

GOLPH3 and GOLPH3L maintain Golgi localization of LYSET and a functional mannose 6-phosphate transport pathway

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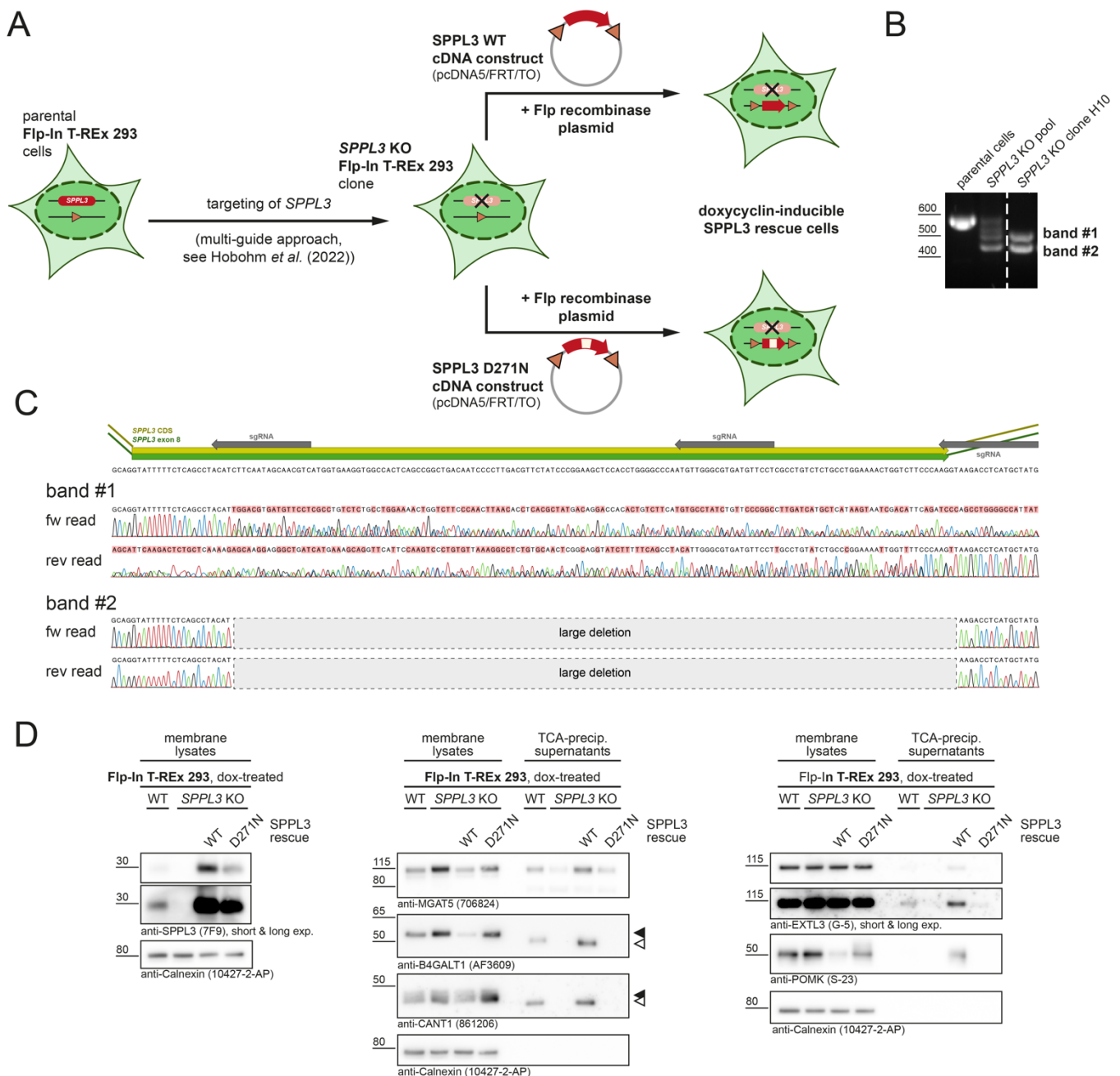
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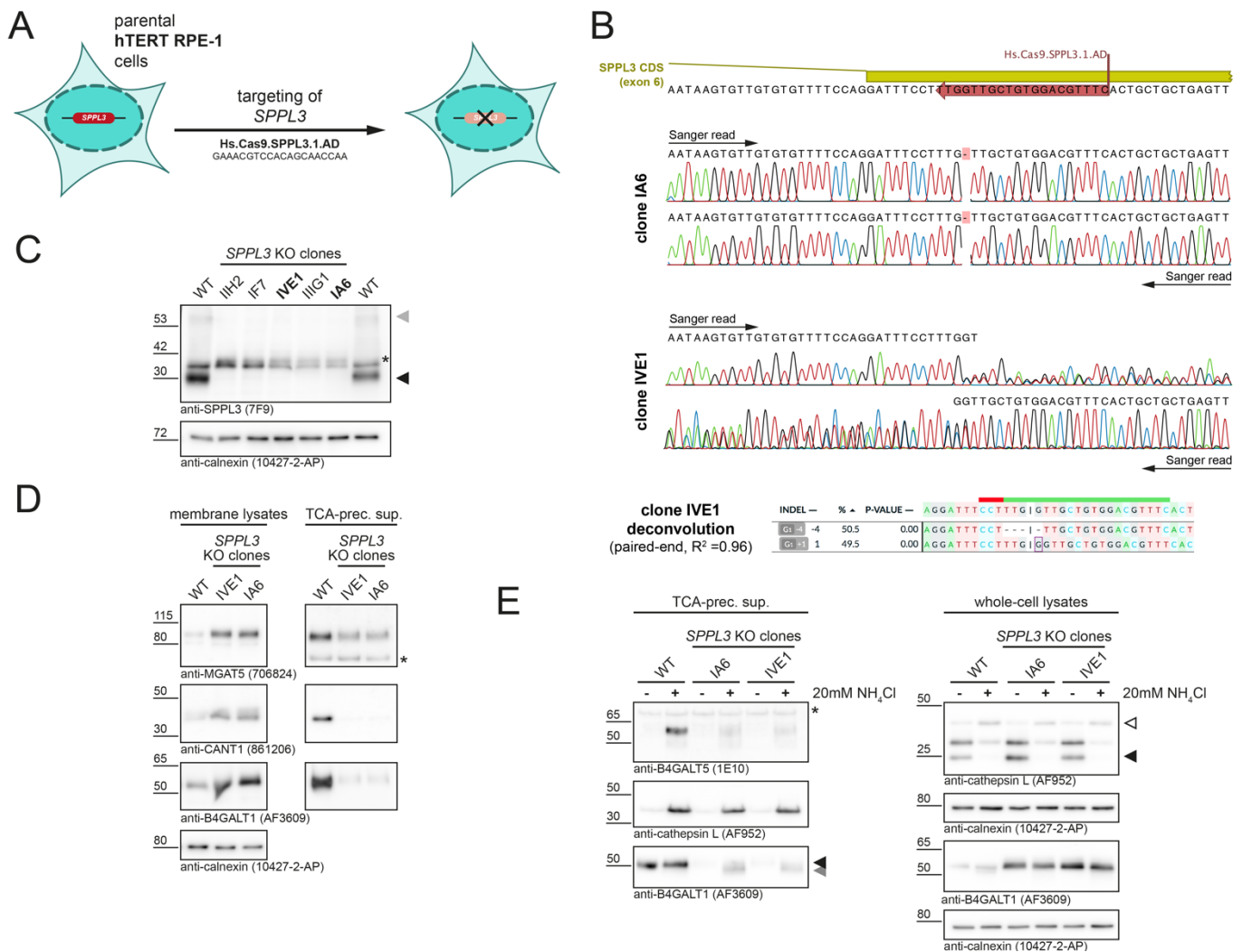
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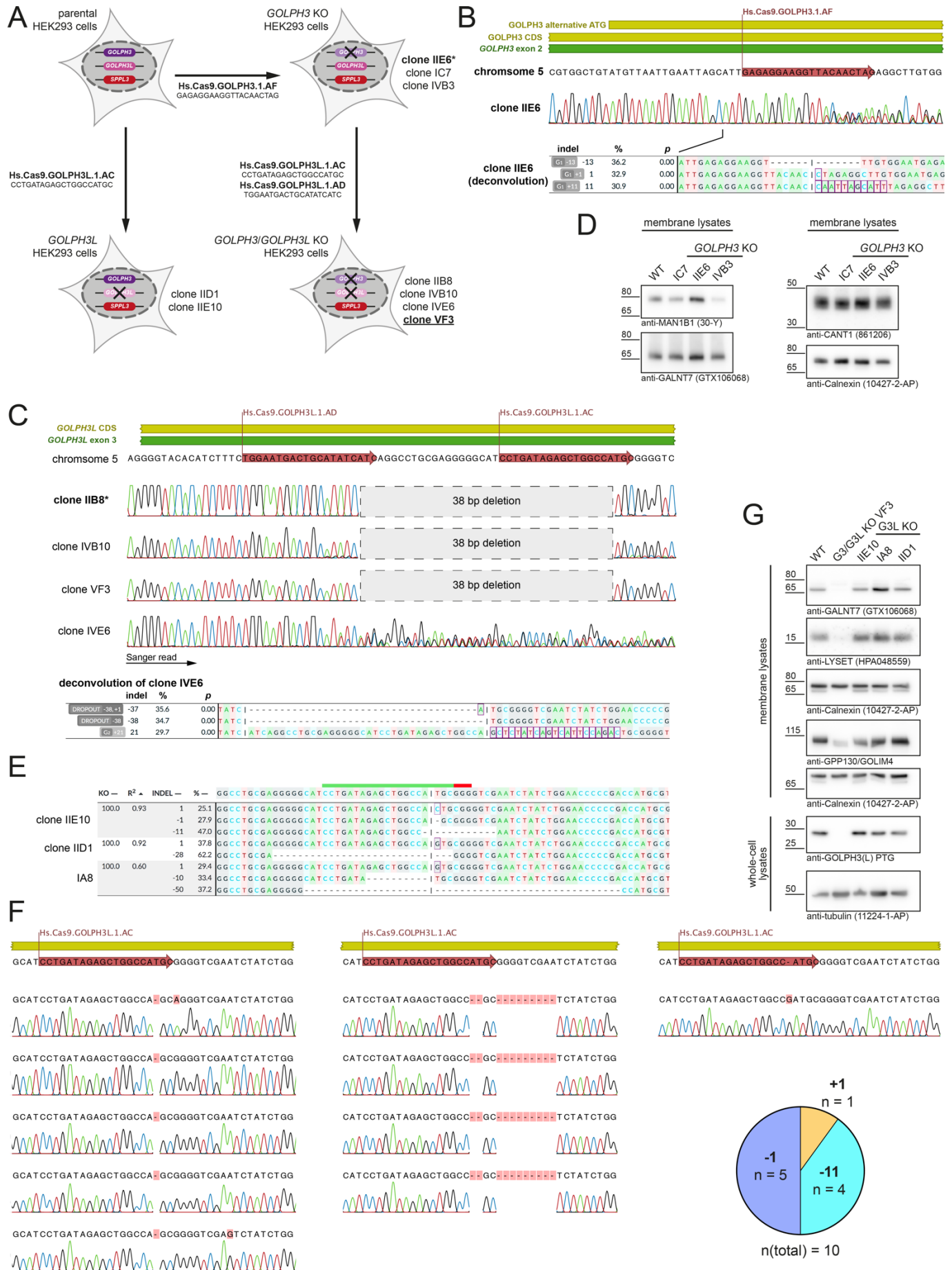
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Appendix Fig. S1: Generation of a *SPPL3* activity-based rescue model in *SPPL3*-deficient Flp-In T-REx 293 cells. (A) Schematic overview of the generation of *SPPL3*-deficient Flp-In T-REx 293 cells with a doxycycline-inducible re-expression of WT human *SPPL3* or an inactive variant (D271N). Flp-In T-REx 293 cells were first edited in *SPPL3* using three sgRNAs binding in close proximity to each other in *SPPL3* exon 8 as detailed elsewhere (Hobohm *et al.*, 2022). Next, cDNA constructs encoding human *SPPL3* WT (top) and catalytically inactive *SPPL3* D271N (bottom) were integrated into the validated *SPPL3* KO clone (H10) using Flp-mediated integration. (B) PCR amplification of a region comprising the sgRNA binding sites in *SPPL3* exon 8 from clone H10. (C) Alignment of Sanger sequencing reads to the *SPPL3* reference sequence. Reads were obtained following purification of the two distinctive bands observed for H10 genomic DNA. (D) Immunoblot analyses of membrane lysates and TCA-precipitated conditioned supernatants of clones derived from parental (WT) Flp-In T-REx 293 cells as depicted schematically in (A). Cells were induced with doxycycline (dox; 100 ng/ml) for 48 hrs before medium was changed to doxycycline-supplemented OptiMEM to collect secreted proteins overnight. Membrane lysates and TCA-precipitated conditioned supernatants were probed for abundance of the indicated proteins.

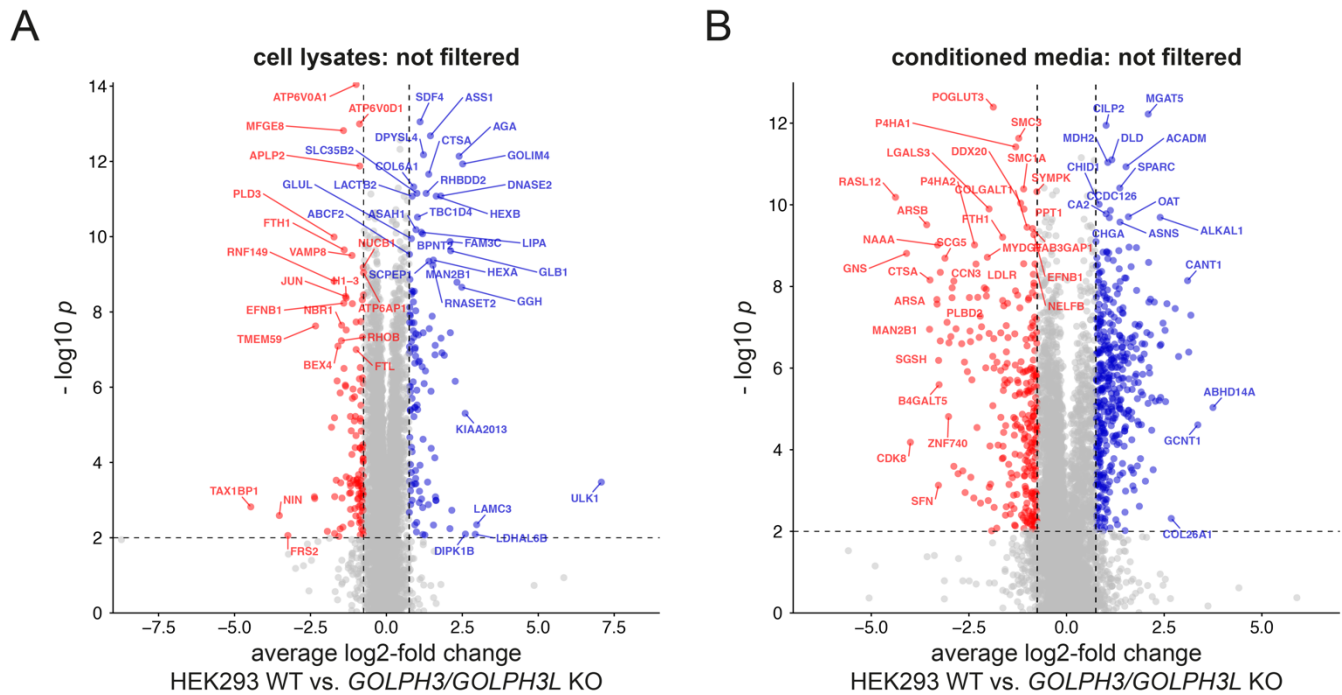


Appendix Fig. S2: Genome editing in *SPPL3* and *B4GALT5* secretion from hTERT RPE-1 cells. (A) Schematic overview of the SPPL3 targeting strategy pursued. (B) Sanger sequencing results of two hTERT RPE-1 clones successfully edited in SPPL3. The reads cover the target region and the crRNA binding site is indicated. Clone IA6 was homozygous for a deletion (top reads). Clone IVE1 was heterozygous based on the Sanger reads (lower reads) and read deconvolution using DECODR predicted two distinct frame-shifting alleles. (C) SPPL3 immunoblots of membrane lysates. Clones IVE1 and IA6 used for further experiments are highlighted in bold. Calnexin was used as a loading control. Black arrowheads denote the expected full-length protein bands, the grey arrowhead points to a likely SPPL3 dimer. (D) Golgi enzyme levels in membrane lysates and TCA-precipitated conditioned supernatants of parental (WT) or SPPL3 KO hTERT RPE-1 clones. Note the reduced secretion and intracellular accumulation of SPPL3 substrates in the SPPL3 KO clones. (E) Ammonium chloride-induced secretion of B4GALT5. Cells were treated as indicated and TCA-precipitated conditioned supernatants were analysed by immunoblotting using the indicated antibodies.

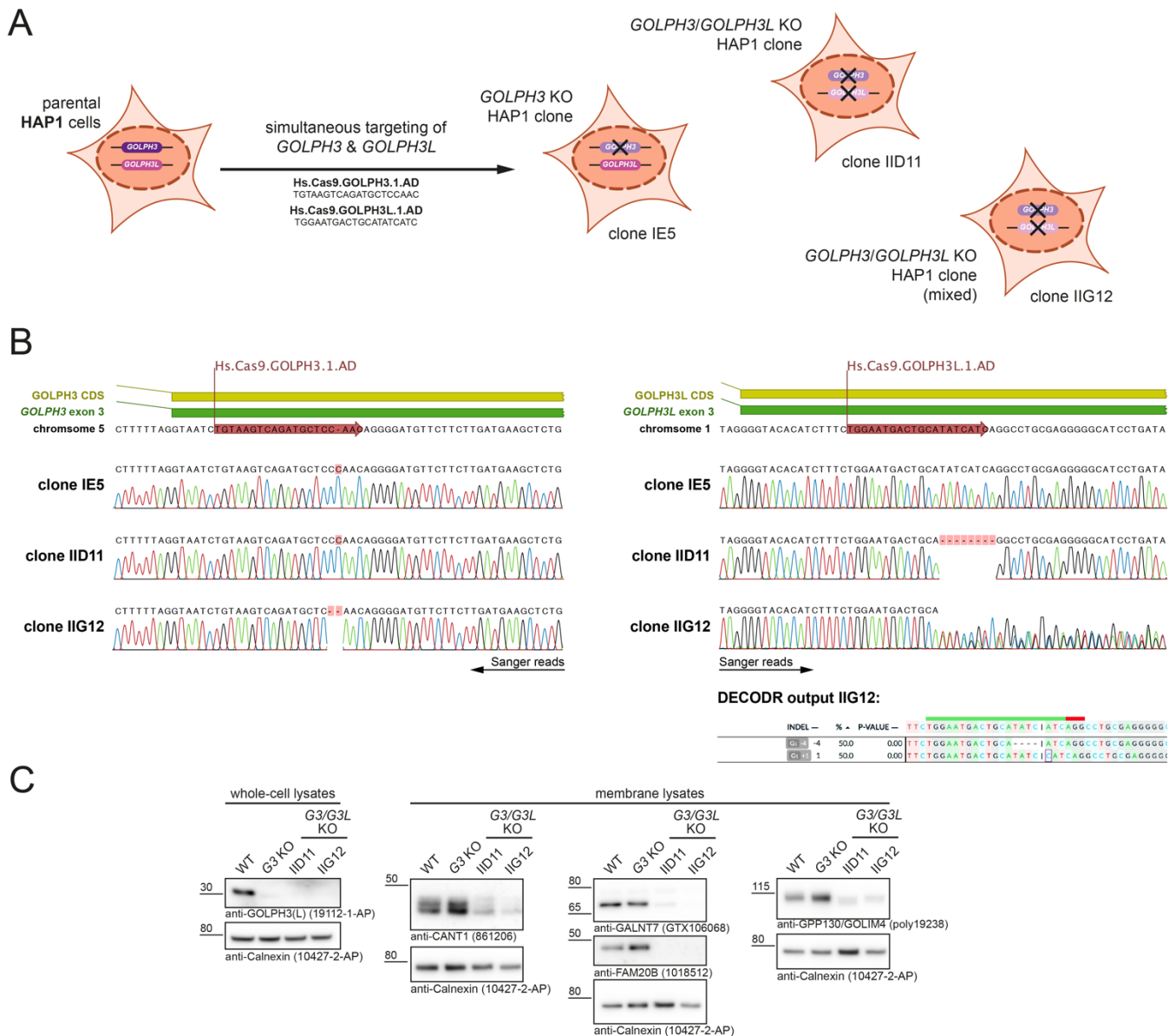


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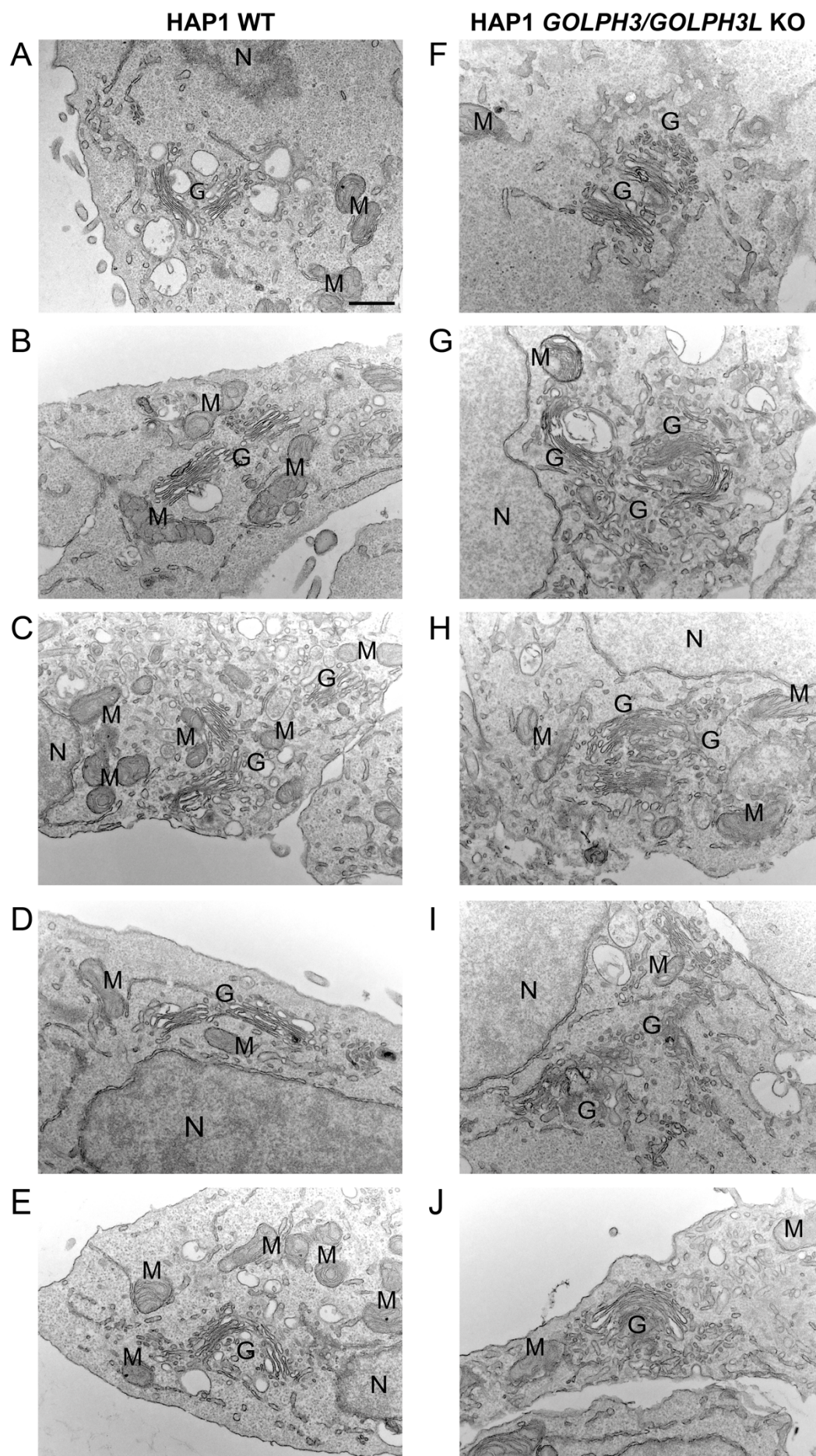
Appendix Fig. S3: Generation of GOLPH3/GOLPH3L-deficient HEK293 cells. (A) Overview of the genome editing strategy for successive depletion of *GOLPH3* and *GOLPH3L* with indicated targeting sequences. Clones validated and used in experiments in this study are shown. Clones in bold and labelled with an asterisk are the clones subjected to the second round of genome editing. The *GOLPH3/GOLPH3L* KO clone VF3 (bold and underlined) was used to establish stable cell pools with a cumate-inducible *GOLPH3* rescue. (B) Sanger read obtained from clone IIE6 following genome editing in *GOLPH3* aligned to the Cas9-targeted region in *GOLPH3* (top). Deconvolution of the same read conducted with DECODR (Bloh *et al*, 2021) (bottom). Absence of *GOLPH3* protein was confirmed by immunoblotting (Fig. 3E) and clone IIE6 was used to subsequently knock-out *GOLPH3L*. (C) Alignment of Sanger reads obtained from the indicated HEK293 clones after the second round of genome editing to the *GOLPH3L* target region. Clone IVE6 was not homozygous for the 38bp deletion and was analysed by deconvolution (bottom). (D) Cell-endogenous levels of select established type II membrane protein *GOLPH3/GOLPH3L* clients in membrane lysates of three *GOLPH3* KO clones and parental HEK293 cells. Calnexin was used as loading control. (E) Results of the paired-end deconvolution of forward and reverse Sanger reads of the *GOLPH3L* target region PCR-amplified from DNA of the indicated clones conducted with DECODR (Bloh *et al*, 2021). (F) Confirmation of the deconvolution results of *GOLPH3L* KO clone IIE10. The target locus was amplified using primers extended with restriction enzyme cleavage sites and was subsequently cloned into a pcDNA3-derived plasmid. Plasmid DNA was isolated from individual *E. coli* clones and subjected to Sanger sequencing. Only reads of frameshifting indel alleles were observed. (G) Abundance of LYSET and select Golgi type II membrane proteins in HEK293 clones edited in only *GOLPH3L* and compared to parental and *GOLPH3/GOLPH3L* KO HEK293 cells. Calnexin was used as loading control.



Appendix Fig. S4: Whole-cell proteome and secretome analyses of *GOLPH3/GOLPH3L* KO and parental HEK293 cells. Four technical replicates of *GOLPH3/GOLPH3L* KO clone VF3 and parental HEK293 cells were analysed. Volcano plots are depicting changes in protein abundance for all proteins quantified in whole-cell lysates (**A**) and conditioned cell culture supernatants (**B**). Significantly ($p < 0.01$) changed ($\log_2$ -fold change < -0.75 and > 0.75) proteins are coloured in red and blue, respectively, and select data points are labelled with gene names.

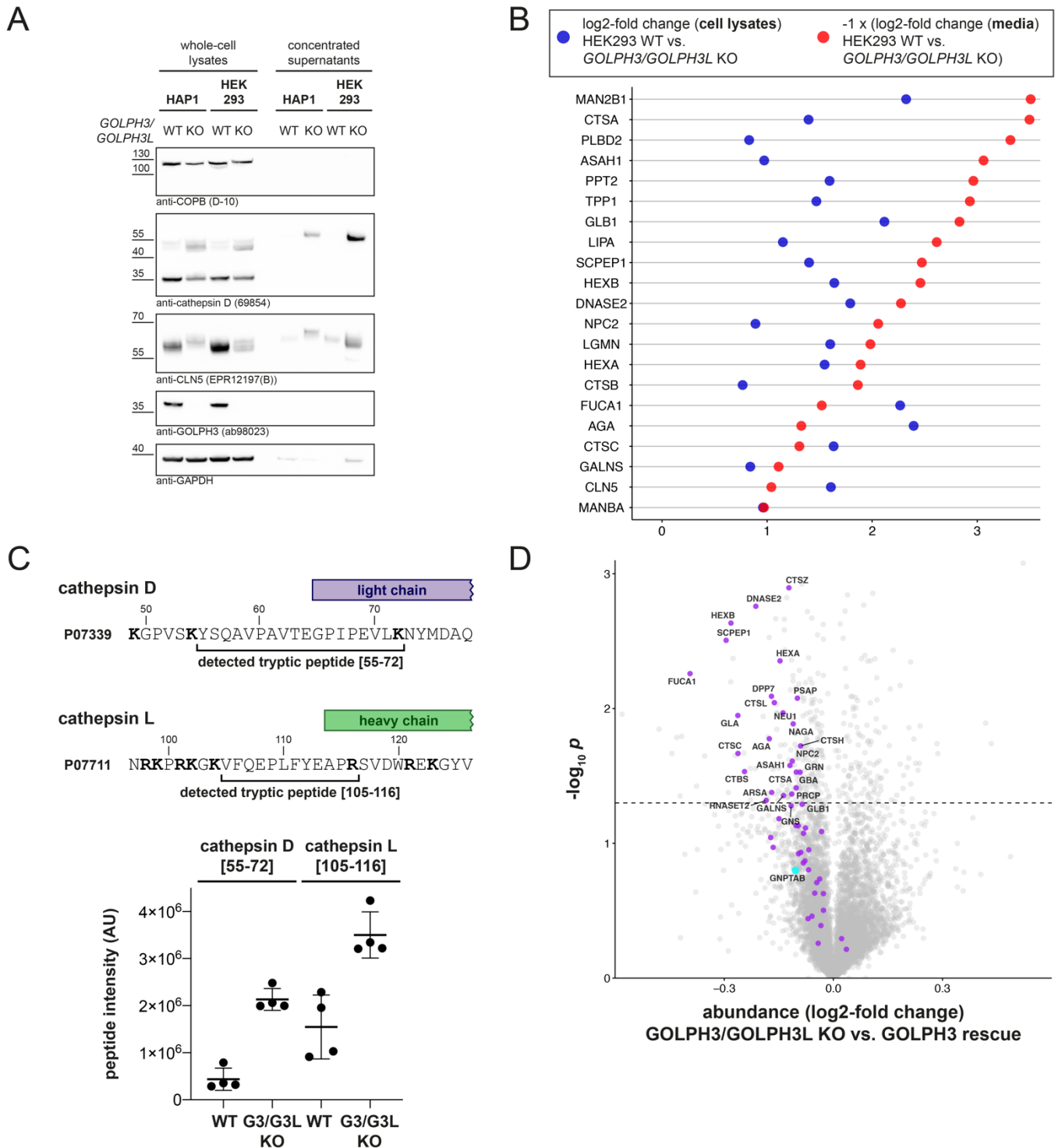


Appendix Fig. S5: Generation of *GOLPH3*/*GOLPH3L*-deficient HAP1 cells. (A) Overview of the genome editing strategy in HAP1 cells. *GOLPH3* and *GOLPH3L* were targeted simultaneously (crRNA binding sites are indicated). **(B)** Genotyping of obtained HAP1 clones using PCR amplification and Sanger sequencing demonstrated the presence of frame-shifting +1, +1 and -2 indels in *GOLPH3* in clones IE5, IID11 and IIG12 and no indel mutation in IE5 and a frame-shifting 8-bp deletion in IID11 in *GOLPH3L*. The *GOLPH3L* read for clone IIG12 was ambiguous and deconvolution suggests equal abundance of two distinct indels. IIG12 may thus represent a mixed clone or may have lost haploidy. Both indels detected are frame-shifting and immunoblotting supported absence of *GOLPH3L* protein (Fig. 3F). **(C)** Levels of select established type II membrane protein *GOLPH3*/*GOLPH3L* clients in membrane lysates of WT, *GOLPH3* KO and *GOLPH3*/*GOLPH3L* KO HAP1 cells. Calnexin was used as loading control.



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Appendix Fig. S6: Ultrastructural analysis of GOLPH3/GOLPH3L-deficient cells. Representative high-resolution electron micrographs of Golgi complexes in WT and *GOLPH3/GOLPH3L* KO (clone IID11) HAP1 cells. In HAP1 WT cells, flattened membrane stacks (cisternae) and associated transport vesicles as well as larger secretory vesicles are well preserved. In *GOLPH3/GOLPH3L* KO cells the Golgi stacks are dissolved, almost not discernible and surrounded by large numbers of vesicles. G, Golgi apparatus; N, nucleus; M, mitochondrion; scale bar = 500 nm.

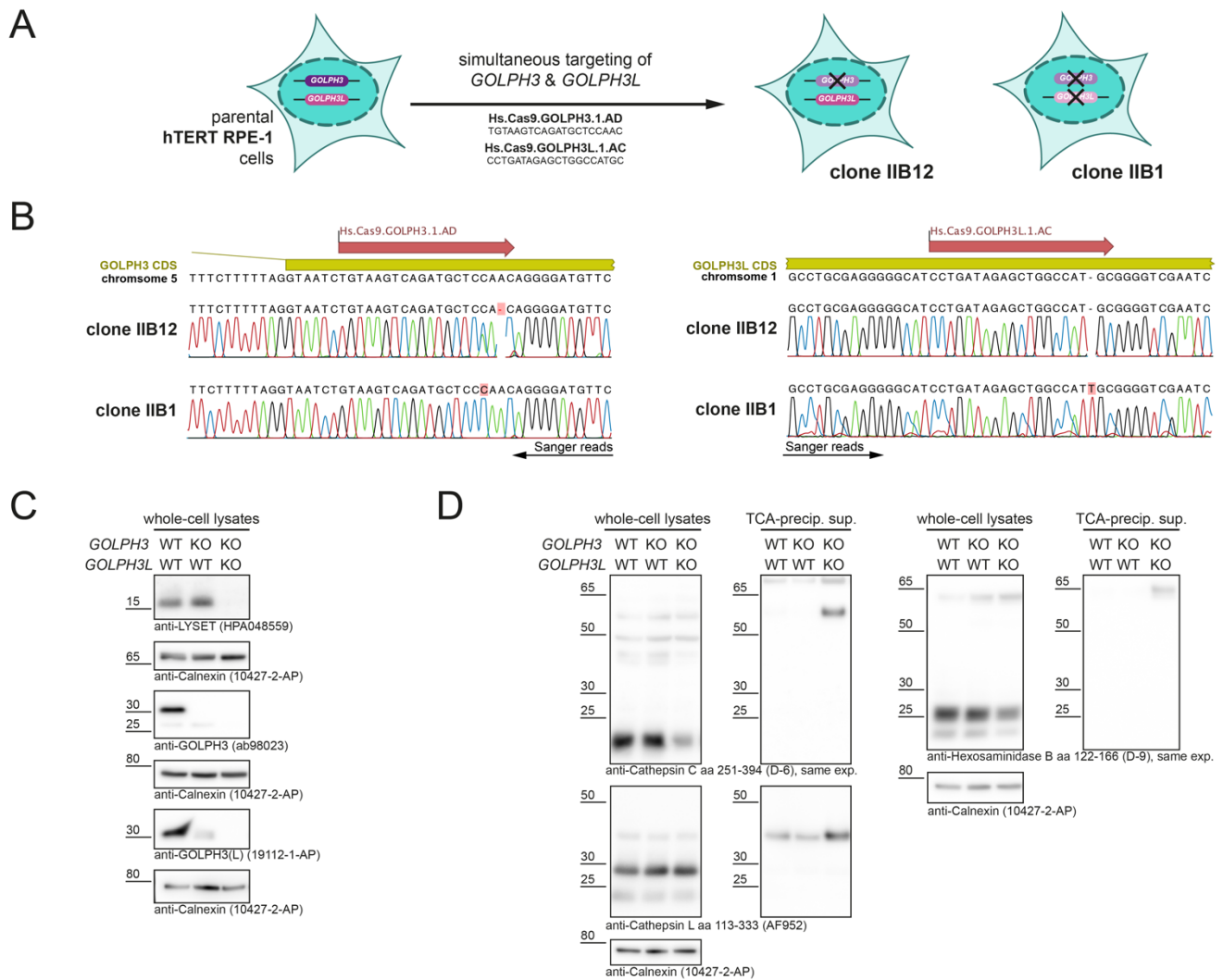


Appendix Fig. S7: Lysosomal proteins in *GOLPH3/GOLPH3L* KO cells. (A) CLN5 and cathepsin D levels in whole-cell lysates (left) and concentrated conditioned supernatants (right) of WT and *GOLPH3/GOLPH3L* KO HAP1 and HEK293 cells. (B) Comparison of absolute log₂-fold changes for luminal lysosomal enzymes (labelled by gene name) significantly changed in both proteome (Fig. 4C) and secretome (Fig. 4D) datasets obtained for *GOLPH3/GOLPH3L*-deficient HEK293 cells. (C) Tryptic cathepsin-derived peptides quantified in the secretome dataset (Suppl. Fig. 4B) supporting the predominant secretion of immature lysosomal enzymes from *GOLPH3/GOLPH3L* KO cells. Quantified cathepsin-derived tryptic peptides were manually searched for tryptic peptides spanning regions known to undergo (auto-)proteolytic cleavage events during maturation. Two peptides, mapping to such regions in cathepsin D (Richo & Conner, 1994) and cathepsin L (Ménard *et al.*, 1998) were identified and intensity values obtained for these peptides in conditioned media of HEK293 WT and *GOLPH3/GOLPH3L* KO cells are plotted ($\pm$ SD). (D) Depletion of lysosomal proteins from *GOLPH3/GOLPH3L*-deficient U2-OS cells. Published proteome data were retrieved from (Welch *et al.*,

2021). Well-characterized soluble lysosomal proteins (Markmann *et al.*, 2017) are shown in purple and labelled with the corresponding gene name in case of a p-value < 0.05 (dashed black line). The α/β subunit of the GNPT complex (GNPTAB) is labelled in cyan. Note that LYSET/TMEM251 was not present in the dataset.

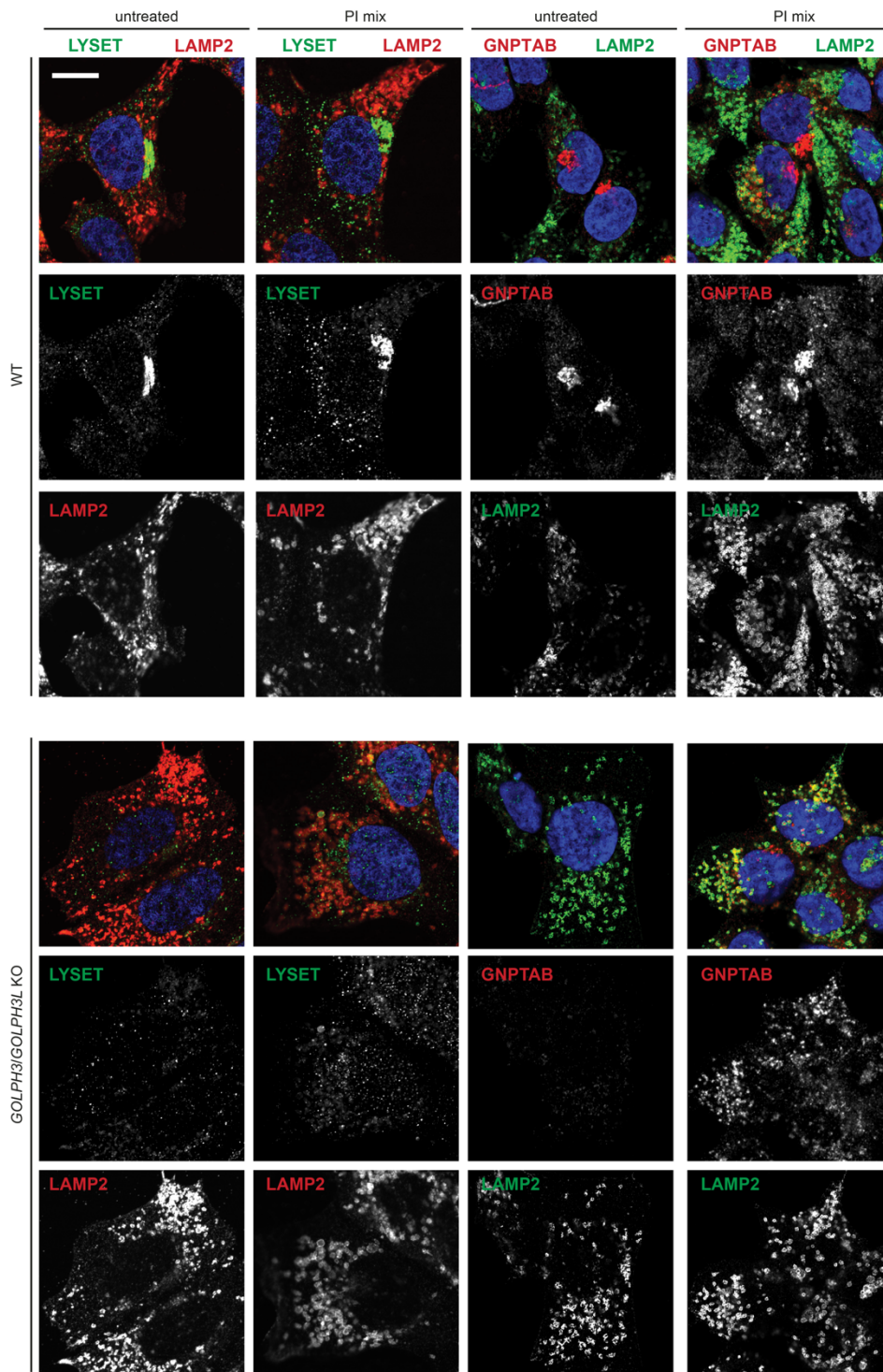
References (not included in main reference list)

- Ménard R, Carmona E, Takebe S, Dufour É, Plouffe C, Mason P & Mort JS (1998) Autocatalytic Processing of Recombinant Human Procathepsin L: Contribution of both intermolecular and unimolecular events in the processing of procathepsin L in vitro. *J Biol Chem* 273: 4478–4484
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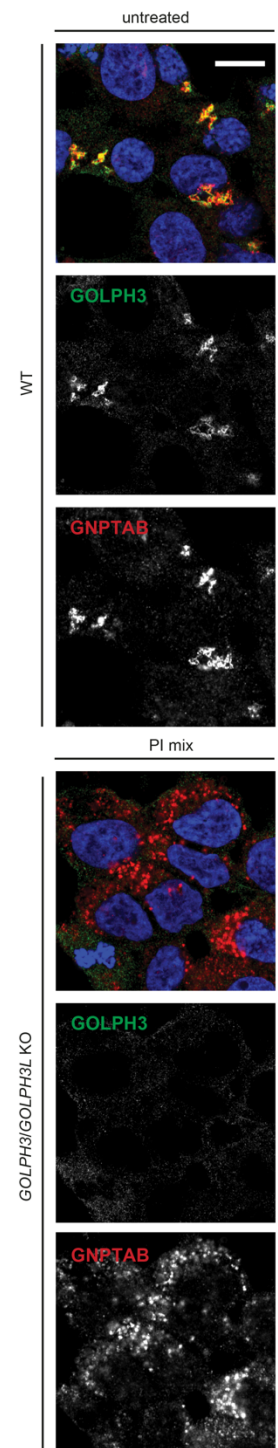


Appendix Fig. S8: Generation of *GOLPH3* and *GOLPH3/GOLPH3L* KO hTERT RPE-1 cells. (A) Genome editing strategy used to simultaneously target *GOLPH3* and *GOLPH3L*. The schematically shown single-cell-derived clones IIB12 and IIB1 were selected and were found to carry biallelic frame-shifting indels in *GOLPH3* only and in both *GOLPH3* and *GOLPH3L*, respectively. (B) Sanger sequencing reads obtained from clones IIB12 and IIB1 aligned to the *GOLPH3* (top) and *GOLPH3L* (bottom) target regions. (C) Immunoblot analysis of LYSET and *GOLPH3/GOLPH3L* levels in whole-cell lysates of parental, *GOLPH3* KO and *GOLPH3/GOLPH3L* KO hTert RPE-1 cells. Calnexin was used as loading control. (D) Immunoblot analysis abundance and maturation of cathepsin C, cathepsin L and hexosaminidase B in whole-cell lysates and TCA-precipitated conditioned cell culture supernatants of parental, *GOLPH3* KO and *GOLPH3/GOLPH3L* KO hTert RPE-1 cells. Calnexin was used as loading control.

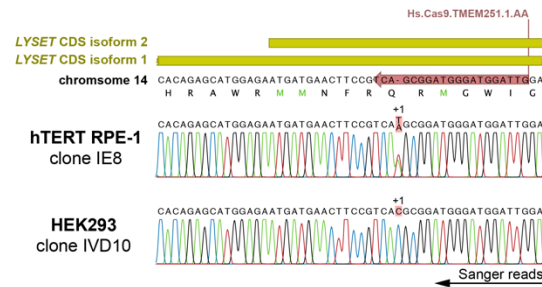
A



B



Appendix Fig. S9: Immunofluorescence data related to Fig. 5. Single-channel images corresponding to overlay images shown in Fig. 5G (A) and Fig. 5H (B). DNA staining (Hoechst 33342) is shown in blue; scale bar = 10 μm.



Appendix Fig. S10: Genotyping of newly established *LYSET* KO hTERT RPE-1 and HEK293 cells. The *LYSET* target region was PCR-amplified from genomic DNA isolated from the indicated clones and subjected to Sanger sequencing. Obtained reads were aligned to the corresponding region in the human reference genome. Frame-shifting +1 insertions in both clones are highlighted.

Appendix Tab. S1: crRNAs and oligos used for CRISPR/Cas9-mediated genome editing and genotyping at target loci. All crRNAs were purchased from Integrated DNA Technologies and the manufacturer's reference ID is provided. All sequences provided are given in 5'>3' orientation. *, Binding site of the two GOLPH3L crRNAs are in close proximity and the very same genotyping strategy was pursued.

Target locus	crRNA ID	crRNA target sequence genotyping oligo #1 sequence genotyping oligo #2 sequence
GOLPH3	Hs.Cas9.GOLPH3.1.AF	GAGAGGAAGGTTACAAC TAG TGGAAAGATAAGCCCCGAAAA TGGCCGGTTACCTTAACAATCA
GOLPH3	Hs.Cas9.GOLPH3.1.AD	TGTAAGTCAGATGCTCCAAC GATACTACATGAAAGCTTGAGGC ACCATTACTTCCCCACTCTATGAC
GOLPH3L	Hs.Cas9.GOLPH3L.1.AC	CCTGATAGAGCTGGCCATGC ACAGCAAACCATGGAAATTC ACT* ACCTGTTTAGACAATGGA ACTGC*
GOLPH3L	Hs.Cas9.GOLPH3L.1.AD	TGGAATGACTGCATATCATC ACAGCAAACCATGGAAATTC ACT* ACCTGTTTAGACAATGGA ACTGC*
SPPL3	Hs.Cas9.SPPL3.1.AB	ACACTTGACTGGAATCCACC CCTCATCACCGAGCAAAACCA AGAAGTGTTTAACAGCAGCCAAT
SPPL3	Hs.Cas9.SPPL3.1.AD	GAAACGTCCACAGCAACCAA ATGGAGATGCTGAACGCACTTA AGGTGGGTAAGGGGAATGACC
LYSET	Hs.Cas9.TMEM251.1.AA	CAATCCATCCCATCCGCTGA TTGGAGTAGTTGCCGTGACAG CAGTAGCCCACTGTTTTGGGA HDR donor: TTTAATTTGAAGGCACAGAGCATGGAGAATGATGAACTTCGCTCAGGCCATGGGATGGATTGGAGTGGGATTGTATCT GTTAGCCAGTG

Appendix Tab. S2: Oligos used for molecular cloning. In case of oligos used for mutagenesis, bases in lower case correspond to introduced mutations.

Gene	Purpose of PCR amplification	Oligonucleotide sequences (forward/reverse), 5'>3'
SPPL3	Site-directed mutagenesis to introduce the D271N active site mutant	GGGCATCGGAaACATCGTTAT
		AACATGGAGAAGTGGCTG
GOLPH3	Amplification of CDS without C-terminal tag, introduction of HindIII and XhoI sites for insertion into pcDNA3.1 backbone	CTTAAGCTTACCATGACCTCGCTGACCCAGC
		GACTCGAGTCACTTGGTGAACGCCGCCAC
	Site-directed mutagenesis to introduce the R14A/R15A mutant	CCTGGTGCAGgctgcACCGAGGCCTCCCGC
		CCGGAGCTGCGCTGGGTC
	Site-directed mutagenesis to introduce the R90L mutant	TCTGGATTACTgGGCTGTATGTTAATTG
		TGATATACAGTCATTCCAAAATG
	Amplification of CDS without C-terminal tag, introduction of NheI and NotI sites for insertion into pBQM812A backbone	AGAGCTAGCACCATGACCTCGCTGACCCAGCGCAG
		ATAGCGGCCGCTCACTTGGTGAACGCCGCCAC
LYSET isoform 1	Amplification of CDS, introduction of HindIII and BamHI sites for insertion into pcDNA6 myc/His backbone	CCCAAGCTTATGCCAAAGCCACCCGATTATTC
		CGCGGATCCTTACGTGTCAATCAGTTGTAGTC
	Side-directed mutagenesis: R22A	GGGAACAGGAgcgTTTTCGGGACCATTG
		ACGGCAAGCGTTAAAGAGTCAC
	Side-directed mutagenesis: R29A	ACCATTGCACgcgGCATGGAGAATG
		CATTCTCCATGCcgCGTGCAATGGT
	Side-directed mutagenesis: R32A	CAGAGCATGGGCGATGATGAACTTCC
		TGCAATGGTCCCCGAAAATCTTCC
	Side-directed mutagenesis: R37A	GATGAACTTCgcTCAGCGGATGGGATG
		ATTCTCCATGCTCTGTGC
	Side-directed mutagenesis: R39A	CTTCCGTCAGGCGATGGGATGGATTGG
		TTCATCATTCTCCATGCTCTGTGC
	Side-directed mutagenesis: R39W	CTTCCGTCAGtGGATGGGATG
		TTCATCATTCTCCATGCTCTG
	Side-directed mutagenesis: R37A/R39A	ggctATGGGATGGATTGGAGTG
		tgagcGAAGTTCATCATTCTCCATG
	Side-directed mutagenesis: R37K/R39K	gaagATGGGATGGATTGGAGTG
		tgcttGAAGTTCATCATTCTCCATG
	Site-directed mutagenesis: R22A/R37A	GATGAACTTCGCGCAGCGGATGGG

	Site-directed mutagenesis: R29A/R37A	ATTCTCCATGCTCTGTGCAATGG
		GATGAACTTCGCGCAGCGGATGGG
		ATTCTCCATGCTCTGTGCAATGG
	Site-directed mutagenesis: R32A/R37A	GATGAACTTCGCGCAGCGGATGGG
		ATCGCCCATGCTCTGTGCAATGG
	Site-directed mutagenesis: R22A/R29A	ACCATTGCACgcgGCATGGAGAATG
		CCCGAAAACGCTCCTGTTCCAC
	Site-directed mutagenesis: R22A/R32A	CAGAGCATGGGCGATGATGAACTTCC
		TGCAATGGTCCCGAAAATCTTCC
LYSET isoform 2	Amplification of CDS, introduction of HindIII and BamHI sites for insertion into pcDNA6 myc/His backbone	CGCGGCATGGGCGATGATGAACTTC
		CATTCTCCATGCcgGTGCAATGGT
		CCCAAGCTTATGATGAACTCCGTCAGCGG
B4GALT5	Amplification of CDS without C-terminal stop codon, introduction into pcDNA3.1-derived backbone with an in-frame C-terminal V5 tag via HindIII and BamHI	CGCGGATCCTTACGTGTCAATCAGTTGTAGTC
		CTTAAGCTTACCATGCGCGCCCGCGGGGGCTG
	Amplification of CDS without C-terminal stop codon, to insert in-frame into Str-KDEL_ST-SBP-mCherry	AGAGGATCCGTA CTCTGTTACCTGAGC
		GAGGCGCGCCATGCGGGCCCGCGGGGGCTGCTG
B4GALT1	Amplification of CDS without C-terminal stop codon, introduction into pcDNA3.1-derived backbone with an in-frame C-terminal V5 tag via HindIII and BamHI	CATGGAATTCCCGTACTCGTTACCTGAGCCAGCT
		AACCTAAGCTTACCATGAGGCTTCGGGAGCCGCTC
		GCCGGATCCGCTCGGTGTCCCGATGTC
GNPTAB	Amplification of CDS without C-terminal stop codon, introduction of 20bp overlapping ends with EcoRV-digested pLenti_CMVTRE3G-Puro-DEST and the synthesized myc-IRES-LYSET-FLAG fragment for Gibson Assembly	TGTGGTGAATTCTGCAGATACCATGCTGTTCAAGCTCCTGCAGAGAC
		TCTGAGATGAGTTTTTGTCTACTCTGATTGATTGGGACTAGCTTCTTG

Appendix Tab. S3: Small molecule inhibitors used.

Inhibitor	Target(s)/target process	Source, catalogue number
Ammonium chloride	Lysosomal acidification, M6P/M6P receptor interaction	Carl Roth
Bafilomycin A1	Lysosomal acidification	Cayman Chemical
E-64d	Cysteine proteases	Cayman Chemical Sigma-Aldrich
Leupeptin (hemisulfate)	Cystein/serine/threonine proteases	Carl Roth Sigma-Aldrich
Pepstatin A	Aspartyl proteases	Carl Roth Roche

Appendix Tab. S4: Primary antibodies used in this study and purchased from commercial sources. For monoclonal antibodies clone name/number are provided, for polyclonal antibodies the vendor's respective catalogue number. WB, Western blot; IF, immunofluorescence.

Antigen	Source	Clone / Catalogue #	Host species	Application & dilution
Actin	Sigma-Aldrich	A5316	mouse	WB: 1:5000
B4GAT1	R&D systems	724057	mouse	WB: 1:1,000
B4GALT1	R&D systems	AF3609	goat	WB: 1:2,000
Calnexin	Protein Tech Group	10427-2	rabbit	WB: 1:25,000
CANT1	R&D systems	861206	mouse	WB: 1:1,000
cathepsin B	Biolegend	W20149K	rat	WB: 1:1,000
cathepsin C	Santa Cruz Biotechnologies	D-6	mouse	WB: 1:1,000
Cathepsin D	Cell Signaling Technology	69854	rabbit	WB: 1:1,000
Cathepsin L	R&D systems	AF952	goat	WB: 1:1,000
CLN5	Abcam	EPR12197(B)	rabbit	WB: 1:1,000
COPB	Santa Cruz Biotechnologies	D-10	mouse	WB: 1:1,000
COG3	Protein Tech Group	11130-1-AP	rabbit	WB: 1:2,000
EXTL3	Santa Cruz Biotechnologies	G-5	mouse	WB: 1:1,000
FLAG tag	Sigma-Aldrich	M2 / F1804	mouse	WB: 1:1,000
GALNT2	Sigma-Aldrich	HPA011222	rabbit	WB: 1:1,000
GALNT7	Genetex	GTX106068	rabbit	WB: 1:3,000
GM130	BD Biosciences	35	mouse	WB: 1:1,000 IF: 1:100
GM130	Cell Signaling Technology	12480	rabbit	WB: 1:1,000 IF: 1:100
GOLGA3	Protein Tech Group	21193-1-AP	rabbit	WB: 1:3,000
GOLGA5	Genetex	GT2726	mouse	WB: 1:1,000
GOLPH3	Abcam	ab98023	rabbit	WB: 1:1,000 IF: 1:100
GOLPH3/GOLPH3L	Protein Tech Group	19112-1-AP	rabbit	WB: 1:2,000 IP: see methods section
Golgin 97	Thermo Fisher Scientific	A21270	mouse	WB: 1:500
GPP130/GOLIM4	Biolegend	poly19238 / #923801	rabbit	WB: 1:1,000
GRASP55	Protein Tech Group	10598-1-AP	rabbit	WB: 1:1,000

GRASP65	Santa Cruz Biotechnologies	D12	mouse	WB: 1:1,000
GS27	BD Biosciences	25	mouse	WB: 1:1,000
GS28	BD Biosciences	1	mouse	WB: 1:1,000
HEXB	Santa Cruz Biotechnologies	D-9	mouse	WB: 1:1,000
LAMP1	Developmental Studies Hybridoma Bank	H4A3	mouse	WB: 1:1,000
LAMP1	Abcam	ab24170	rabbit	WB 1:1,000
LAMP2	Developmental Studies Hybridoma Bank	H4B4	mouse	WB: 1:1,000
LYSET (TMEM251)	Thermo Fisher Scientific	PA5-61769	rabbit	WB: 1:1,000 IF: 1:100
LYSET (TMEM251)	Human Proteome Atlas	HPA048559	rabbit	WB: 1:1,000
MAN1B1	Santa Cruz Biotechnologies	30-Y	mouse	WB: 1:500
mCherry	Chromotek	6G6	mouse	WB: 1:1,500
MGAT5	R&D systems	706824	mouse	WB: 1:1,000
myc tag	Cell Signaling Technology	9B11 / #2276	mouse	WB: 1:1,000
POMK	Santa Cruz Biotechnologies	S-23	mouse	WB: 1:1,000
SPPL3	Sigma-Aldrich	7F9	mouse	WB: 1:1,000
TGN46	Abcam	ab50595	rabbit	WB: 1:1,000
TM9SF2	Thermo Fisher Scientific	PA5-48517	rabbit	WB: 1:1,000
TM9SF4	Protein Tech Group	25595-1-AP	rabbit	WB: 1:1,000
tubulin	Protein Tech Group	11224-1-AP	rabbit	WB: 1:20,000
V5 tag	Thermo Fisher Scientific	R960-25	rabbit	WB: 1:5,000