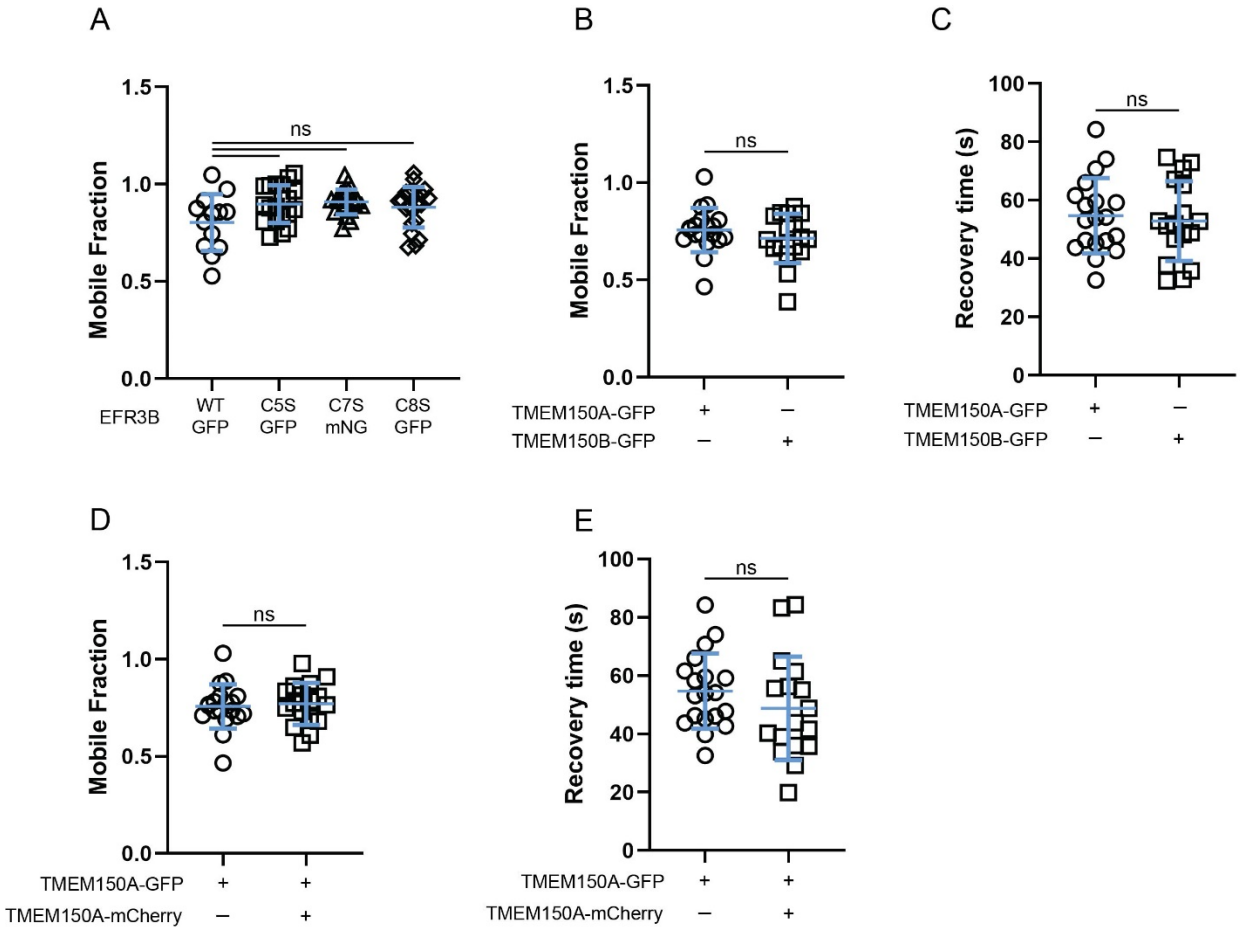

Fig. S2. The mobile fractions of WT and CxS EFR3B are the same. (A) Mobile fractions (mean ± SD) of the indicated EFR3B-GFP (or, for C7S, -mNG) construct, measured by FRAP. Note that EFR3B(C7S)-mNG was used due to undesired clustering of EFR3B(C7S)-GFP. n=13–22; ns, not significant. (B) The mobile fraction (mean ± SD) of TMEM150A-GFP and TMEM150B-GFP, measured by FRAP. (C) The recovery times ($t_{1/2}$, mean ± SD) of TMEM150A-GFP and TMEM150B-GFP, measured by FRAP. n=17–19; ns, not significant. (D) Mobile fractions (mean ± SD) of TMEM150A-GFP when expressed alone or together with additional amounts of TMEM150A-mCherry. (E) Recovery times ($t_{1/2}$, mean ± SD) of TMEM150A-GFP when expressed alone or together with TMEM150A-mCherry. Note that overexpression of TMEM150A-mCherry does not influence the mobile fraction or diffusion of TMEM150A-GFP, suggesting that substantial oligomerization does not occur by increased amount of TMEM150A, as might be expected by mass action. n=14–17; ns, not significant.

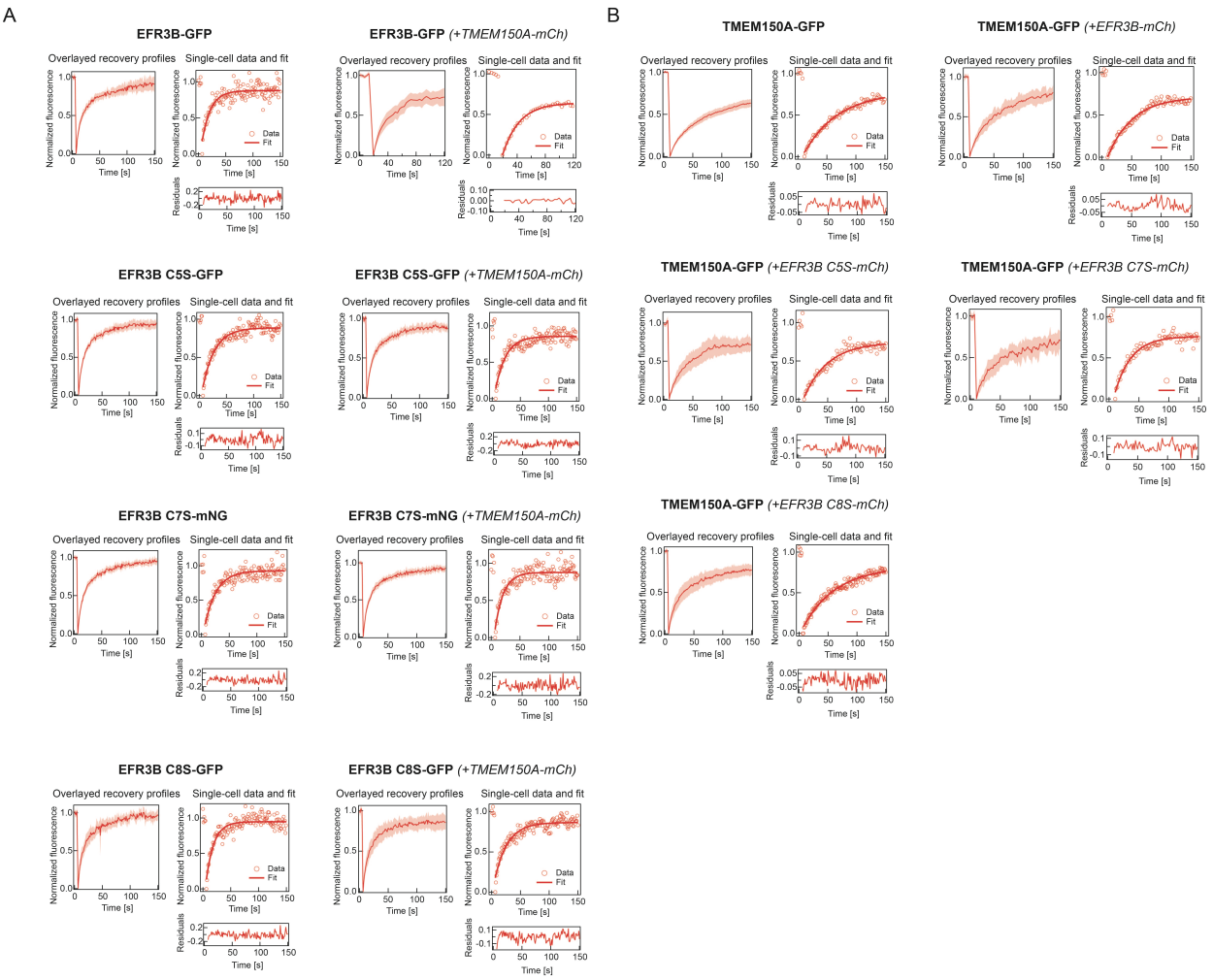

Fig. S3. FRAP data follow a single-exponential recovery curve. (A) FRAP recovery profiles, single-exponential fit, residuals and single-cell representative data are shown for FRAP experiments involving GFP-tagged EFR3B (WT, CxS mutants) in the absence or presence of TMEM150A-mCh. (B) FRAP recovery profiles, single-exponential fit, residuals and single-cell representative data are shown for FRAP experiments involving GFP-tagged TMEM150A in the absence or presence of EFR3B-mCh (WT, CxS mutants).

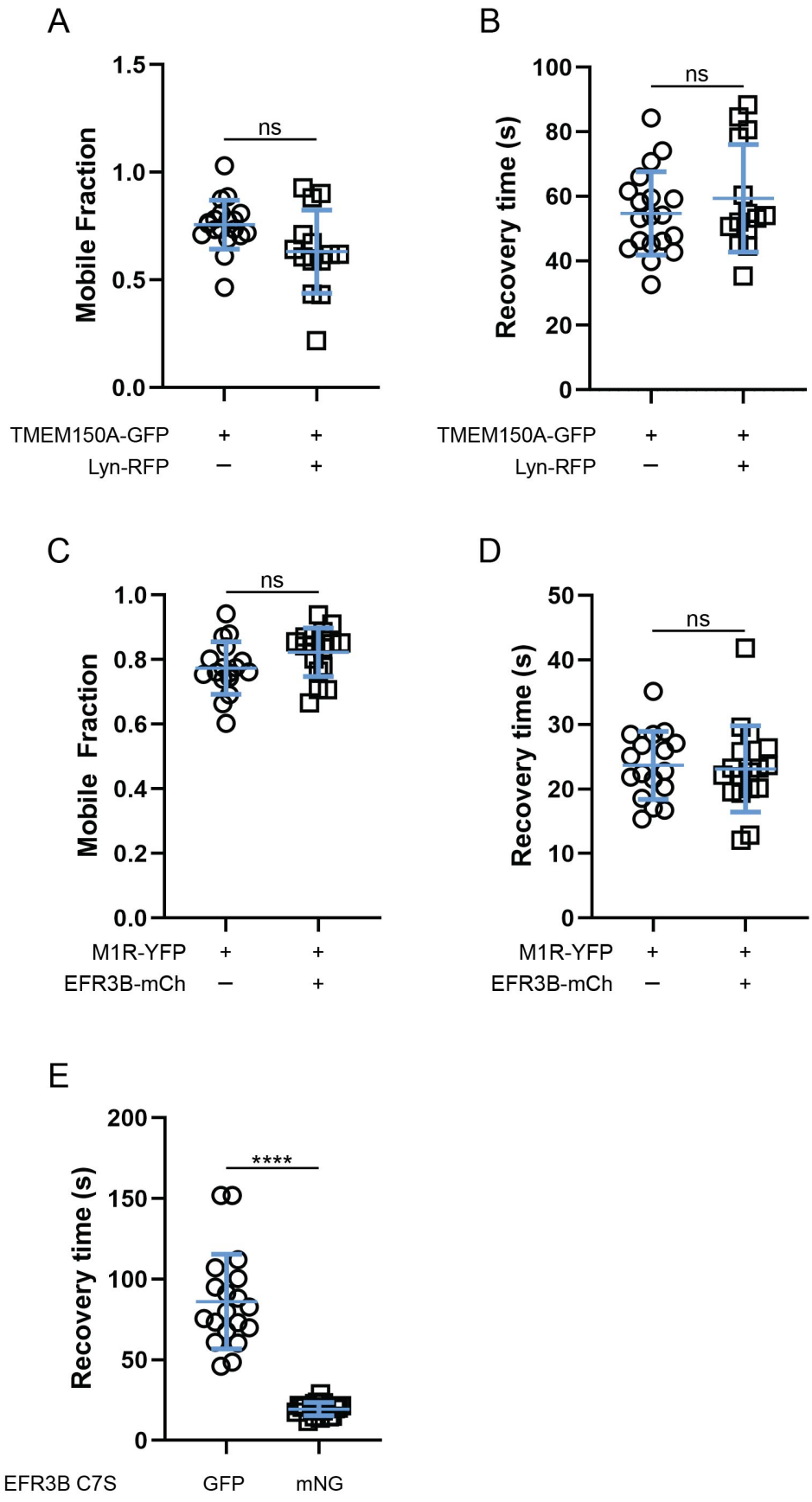

Fig. S4. TMEM150A-GFP does not engage in nonspecific interactions with Lyn, a different palmitoylated protein than EFR3B. (A) Mobile fractions (mean ± SD) of TMEM150A-GFP when expressed alone or in presence of Lyn-RFP, a model palmitoylated and myristoylated membrane anchor. (B) Recovery times ($t_{1/2}$, mean ± SD) for TMEM150A-GFP in the presence of absence of Lyn-RFP. The mobile fraction and recovery time of TMEM150A are not influenced by the presence of Lyn-RFP, indicating that the diffusion properties of TMEM150A as measured by FRAP are not sensitive to overexpression of a palmitoylated protein unrelated to the PI4KIIIα complex. n=14–19; ns, not significant. (C) The mobile fraction (mean ± SD) of M1R-YFP in the presence of absence of EFR3B-mCherry measured by FRAP. (D) The recovery time ($t_{1/2}$, mean ± SD) of M1R-YFP in the presence and absence of EFR3B-mCherry measured by FRAP. Note that the mobile fraction and recovery time of M1R-YFP is not modified by the overexpression of EFR3B, suggesting that the effect of EFR3B on TMEM150A diffusion is specific. n=17; ns, not significant. (E) Recovery times (mean ± SD) of EFR3B(C7S)-GFP and EFR3B(C7S)-mNG, measured by FRAP. Note that the GFP-tagged construct, which forms clusters visible by confocal microscopy, exhibits much slower diffusion. By contrast, the mNG construct, which does not exhibit such clustering, results in diffusion properties similar to the other GFP-tagged CxS mutants (see Fig. 2E). n=19–20; ****, $p < 0.001$

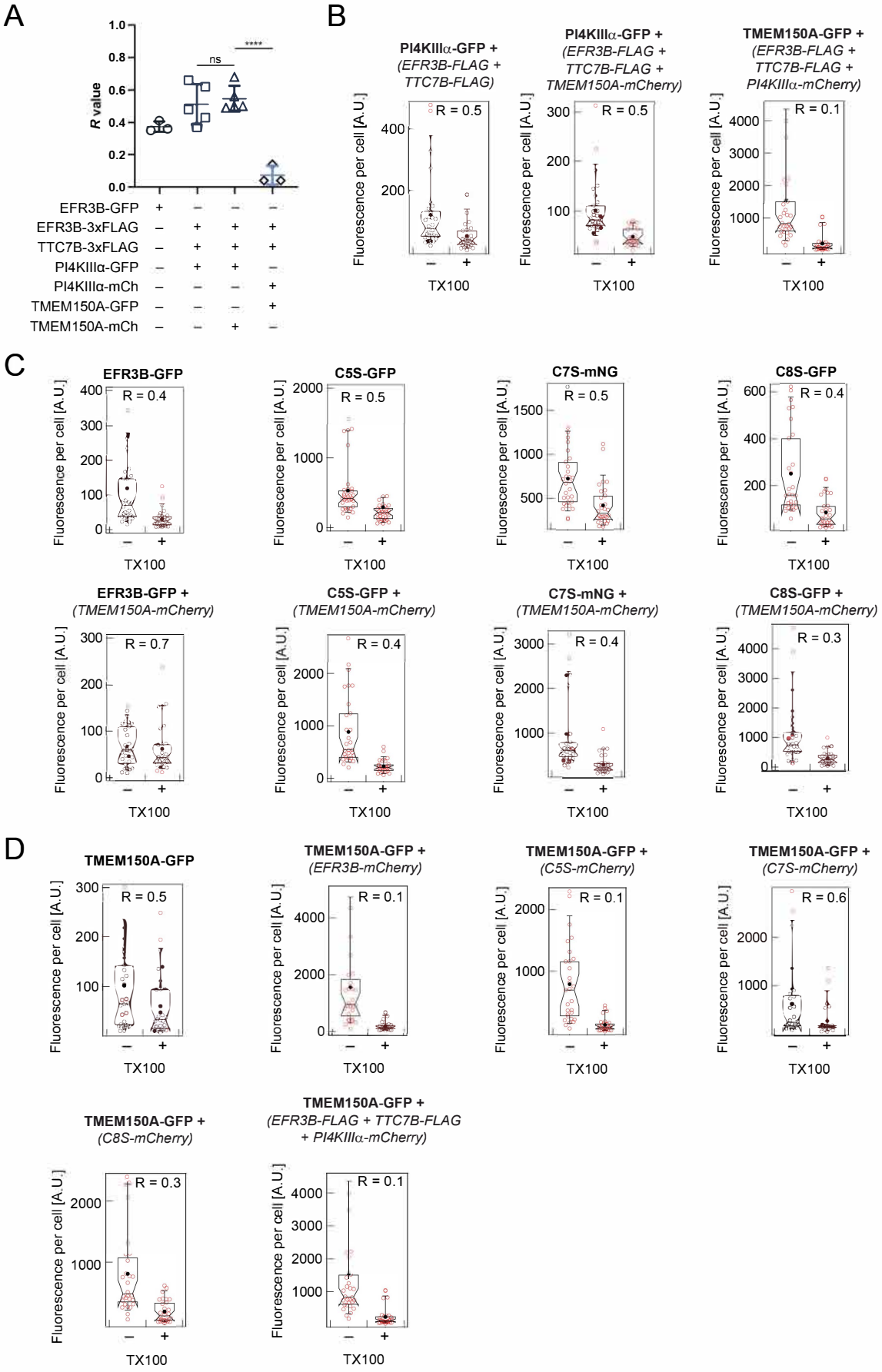

Fig. S5. The TTC7B-containing Complex I and TMEM150A-containing Complex II co-exist in the PM. (A) *R* values (mean ± SD) from iDRM assays showing the fraction of retained GFP fluorescence from RBL-2H3 cells expressing the indicated combination of constructs, after a mild wash with 0.04% TX-100. Lane 1: EFR3B-GFP is moderately detergent resistant when expressed alone. Lanes 2–3: PI4KIIIα is highly detergent resistant in both the TTC7B-containing Complex I (lane 2) and TMEM150A-containing Complex II (lane 3). Lane 4: TMEM150A is highly sensitive to detergent even when Complex II is formed. n=3–5; ****, $p<0.001$; ns, not significant. (B) Mean fluorescence values of representative cells expressing the indicated constructs (*R* values shown in panel **A**) after TX-100 treatment or control treatment. (C-D) Mean fluorescence values of representative cells expressing EFR3B WT or CxS-GFP (C) or TMEM150A-GFP (D) with additional indicated constructs after TX-100 or control treatment.

Table S1. Sources of reagents, primers, antibodies, and plasmids.

Reagents	Source	Catalog number
DMEM	Corning	10-017-CV
FBS	VWR	45000-734
Dialyzed FBS	VWR	97065-302
Penicillin/Streptomycin	VWR	45000-652
Gentamicin	Thermo Fisher	15750078
Transfectagro	VWR	71002-816
Lipofectamine 2000	Invitrogen	11668019
cOmplete protease inhibitor	Millipore Sigma	5056489001
Fugene	Promega	E2312
Phorbol 12,13-dibutyrate (PDB)	Sigma-Aldrich	P1269
BCA assay	Pierce	23225
TCEP hydrochloride	Cayman Chemical Company	14329
N-ethylmaleimide	Alfa Aesar	40526
Hydroxylamine	Allied Chemical	1789
Methoxypolyethylene glycol maleimide (5 kDa)	Sigma-Aldrich	63187
Lactacystin	Santa Cruz Biotechnology	sc-3575
Cycloheximide	Amresco	94271
Ezview Red Anti-FLAG M2 Affinity beads	Sigma-Aldrich	F2426
17-Octadecynoic acid (alk-16)	Cayman Chemical Company	90270
Cy5.5 azide	Click Chemistry Tools	1059-1
THPTA (tris-hydroxypropyltriazolylmethylamine)	Click Chemistry Tools	1010
Sodium L-ascorbate	Chem Impex	01436
Cupric sulfate	Mallinckrodt	4844
Clarity ECL reagent	Bio-Rad	1705061
Oxotremorine M	Santa Cruz	Sc-203656

DMEM	Corning	10-017-CV
FBS	VWR	45000-734
Dialyzed FBS	VWR	97065-302
Penicillin/Streptomycin	VWR	45000-652
Gentamicin	Thermo Fisher	15750078
Transfectagro	VWR	71002-816
Lipofectamine 2000	Invitrogen	11668019
cOmplete protease inhibitor	Millipore Sigma	5056489001
Fugene	Promega	E2312
Phorbol 12,13-dibutyrate (PDB)	Sigma-Aldrich	P1269
BCA assay	Pierce	23225
TCEP hydrochloride	Cayman Chemical Company	14329
N-ethylmaleimide	Alfa Aesar	40526
Hydroxylamine	Allied Chemical	1789
Methoxypolyethylene glycol maleimide (5 kDa)	Sigma-Aldrich	63187
Lactacystin	Santa Cruz Biotechnology	sc-3575
Cycloheximide	Amresco	94271
Ezview Red Anti-FLAG M2 Affinity beads	Sigma-Aldrich	F2426
17-Octadecynoic acid (alk-16)	Cayman Chemical Company	90270
Cy5.5 azide	Click Chemistry Tools	1059-1
THPTA (tris-hydroxypropyltriazolylmethylamine)	Click Chemistry Tools	1010
Sodium L-ascorbate	Chem Impex	01436
Cupric sulfate	Mallinckrodt	4844
Clarity ECL reagent	Bio-Rad	1705061
Oxotremorine M	Santa Cruz	Sc-203656

Atropine	TCI	A0550
Primers	**Source**	**Sequence**
EFR3B C5S-3xFLAG	IDT	5'-agggcaccgcagcagccagacacaccgtac-3' 5'-gtacggtgtgtctggctgctgcggtgccct-3'
EFR3B C7S-3xFLAG	IDT	5'-ggtgtgtgtggcagctgcggtgccc-3' 5'-gggcaccgcagctgccacacacacc-3'
EFR3B C8S-3xFLAG	IDT	5'-gtgtgtggctgcagcggtgcccttc-3' 5'-gaagggcaccgctgcagccacacac-3'
EFR3B C5,7S-3xFLAG	IDT	5'-tgtgtctggctcctgcggtgcccttc-3' 5'-gaagggcaccgcaggagccagacaca-3'
EFR3B C5,8S-3xFLAG	IDT	5'-tgtgtctggctcctgcggtgcccttc-3' 5'-agggcaccggagcagccagacacac-3'
EFR3B C7,8S-3xFLAG	IDT	5'-ggtgtgtgtggcagcagcggtgcccttc-3' 5'-gaagggcaccgctgctgccacacacacc-3'
EFR3B C5S-GFP/mCherry	Eurofins Genomics IDT	5'-catgtacggtgtgagtggctgctgcgg-3' 5'-agaggtaccgagtatacacacagatcaggaaacttcatctcatagac-3'
EFR3B C7S-GFP/mCherry	IDT	5'-cagagaattcaccatgtacggtgtgtgtggctcctgc-3' 5'-agaggtaccgagtatacacacagatcaggaaacttcatctcatagac-3'
EFR3B C8S-GFP/mCherry	IDT	5'-cagagaattcaccatgtacggtgtgtgtggctgctccg-3' 5'-agaggtaccgagtatacacacagatcaggaaacttcatctcatagac-3'
EFR3B C5,7,8S-GFP/mCherry	Eurofins Genomics	5'-cgaagggcaccgctgctgccactcacaccgtacatg-3' 5'-catgtacggtgtgagtggcagcagcggtgcccttcg-3'
M1R-3xFLAG	Eurofins Genomics	5'-aagcggccgcagccaccatgaacacctcagtgccccctgc-3' 5'-actagaatccgcattggcgggaggggtgc-3'
Antibodies	**Source**	**Catalog number**
Rabbit anti-FLAG polyclonal	Sigma-Aldrich	F7425
Mouse anti-FLAG M2 monoclonal	Millipore Sigma	F1804
Anti-GFP	Takara	632375
Anti-GAPDH	Genetex	GTX78213
Anti-Calnexin	Abcam	Ab22595
Anti-rabbit-HRP	Bio-Rad	1706515
Anti-mouse-HRP	Bio-Rad	1706516
Plasmids	**Source**	
EFR3B-3xFLAG	Gift from the De Camilli lab	
EFR3B C5S-3xFLAG	This paper	
EFR3B C7S-3xFLAG	This paper	

EFR3B C8S-3xFLAG	This paper	
EFR3B C5,7S-3xFLAG	This paper	
EFR3B C5,8S-3xFLAG	This paper	
EFR3B C7,8S-3xFLAG	This paper	
EFR3B C5S-GFP	This paper	
EFR3B C7S-GFP	This paper	
EFR3B C7S-mNeonGreen	This paper	
EFR3B C8S-GFP	This paper	
EFR3B C5,7S-GFP	This paper	
EFR3B C5,8S-GFP	This paper	
EFR3B C7,8S-GFP	This paper	
EFR3B C5,7,8S-tdTomato	Gift from the De Camilli lab	
EFR3B C5,7,8S-GFP	This paper	
EFR3B C5,7,8S-3xFLAG	This paper	
TMEM150A-GFP	Gift from the De Camilli lab	
TMEM150A-mCherry	This paper	
TTC7B-mCh	Gift from the De Camilli lab	
GFP-PI4KIIIα	Gift from the De Camilli lab	
iRFP-PH(PLCδ)	Gift from the De Camilli lab	
M1R-YFP	Gift from the De Camilli lab	
M1R-3xFLAG	This paper	