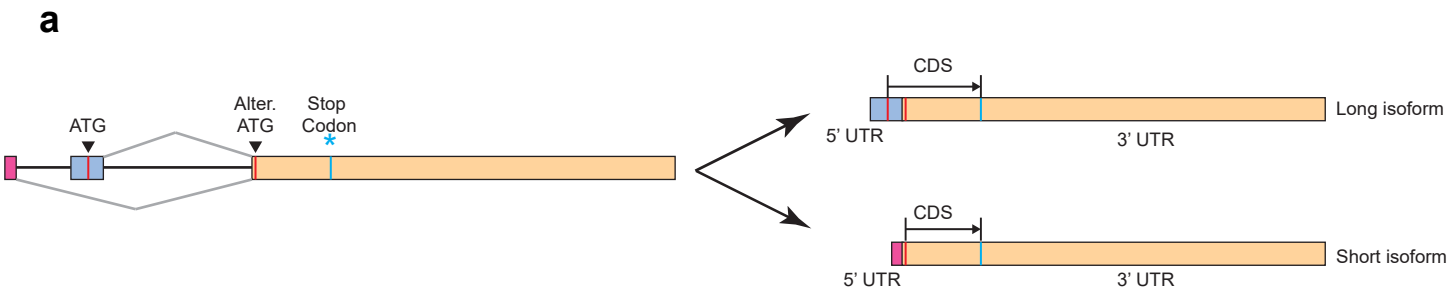
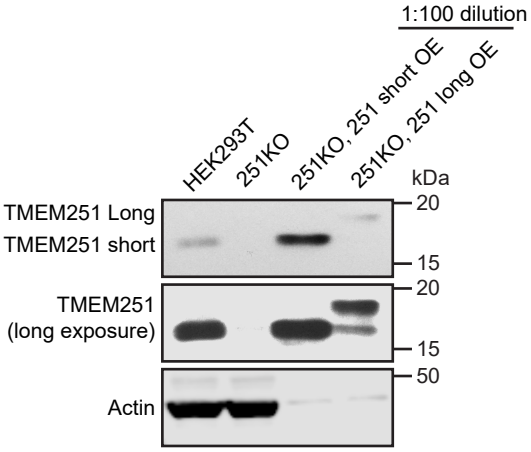
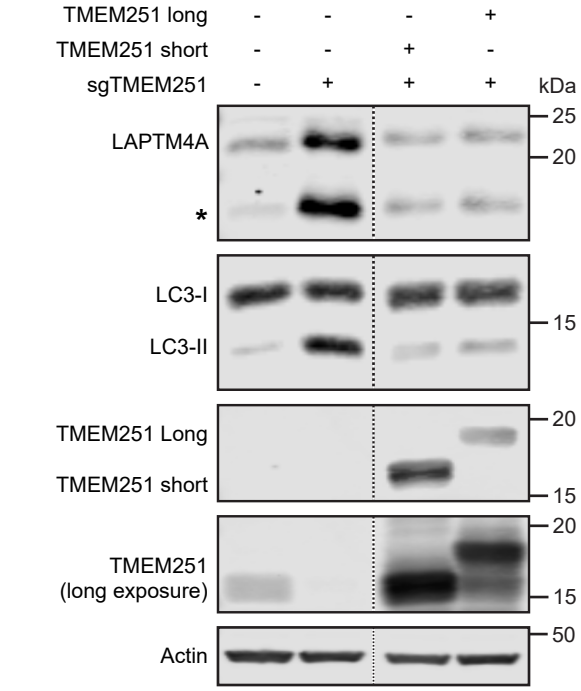
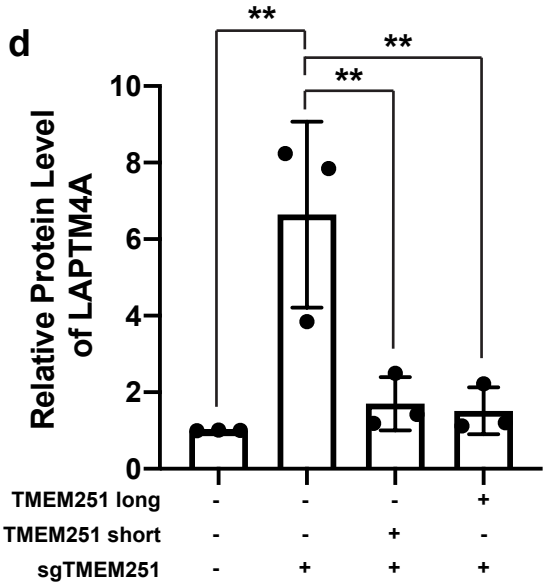
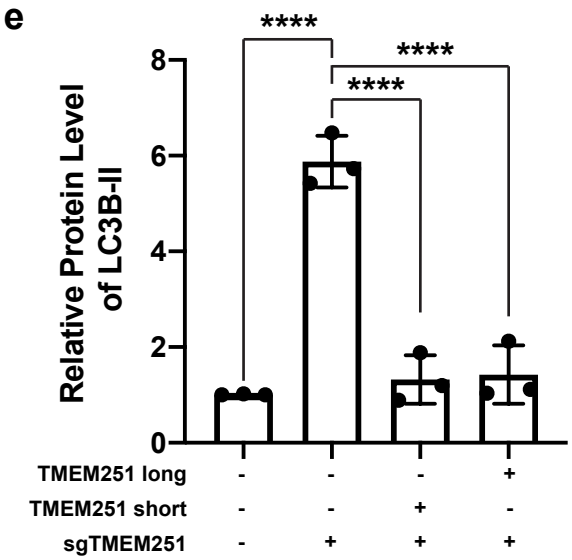

a
ATG
Alter. ATG
Stop Codon
CDS
5' UTR
3' UTR
Long isoform
CDS
5' UTR
3' UTR
Short isoform

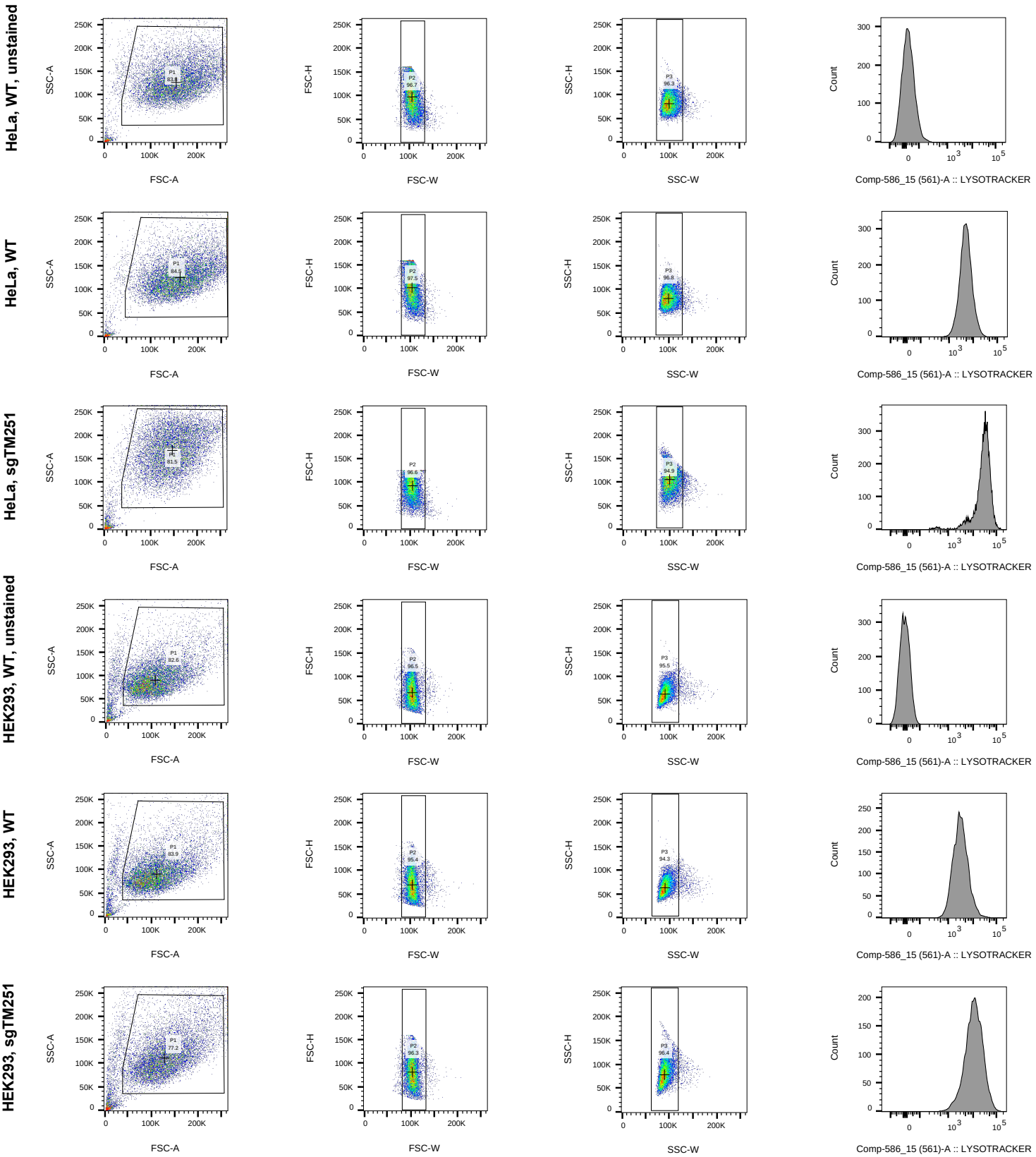

b
1:100 dilution
HEK293T
251KO
251KO, 251 short OE
251KO, 251 long OE
kDa
TMEM251 Long
TMEM251 short
TMEM251 (long exposure)
Actin
20
15
20
15
50


c
TMEM251 long - - - +
TMEM251 short - - + -
sgTMEM251 - + + +
kDa
LAPTM4A
*
LC3-I
LC3-II
TMEM251 Long
TMEM251 short
TMEM251 (long exposure)
Actin
25
20
15
20
15
20
15
50

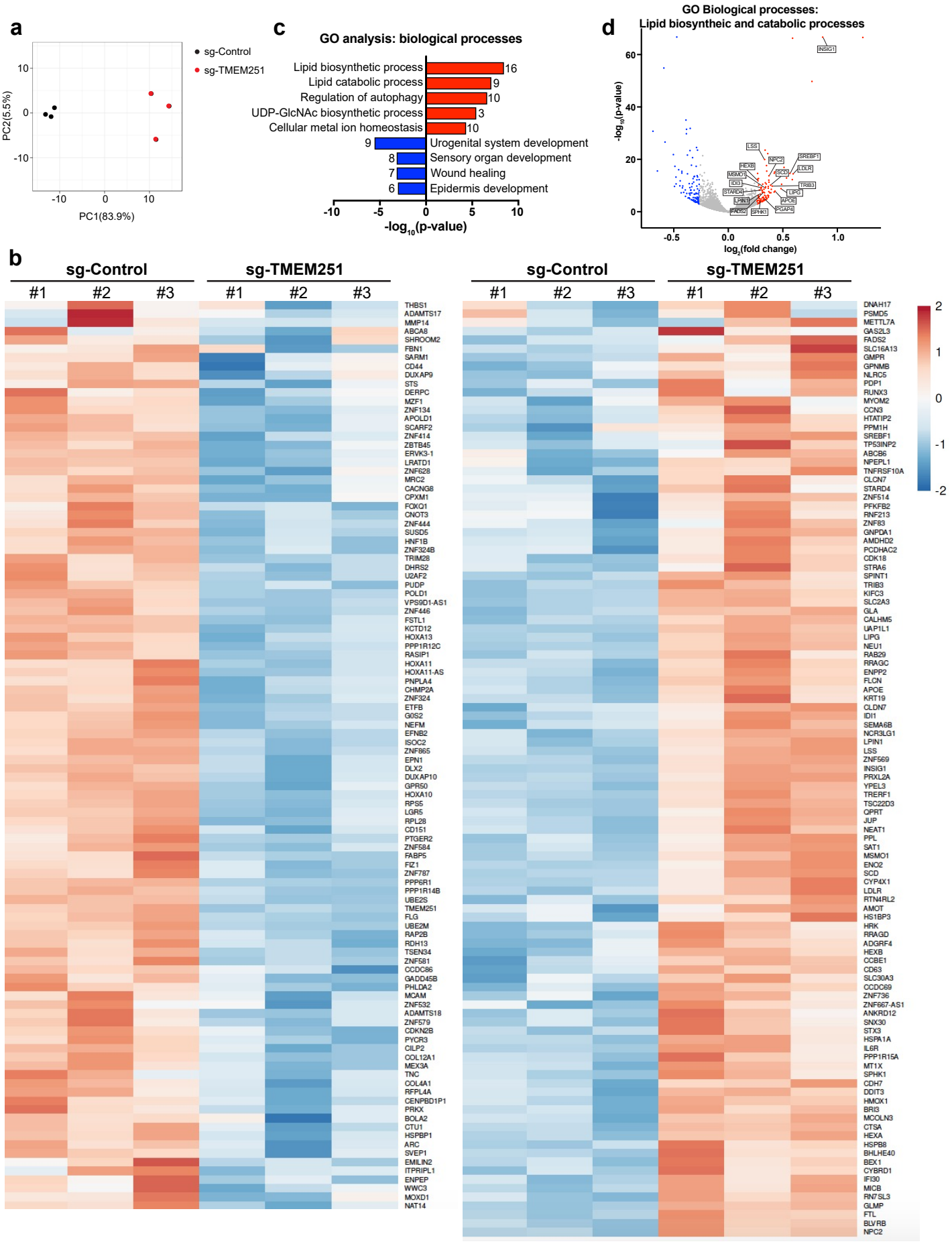

d
Relative Protein Level of LAPTM4A
**
**
**
10
8
6
4
2
0
TMEM251 long - - - +
TMEM251 short - - + -
sgTMEM251 - + + +


e
Relative Protein Level of LC3B-II
****
****
****
8
6
4
2
0
TMEM251 long - - - +
TMEM251 short - - + -
sgTMEM251 - + + +

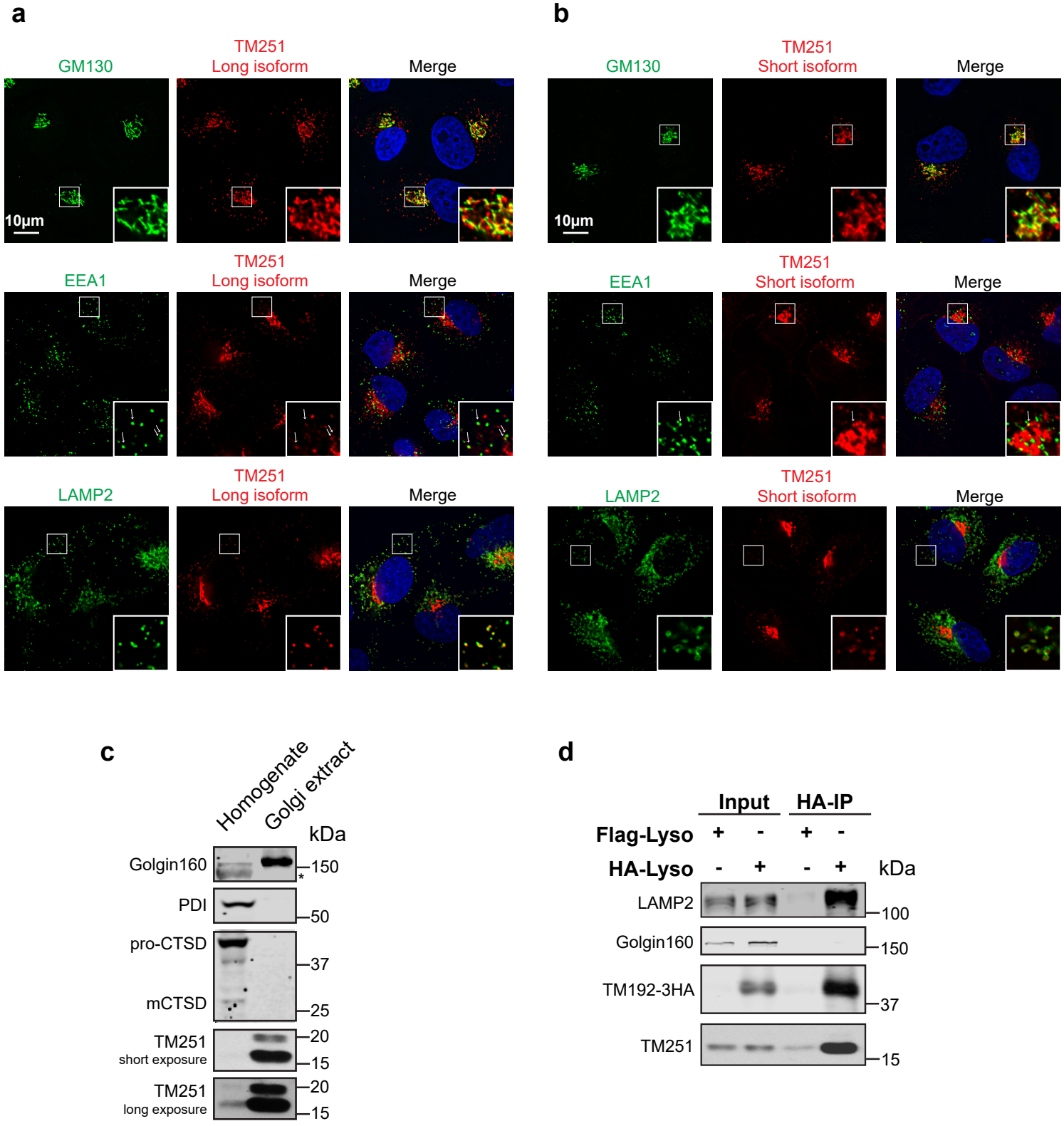
Supplementary Figure 1: Transcriptional variants of TMEM251. (a) A schematic representation of the alternative splicing of TMEM251 mRNA. **(b)** Endogenous TMEM251 and the overexpression of different isoforms of TMEM251 in HEK293T cells (n=3 independent replicates). The overexpression samples were diluted to make them comparable to the endogenous TMEM251. **(c)** Overexpression of either short or long isoform of TMEM251 rescues LAPTM4A and LC3B-II protein levels in TMEM251-deficient cells. * highlights the cleavage product of LAPTM4A. **(d)** Quantification of LAPTM4A protein level in c. Mean of n=3 independent replicates is shown. Error bars represent standard deviation. **: $p \leq 0.01$. **(e)** Quantification of LC3B-II protein level in c. Mean of n=3 independent replicates is shown. Error bars represent standard deviation. ****: $p \leq 0.0001$. See source data file for exact P values. Related to Figure 2.


HeLa, WT, unstained
HeLa, WT
HeLa, sgTM251
HEK293, WT, unstained
HEK293, WT
HEK293, sgTM251
SSC-A
FSC-A
FSC-H
FSC-W
SSC-H
SSC-W
Count
Comp-586_15 (561)-A :: LYSOTRACKER

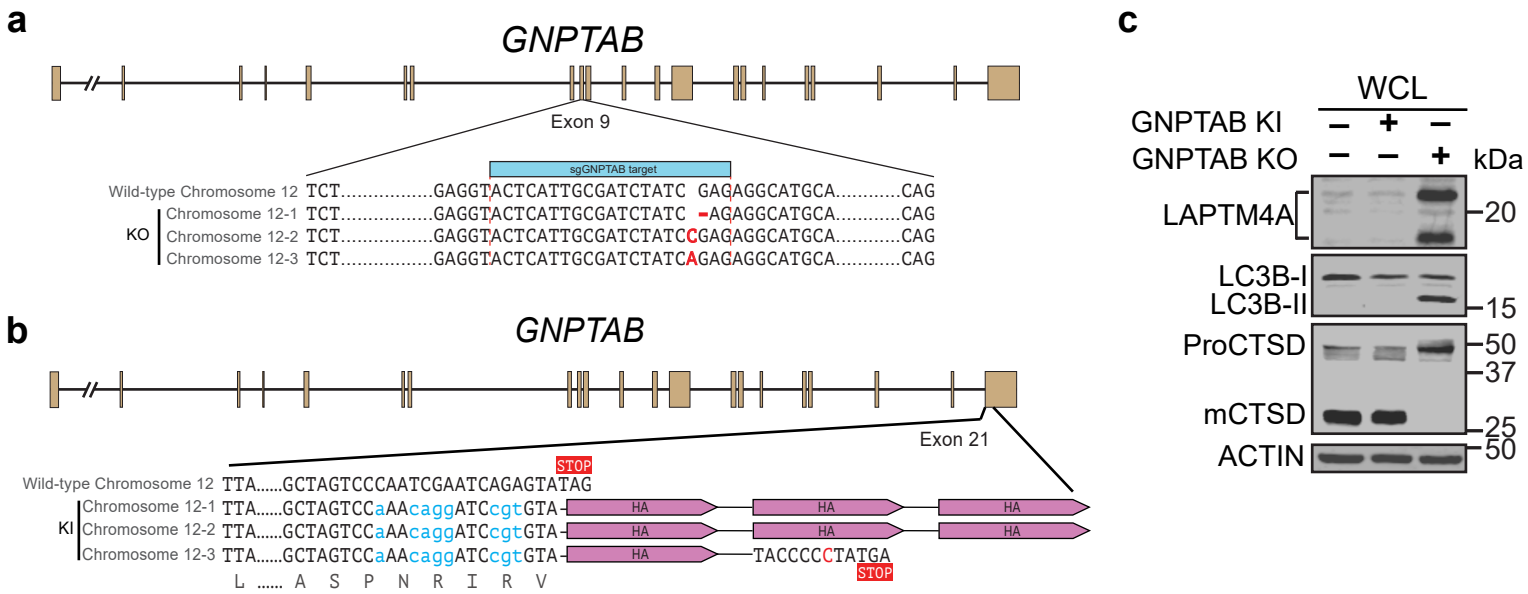
Supplementary Figure 2: Gating strategy for the flow cytometry analysis after LysoTracker staining. Related to Figure 3.


a
sg-Control
sg-TMEM251
PC2(5.5%)
PC1(83.9%)
c
GO analysis: biological processes
Lipid biosynthetic process
Lipid catabolic process
Regulation of autophagy
UDP-GlcNAc biosynthetic process
Cellular metal ion homeostasis
Urogenital system development
Sensory organ development
Wound healing
Epidermis development
-log10(p-value)
d
GO Biological processes:
Lipid biosyntheic and catabolic processes
-log10(p-value)
log2(fold change)
b
sg-Control
sg-TMEM251
#1
#2
#3

Supplementary Figure 3: RNA sequencing analysis of sgTMEM251 cells vs. control cells. **(a)** Principal component analysis (PCA) of RNA-sequencing. **(b)** A heatmap of 211 DEGs altered in sgTMEM251 vs. control cells. **(c)** GO biological processes analysis of DEGs altered in sgTMEM251 (n=3 independent replicates) vs. control (n=3 independent replicates) cells. The number of genes in each pathway is indicated. **(d)** A Volcano plot of RNA-seq analysis of sgTMEM251 (n=3 independent replicates) vs. control (n=3 independent replicates) cells. Annotated genes are classified in lipid biosynthetic and catabolic processes. Related to Figure 3.


a
GM130
TM251
Long isoform
Merge
10μm
EEA1
TM251
Long isoform
Merge
LAMP2
TM251
Long isoform
Merge
b
GM130
TM251
Short isoform
Merge
10μm
EEA1
TM251
Short isoform
Merge
LAMP2
TM251
Short isoform
Merge
c
Homogenate
Golgi extract
kDa
Golgin160
150
*
PDI
50
pro-CTSD
37
mCTSD
25
TM251
short exposure
20
15
TM251
long exposure
20
15
d
Input
HA-IP
Flag-Lyso + - + -
HA-Lyso - + - +
kDa
LAMP2
100
Golgin160
150
TM192-3HA
37
TM251
15

Supplementary Figure 4: Subcellular localization of TMEM251. (a-b) Immunofluorescence showing the localization of overexpressed long and short isoform of TMEM251 in HeLa cells (n=50 cells from three independent replicates) co-stained with GM130, EEA1, and LAMP2. Scale bar: 10 µm. **(c)** Endogenous TMEM251 in rat liver Golgi extract. n=2 independent replicates. **(d)** Endogenous TMEM251 in purified lysosomes from HEK293T cells. n=3 independent replicates. Related to Figure 5.


a
GNPTAB
Exon 9
sgGNPTAB target
Wild-type Chromosome 12 TCT.....GAGGTACTCATTGCGATCTATC GAGAGGCATGCA.....CAG
KO
Chromosome 12-1 TCT.....GAGGTACTCATTGCGATCTATC -AGAGGCATGCA.....CAG
Chromosome 12-2 TCT.....GAGGTACTCATTGCGATCTATCCGAGAGGCATGCA.....CAG
Chromosome 12-3 TCT.....GAGGTACTCATTGCGATCTATCAGAGAGGCATGCA.....CAG
b
GNPTAB
Exon 21
STOP
Wild-type Chromosome 12 TTA.....GCTAGTCCCAATCGAATCAGAGTATAG
KI
Chromosome 12-1 TTA.....GCTAGTCCaAAcaggATCcgtGTA
Chromosome 12-2 TTA.....GCTAGTCCaAAcaggATCcgtGTA
Chromosome 12-3 TTA.....GCTAGTCCaAAcaggATCcgtGTA
HA
HA
HA
TACCCCCTATGA
STOP
L ..... A S P N R I R V
c
WCL
GNPTAB KI – + –
GNPTAB KO – – +
kDa
LAPTM4A
20
LC3B-I
LC3B-II
15
ProCTSD
50
37
mCTSD
25
ACTIN
50
d
WCL
GNPTAB 3V5 + + + +
TMEM251 OE – + – +
S1P-Flag OE – – + +
kDa
α+β
250
150
100
75
β
50
TMEM251
15
S1P-Flag
150
100
GAPDH
37
e
Processing efficiency (%)
80
60
40
20
0
****
*
***
TM251 WT WT OE OE
S1P WT OE WT OE
f
HeLa WCL
sgTMEM251 – + kDa
GNPTAB-2HA
GNPT α+β
250
150
100
75
GNPT β
50
TMEM251
15
ACTIN
50
g
GNPTAB-2xHA
GPP130
Merge
WT
WT
WT
10μm
sgTMEM251
sgTMEM251
sgTMEM251

Supplementary Figure 5: TMEM251 functions at the cleavage step of GNPT α/β precursor by S1P. (a) Sequencing analysis to confirm the genotype of GNPTAB single colony KO HEK293T cells. All three chromosomes from the same single colony cell contain either 1 bp deletion or insertion (highlighted in red), which leads to a frameshift, indicating a complete KO of GNPTAB protein. **(b)** Sequencing analysis of the GNPTAB-3xHA endogenous knock-in single colony cells. Two chromosomes from the same single colony cell contain 3x HA insertion, while the third chromosome only contains 1x HA insertion due to a premature stop codon generated during recombination. Sequences highlighted in blue lower case showed the silencing mutations designed in the KI template to prevent the cutting from Cas9. Sequence highlighted in red showed one bp insertion, which leads to a premature stop codon in the second HA region. **(c)** Analysis of lysosome function in GNPTAB KO and GNPTAB-3xHA KI cells (n=3 independent replicates). **(d)** Overexpression of TMEM251 stimulated the processing of GNPTAB-3V5. **(e)** Quantification of GNPTAB-3V5 processing efficiency in d. Mean of n=3 independent replicates is shown. Error bars represent standard deviation. *: $p \leq 0.05$, *** $p \leq 0.001$, ****: $p \leq 0.0001$. See source data file for exact P values. **(f)** The processing of overexpressed GNPTAB-2xHA in WT and TMEM251 KO HeLa cells (n=3 independent replicates). **(g)** Immunofluorescence showing the localization of overexpressed GNPTAB-2xHA in WT and TMEM251 KO HeLa cells (n=50 cells from three independent replicates) co-stained with GPP130. Scale bar: 10 μm. Related to Figure 6.

Supplementary Table 1: Hypersecreted lysosomal enzymes in TMEM251-deficient cells. $Log_2FC>1$, $p<0.05$.

AGA	ARSA	ARSK	C3	CPQ
CREG1	CTSA	CTSB	CTSC	CTSH
CTSL	CTSV	DNASE2	EPDR1	GAA
GBA	GGH	GLB1	GNS	GRN
GUSB	HEXA	HEXB	IDS	LGMN
LIPA	MAN2B1	MAN2B2	MANBA	NAGLU
NEU1	NPC2	PLBD2	PRCP	PSAP
RNASET2	SGSH	SIAE	TPP1	

Supplementary Table 2: Mammalian cell lines used in this study		
Cell lines	***Description***	***reference/source***
Human HEK293	CRL-1573	ATCC
Human HEK293T	CRL-3216	ATCC
Human HeLa	CCL-2	ATCC
Human HEK293, GFP-RNF152	pHAGE2-EF1α-EGFP-RNF152-IRES-Puro	Zhang et al., 2021
Human HEK293, GFP-RNF152	pHAGE2-EF1α-EGFP-RNF152-IRES-mCherry	Zhang et al., 2021
Human HEK293, GFP-RNF152, Cas9	pHAGE2-EF1α-EGFP-RNF152-IRES-mCherry, LentiCas9-Blast	This study
Human HEK293, sg1-TMEM251	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1	This study
Human HEK293, sg2-TMEM251	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1	This study
Human HEK293, sg3-TMEM251 (for RNA-seq)	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-3	This study
Human HEK293, sg1-TMEM251, TMEM251 long isoform	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1, pLenti6.3-TMEM251(long)-BLAST	This study
Human HEK293 sg1-TMEM251, TMEM251 short isoform	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1, pLenti6.3-TMEM251(short)-BLAST	This study
Human HEK293T, TMEM251KO	CRISPR-Cas9 KO of TMEM251, sgRNA-1, single colony.	This study
Human HEK293T, sg-TMEM251	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1	This study
Human HEK293T, TMEM251KO, TMEM251 long isoform	TMEM251 CRISPR-Cas9 KO, pLenti6.3-TMEM251(long)-BLAST	This study
Human HEK293T, TMEM251KO, TMEM251 short isoform	TMEM251 CRISPR-Cas9 KO, pLenti6.3-TMEM251(short)-BLAST	This study
Human HEK293T, FLAG-Lyso	pLJC5-TMEM192-2XFLAG-Puro (Addgene 102929)	This study (Abu-Remaileh et al. 2017)

Human HEK293T, HA-Lyso	pLJC5-TMEM192-3XHA-Puro (Addgene 102930)	This study (Abu-Remaileh et al. 2017)
Human HEK293T, GNPTAB KO	CRISPR-Cas9 KO of GNPTAB, single colony.	This study
Human HEK293T, CI-MPR KO	CRISPR-Cas9 KO of CI-MPR, single colony.	This study
Human HEK293T, CI-MPR KO, TMEM251KO	CRISPR-Cas9 KO of CI-MPR and TMEM251, single colony.	This study
Human HEK293T, GNPTAB-3HA knockin	CRISPR-Cas9 knockin of 3HA tag the C-terminus of GNPTAB	This study
Human HEK293T, GNPTAB-3HA knockin, TMEM251KO	CRISPR-Cas9 knockin of 3HA tag the C-terminus of GNPTAB, CRISPR-Cas9 KO of TMEM251	This study
Human HEK293T, GNPTAB-3HA knockin, TMEM251KO, TMEM251OE	CRISPR-Cas9 knockin of 3HA tag the C-terminus of GNPTAB, CRISPR-Cas9 KO of TMEM251, pLenti6.3-TMEM251(short)	This study
Human HeLa, TMEM251KO #1	CRISPR-Cas9 KO of TMEM251, sgRNA-1, single colony #1	This study
Human HeLa, TMEM251KO #2	CRISPR-Cas9 KO of TMEM251, sgRNA-2, single colony #2	This study
Human HeLa, sgTMEM251	Polyclonal TMEM251 CRISPR-Cas9 KO with sgRNA-1	This study
Human HeLa, TMEM251 long isoform	pLenti6.3-TMEM251(long)-BLAST	This study
Human HeLa, TMEM251 short isoform	pLenti6.3-TMEM251(short)-BLAST	This study

Supplementary Table 3: Mammalian plasmids used in this study			
Vector	***Insert***	***description***	***reference/source***
pHEK293Ultra (mamp418)	sHF-LIPA	CMV promoter, N-terminal His_6-FLAG	Jin et al., 2018

pcDNA3.1(-) (mamp415)	CTSD-3FLAG	CMV promoter, C-terminal 3FLAG	This study
pcDNA3.1(-) (mamp416)	CTSZ-3FLAG	CMV promoter, C-terminal 3FLAG	This study
pcDNA4.0 (mamp410)	MBTPS1-FLAG	CMV promoter, C-terminal FLAG	Chen et al., 2021
pcDNA4.0 (mamp411)	MBTPS1 (S414A)-FLAG	CMV promoter, C-terminal FLAG	Chen et al., 2021
pHAGE2-BSD (Mamp300)	TMEM251 long isoform	EF1α promoter, Blasticidin selection	This study
pHPAG2-BSD (Mamp301)	TMEM251 short isoform	EF1α promoter, Blasticidin selection	This study
pLenti6.3-BSD (Mamp317)	TMEM251 (short)-3HA	CMV promoter, Blasticidin selection	This study
pHAGE2-IRES-mCherry (Mamp149)	EGFP-RNF152	EF1α promoter, mCherry selection	Zhang et al., 2021
pLJC5 (Mamp074)	TMEM192-2XFLAG	UbC promoter	Abu-Remaileh et al., 2017 Addgene 102929
pLJC5 (Mamp 075)	TMEM192-3XHA	UbC promoter	Abu-Remaileh et al., 2017 Addgene 102930
pSpCas9(BB)-2A-Puro (PX459) (Mamp010)		CRISPR-Cas9 knockout	Ran et al., 2013 Addgene, 48139
Lenti-multi-CRISPR (Mamp194)		CRISPR-Cas9 knockout	Cao et al., 2016 Addgene 85402
psPAX2 (Mamp094)		Lentiviral packaging plasmid	Addgene 12260
pMD2.G (Mamp093)		VSV-G envelope	Addgene 12259
AG949 (Mamp372)	scFv M6P	N-terminal IL-2 signal sequence and C-terminal Fc region of Rabbit IgG	University of Geneva, This Study

Supplementary Table 4: primers used for F0 knockout in zebrafish	
Names	***Sequences***
tmem251 sgRNA-1	GAAATTAATACGACTCACTATATGAATTTCCGTCAGCGGATGGTTTTAGAGCTAGAAAT

tmem251 sgRNA-2	GAAATTAATACGACTCACTATAGGAGCGCAGGCAAAATGG CCGTTTTAGAGCTAGAAAT
tmem251 sgRNA-3	GAAATTAATACGACTCACTATAGAAACACCTGCAGATAGG GAGTTTTAGAGCTAGAAAT
tmem251 sgRNA-4	GAAATTAATACGACTCACTATATGATTGACACCTGAAGATG TGTTTTAGAGCTAGAAAT
gnptab sgRNA-1	GAAATTAATACGACTCACTATAGGGCTTTACCTGTGTTTCG GGTTTTAGAGCTAGAAAT
gnptab sgRNA-2	GAAATTAATACGACTCACTATAGAAGGGACTCACCGCCCTT TGTTTTAGAGCTAGAAAT
gnptab sgRNA-3	GAAATTAATACGACTCACTATAGGGCTGTGCTAACTCTTGG TGTTTTAGAGCTAGAAAT
gnptab sgRNA-4	GAAATTAATACGACTCACTATAGGAGAGGGATTCAGACTCC GGTTTTAGAGCTAGAAAT

Supplementary References:

Abu-Remaileh M, Wyant GA, Kim C, et al. Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent regulation of amino acid efflux from lysosomes. *Science*. 2017;358(6364):807-813. doi:10.1126/science.aan6298

Cao J, Wu L, Zhang SM, et al. An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. *Nucleic Acids Res*. 2016;44(19):e149. doi:10.1093/nar/gkw660

Chen X, Zhang J, Liu P, et al. Proteolytic processing of secretory pathway kinase Fam20C by site-1 protease promotes biomineralization. *Proc Natl Acad Sci U S A*. 2021;118(32):e2100133118. doi:10.1073/pnas.2100133118

Jin ZC, Kitajima T, Dong W, et al. Genetic disruption of multiple α1,2-mannosidases generates mammalian cells producing recombinant proteins with high-mannose-type *N*-glycans. *J Biol Chem*. 2018;293(15):5572-5584. doi:10.1074/jbc.M117.813030

Zhang W, Yang X, Chen L, et al. A conserved ubiquitin- and ESCRT-dependent pathway internalizes human lysosomal membrane proteins for degradation. *PLoS Biol*. 2021;19(7):e3001361. Published 2021 Jul 23. doi:10.1371/journal.pbio.3001361