Acute GARP depletion disrupts vesicle transport leading to severe defects in sorting, secretion, and O-glycosylation

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Abstract

The GARP complex is an evolutionarily conserved protein complex proposed to tether endosome-derived vesicles at the trans-Golgi network. Prolonged depletion of GARP leads to severe trafficking and glycosylation defects; however, the primary defects associated with GARP dysfunction remain unclear. In this study, the mAID degron strategy was used to rapidly degrade VPS54 in human cells, allowing for the acute disruption of GARP function. This acute dysfunction resulted in the partial mislocalization and degradation of a subset of Golgi-resident proteins, including TGN46, ATP7A, TMEM87A, CPD, C1GALT1, and GS15. Defects in enzyme recycling led to an early onset of O-glycosylation abnormalities. Additionally, the secretion of fibronectin and cathepsin D was altered, though mannose-6-phosphate receptors remained largely unaffected. COPI, AP1, and GGA coats were partially displaced, causing a significant accumulation of vesicle-like structures and large vacuoles. The detection of GARP-dependent vesicles by electron microscopy, along with the identification of specific cargo proteins associated with these trafficking carriers, provides direct experimental evidence for the GARP's role as a vesicular tether. We conclude that the primary defects of GARP dysfunction include the mislocalization of vesicular coats, accumulation of GARP-dependent vesicles, degradation and mislocalization of specific Golgi proteins, and defects in O-glycosylation.

Keywords: GARP complex, Golgi, Endosome-to-Golgi traffic, degron, vesicle tethering, glycosylation

Introduction

Proteins and lipids within the cell are continuously trafficked between the plasma membrane and the *trans*-Golgi network (TGN) via the endosome-to-TGN pathway [1] [2]. This retrograde transport mechanism is crucial for the recycling of protein and lipid cargoes, balancing the anterograde movement of membranes [3] and preventing the degradation of these components in lysosomes [4]. Some of the cargoes that utilize endosome-to-TGN trafficking include the copper transporters ATP7A and ATP7B [5] [6] [7], enzymes carboxypeptidase D and furin [8] [9] [10], putative ion channel TMEM87 [3] and recycling receptors such as mannose-6-phosphate receptors (MPRs) [11], sortilins [12] [13] and TGN46 [1]. This trafficking step is also exploited by multiple pathogens, including cholera [14], Shiga [15] and SubAB [16] toxins.

Cargo transport between cellular compartments begins with the selection and packaging of cargo into small membrane intermediates (vesicles or tubules) at the donor compartment [17]. This is followed by the tethering of these vesicles and their subsequent fusion with the acceptor compartment [18] [19]. At the TGN, cargo-laden vesicle tethering is mediated by long coiled-coil proteins known as Golgins [20] [21] and the multisubunit tethering complex (MTC) Golgi Associated Retrograde Protein (GARP) [22] [11] [23]. The GARP complex is evolutionarily conserved across a range of organisms, including humans, mice, and plants [24] [25] [26]. GARP belongs to the CATCHR (Complexes associated with tethering containing helical rods) family of MTCs and is thought to tether retrograde transport vesicles originated from endosomes, facilitating their fusion with the TGN [27] [28] [29]. The GARP complex is composed of four subunits: VPS51, VPS52, VPS53, and VPS54 [23]. Of these, VPS51, VPS52, and

VPS53 are shared with the EARP (endosome-associated recycling protein) complex, while VPS54 is unique to GARP [30]. In mammalian cells, GARP's localization to the TGN relies on small GTPases ARFRP1 and ARL5 [31]. GARP role in retrograde trafficking is supported by multiple interactions with other components of the endosome-TGN trafficking machinery [32] [33] [34]. However, the mechanism of GARP's action remains unclear. Mutations in the VPS51 have been associated with abnormal glycosylation patterns in patients [30]. Similarly, knockout (KO) of VPS53 and VPS54 in tissue culture cells causes severe defects in both N- and O-linked protein glycosylation resulting from mislocalisation and degradation of multiple Golgi enzymes [35] [36]. Moreover, GARP-KO led to significant mislocalization of COPI, AP1, and GGA vesicle coats, displacement of ARF1 GEFs (GBF1 and BIG1), and severe alterations in Golgi morphology. Although the expression of missing GARP subunits rescues all observed defects, some of these defects may be secondary, arising from the persistent mistargeting of receptors and cellular trafficking machinery, or from cellular adaptation to the loss of the GARP complex.

To investigate the primary defects caused by GARP dysfunction, we developed a novel cellular system that enables the acute depletion of VPS54, a key subunit of the GARP complex, using the auxin-inducible degron (mAID) technology [37] [38] [39] [40]. A combination of biochemical and microscopic techniques was used to analyze the impact of acute VPS54 depletion on Golgi morphology, stability of other GARP subunits, GARP-interacting membrane trafficking partners, glycosylation enzymes, and other Golgi resident proteins. This study provides a comprehensive view of the primary cellular defects associated with GARP dysfunction in human cells.

Materials and Methods

Cell Culture

hTERT-RPE1 (retinal pigment epithelial, RPE1) and HEK293T cells used for all experiments were purchased from ATCC. RPE1 VPS54-KO cells were described previously [36]. HeLa-KO cells were obtained from Bonifacino lab (NIH) [31]. RPE1, HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing Nutrient mixture F-12 (Corning) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher). Cells were incubated in a 37°C incubator with 5% CO2 and 90% humidity. All DNA plasmids used in this work are listed in Tables 1.

Preparation of mVPS54-mAID expressing cells

hTERT RPE1 VPS54-KO cells were rescued with mVPS54-13-myc–mAID-mClover. For convenience, we will use VPS54-mAID hereafter.

Briefly, mVPS54 in pENTR1A 48-1 was amplified using VPS54-Xba1-Forward (CGGCCGCACTCGAGATATCTAGACCCAG) and VPS54-BamH1-Reverse (ATTGGATCCGTGGTGATGGTGGTGGTGATG) primers.

PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) following the standard protocol. To create VPS54-mAID in pENTR1A, mVPS54 in pENTR1A (48-1) and MK289 (mAID-mClover-NeoR) were digested with BamHI and XbaI, and ligated. This construct was then recombined with the pLentiCOG4$_{pr}$-Neo–DEST plasmid, using Gateway LR Clonase II Enzyme Mix (Thermo Fisher). The recombined plasmid was transformed into Stbl3 competent cells as per the manufacturer's instructions, and DNA

was extracted using the QIAprep Spin Miniprep Kit. VPS54-mAID pLenti clones were verified by restriction analysis. The expression of mVPS54-mAID was validated by transfecting HEK293T cells with the selected pLenti plasmids and performing Western blot (WB) analysis using an anti-myc antibody.

To produce lentiviral particles, HEK293FT cells were co-transfected with equal amounts of lentiviral packaging plasmids (pMD2.G, pRSV-Rev, pMDLg/pRRE) and the mVPS54-mAID pLenti plasmid using Lipofectamine 3000, following the manufacturer's protocol as previously described [36]. hTERT-RPE1 VPS54-KO cells were transduced with the lentivirus expressing mVPS54-mAID. Single-cell clones were isolated by serial dilution, expanded, and validated by WB and immunofluorescence (IF) for stable expression of mVPS54-mAID.

Construction of cells that co-express mVPS54-mAID and OsTIR1 (F74G)-V5

hTERT-RPE1 VPS54-KO cells expressing mVPS54-mAID were transduced with lentiviral AAVS1 CMV-OsTIR1F74G. Briefly, OsTIR1 (F74G)-V5 was amplified using OsTIR1 (F74G)-V5 SAL1 Forward (GAGGTCGACATGACATACTTTCCTGAAGA) and OsTIR1 (F74G)-V5 Kpn1 Reverse (GATGGTACCTCACGTAGAATCGAGACCGA) primers.

OsTIR1 (F74G)-V5 PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) following the standard protocol. To generate OsTIR1 (F74G)-V5 in pENTR1A, the OsTIR1 (F74G)-V5 PCR product was subcloned into the pENTR1A no ccDB (w48-1) entry vector using Sal1 and KpnI restriction sites. The OsTIR1 (F74G)-V5 in pENTR1A was then recombined with the pLenti CMV-Neo-DEST (705-1) vector

under the CMV promoter using Gateway LR Clonase II Enzyme Mix according to the manufacturer's instructions. The OsTIR1 (F74G)-V5 lentiviral particles were prepared as described previously. This lentivirus was used to transduce hTERT-RPE1 VPS54-KO cells expressing mVPS54-mAID. The transduced cells were tested for mVPS54-mAID /OsTIR1 (F74G)-V5 co-expression by WB and IF. Single-cell clones were then isolated by serial dilution, expanded, and characterized. To induce rapid VPS54 depletion in resulting cells, the auxin analog 5-phenyl-indole-3-acetic acid (5-Ph-IAA) (10 µM) was added at various time points. For convenience, auxin analog 5-phenyl-indole-3-acetic acid (5-Ph-IAA) will be named as AA hereafter.

HeLa VPS54-mAID OsTIR1 (F74G)-V5 cells were generated using a slightly different procedure. Briefly, HeLa VPS54-KO cells were co-transfected with AAVS1-Tet-OsTIR1 (F74G)-V5 and AAVS1 T2 CRISPR in pX330. After 48 hours, selection was performed with 2 µg/ml puromycin. Single-cell sorting was conducted to isolate OsTIR1 (F74G)-V5 positive clones. Once these clones were established, they were transduced with mVPS54-mAID lentiviruses. Single-cell clones expressing mVPS54-mAID OsTIR1 (F74G)-V5 were isolated by serial dilution. Expression of OsTIR1 (F74G)-V5 was induced by doxycycline (2 µg/ml) for 24 hours before the experiment. To induce rapid VPS54 depletion in HeLa cells, the auxin analog AA (10 µM) was added at various time points.

Construction of RPE1 cell lines stably expressing MPR-mNeonGreen and mScarlet-GS15

MPR-mNeonGreen-P2A-mScarlet3-GS15-pUC57 construct synthesized by Genescript initially subcloned into pENTR1A using BamH1 and XhoI sites. This construct was then recombined into the pLenti-COG4$_{pr}$-Neo-DEST plasmid, using Gateway LR Clonase II Enzyme Mix (Thermo Fisher). The recombined plasmid was transformed into Stbl3 competent cells as per the manufacturer's instructions, and DNA was extracted using the QIAprep Spin Miniprep Kit. Correct MPR-mNGP2A-mScarlet-GS15 pLenti clones were verified by restriction analysis. The expression of MPR-mNG .and mScarlet-GS15 was validated by WB and IF analysis of transfected HEK293T cells. The MPR-mNG-P2A-mScarlet-GS15 lentivirus was prepared as described previously and RPE1 mVPS54-mAID expressing cells were transduced and sorted for single cell clones.

Preparation of cell lysates and Western blot analysis

For preparation of cell lysates, cells grown on tissue culture dishes were washed twice with PBS and lysed in 2% SDS that was heated for 5 min at 70ºC. Total protein concentration in the cell lysates was measured using the BCA protein assay (Pierce). The protein samples were prepared in 6X SDS sample buffer containing beta-mercaptoethanol and denatured by incubation at 70°C for 10 minutes. 10-30 µg of protein samples were loaded onto Bio-Rad (4-15%) gradient gels or Genescript (8-16%) gradient gels. Gels were transferred onto nitrocellulose membranes using the Thermo Scientific Pierce G2 Fast Blotter. Membranes were rinsed in PBS, blocked in Odyssey blocking buffer (LI-COR) for 20 min, and incubated with primary antibodies overnight at 4°C. Membranes were washed with PBS and incubated with secondary

fluorescently tagged antibodies diluted in Odyssey blocking buffer for 60 min. Blots were then washed and imaged using the Odyssey Imaging System. Images were processed using the LI-COR Image Studio software. Primary and secondary antibodies used in this work are listed in Table 2.

Lectin blotting and staining

To perform blots with fluorescent lectins, 10 µg of cell lysates were loaded onto Bio-Rad (4-15%) gradient gels and run at 160V. Next, proteins were transferred to nitrocellulose membrane using the Thermo Scientific Pierce G2 Fast Blotter. The nitrocellulose membrane was blocked with 3% bovine serum albumin (BSA) for 30 minutes. The lectins Helix Pomatia Agglutinin (HPA) or Galanthus Nivalis Lectin (GNL) conjugated to Alexa 647 fluorophore were diluted 1:1000 in 3% BSA from their stock concentration of 1 µg/µl and 5 µg/µl, respectively. Blots were incubated with lectin solutions for 30 min and then washed in PBS four times for four minutes each and imaged using the Odyssey Imaging System.

Immunofluorescence microscopy

Cells were plated on glass coverslips to 80-90% confluency and fixed with 4% paraformaldehyde (PFA) (freshly made from 16% stock solution) in phosphate-buffered saline (PBS) for 15 minutes at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for one minute followed by treatment with 50 mM ammonium chloride for 5 minutes and washed with PBS. After washing and blocking twice with 1% BSA, 0.1% saponin in PBS for 10 minutes, cells were incubated with primary antibody

(diluted in 1% cold fish gelatin, 0.1% saponin in PBS) for 40 minutes, washed, and incubated with fluorescently conjugated secondary antibodies for 30 minutes. Cells were washed four times with PBS, then coverslips were dipped in PBS and water 10 times each and mounted on glass microscope slides using Prolong® Gold antifade reagent (Life Technologies). Cells were imaged with a 63× oil 1.4 numerical aperture (NA) objective of a LSM880 Zeiss Laser inverted microscope and Airyscan super resolution microscope using ZEN software. Quantitative analysis was performed using single-slice confocal images. All the microscopic images shown are Z-stacked Maximum Intensity Projection images.

Live cell microscopy

Cells were plated on 35 mm glass bottom dishes with No. 1.5 coverglass (MatTek Corporation). Transfection was performed using Lipofectamine 3000. After 16–18 hours, just before imaging, the media was replaced with warm FluoroBrite™ DMEM Media (Gibco, Cat # A1896701) supplemented with 10% FBS. Imaging was conducted on an LSM880 Zeiss inverted microscope equipped with confocal optics, using a 63× oil objective with a 1.4 numerical aperture (NA) and Airyscan. During imaging, the environment was maintained at 37°C, 5% CO2, and 90% humidity.

Cell fractionation

Cells grown to 90% confluency in 15 cm dishes were washed with PBS and collected by trypsinization, followed by centrifugation at 400×g for 5 minutes. The cell pellet was resuspended in 1.5 ml of cell collection solution (0.25 M sucrose in PBS) and

centrifuged again at 400×g for 5 minutes. The pellet was then resuspended in 1.5 ml of hypotonic lysis solution (20 mM HEPES, pH 7.2, with a protein inhibitor cocktail and 1 mM PMSF) and passed through a 25 G needle 20 times to lyse the cells. Cell lysis efficiency was assessed under a phase-contrast microscope. Subsequently, KCl (to a final concentration of 150 mM) and EDTA (to a final concentration of 2 mM) were added. Unlysed cells and nuclei were removed by centrifugation at 1000×g. The post nuclear supernatant (PNS) was transferred to a 1.5 ml Beckman tube, and the Golgi-enriched fraction (P30) was pelleted by centrifugation at 30,000×g for 10 minutes. The supernatant (S30) was then transferred to a new Beckman tube, and the vesicle-enriched fraction was isolated by centrifugation at 100,000×g for 1 hour at 4°C using a TLA-55 rotor.

Vesicle Immunoprecipitation (GS15 IP)

Cells grown to 90% confluency in 15 cm dishes were washed with PBS and collected by trypsinization, followed by centrifugation at 400×g for 5 minutes. The cell pellet was resuspended in 1.5 ml of cell collection solution (0.25 M sucrose in PBS) and centrifuged again at 400×g for 5 minutes. The pellet was then resuspended in 1.5 ml of hypotonic lysis solution (20 mM HEPES, pH 7.2, with a protein inhibitor cocktail and 1 mM PMSF) and passed through a 25 G needle 20 times to lyse the cells. Cell lysis efficiency was assessed under a phase-contrast microscope. Subsequently, KCl (to a final concentration of 150 mM) and EDTA (to a final concentration of 2 mM) were added. Unlysed cells and nuclei were removed by centrifugation at 1000×g. The

postnuclear supernatant (PNS) was transferred to a 1.5 ml Beckman tube, and the Golgi-enriched fraction (P30) was pelleted by centrifugation at 30,000×g for 10 minutes. The supernatant (S30) was transferred to a new tube containing 10 µl of GS15 antibody and incubated at room temperature on a rotating platform for 2 hours. Subsequently, 30 µl of Dyna Protein G magnetic beads (ThermoFisher Scientific #10004D) were added to the tube with the S30 and GS15 antibody mixture. This mixture was rotated at room temperature for an additional 1 hour. The protein bound to the beads were eluted by adding 2x sample buffer with 10% β-mercaptoethanol and heated at 95°C heat block for 5 min.

Secretion assay

hTERT-RPE1-VPS54-mAID expressing cells were plated in three 6-cm dishes and grown to 90-100% confluency. Cells were then rinsed 3 times with PBS and placed in 2 ml serum-free, chemically defined medium (BioWhittaker Pro293a-CDM, Lonza) with 1× GlutaMAX (100× stock, Gibco) added per well for 48 hours. 42 hours post-incubation of cells in serum-free, chemically defined medium, one of the wells was treated with 10 µM of AA and the other well was used as control. After completion of 48 hours incubation, the supernatant was collected and spun down at 3,000xg to remove floating cells. The supernatant was concentrated using a 10k concentrator (Amicon® Ultra 10k, Millipore); final concentration was 10× that of cell lysates.

High-pressure freezing, freeze substitution, and Electron Microscopy

High-pressure freezing/freezing substitution

Sapphire disks were initially coated with a 10 nm carbon layer, followed by a collagen (Corning) coating according to the manufacturer's protocol. The coated disks were sterilized under UV light and transferred into new sterile 3 cm dishes for plating the cells. After the cells reached 80%–100% confluence, they were incubated in fresh media for 2–3 hours at 37°C to equilibrate, then treated with Auxin for 0 hour and 3 hours respectively. High-pressure freezing (HPF) was carried out at designated time points in a cryo-protectant solution (PBS with 2% Type IX ultra-low melt agarose (Sigma-Aldrich), 100 mM D-mannitol, and 2% FBS). This procedure used a Leica EM PACT2 high-pressure freezing unit (Leica Microsystems) equipped with a rapid transfer system, maintaining a high-pressure of 2100 bar. All solutions, bayonets, and sample holders were pre-warmed to 37°C, and every step of the process was performed on a 37°C heating platform to ensure consistent temperature control.

Freeze substitution dehydration

Samples were transferred under liquid nitrogen into cryovials containing anhydrous acetone with 2% osmium tetroxide (OsO4), 0.1% glutaraldehyde, and 1% double-distilled (dd) H_2O. The cryovials were then placed into a freeze-substitution chamber set at −90°C and subjected to the following schedule: maintained at −90°C for 22 hours, warmed at 3°C per hour to −60°C, held at −60°C for 8 hours, then warmed at 3°C per hour to −30°C, and kept at −30°C for 8 hours before warming to 0°C. Afterward, the samples were placed on ice and transferred to a cold room set at 4°C. Followisng three washes with acetone, the samples were stained with a solution of 1% tannic acid and 1% ddH_2O in acetone on ice for 1 hour, followed by another three acetone washes.

Next, the samples were stained with a 1% OsO4 and 1% ddH_2O solution in acetone on ice for 1 hour. Afterward, they were washed three times for 10 minutes each in acetone and dehydrated through a graded ethanol series (25%, 50%, 75%, and 100%) using automatic resin infiltration. protocol for PELCO Bio-Wave Pro laboratory microwave system. Samples were embedded in Araldite 502/Embed 812 resins with a DMP-30 activator and baked at 60°C for 48□h.

Thin section TEM

Thin sections, 50 nm in thickness, were cut using a Leica UltraCut-UCT microtome and subsequently post-stained with aqueous uranyl acetate and Reynold's lead citrate (EMS).

Electron microscopy and image handling

Images were taken using an FEI Tecnai TF20 intermediate-voltage electron microscope operated at 80□keV (FEI Co.). The images were acquired with an FEI Eagle 4□k digital camera controlled with FEI software.

Colocalization analysis

Pearson's correlation coefficient was calculated using "Colocalization" module of Zen Blue software. The colocalization between different proteins was recorded and the graph was made using GraphPad Prism 9.3.0. At least 30 cells were used for quantification of Golgi area per group and Pearson's correlation coefficient was measured.

Statistical analysis

All results are representative of at least 3 independent experiments. Western blot images are representative from 3 repeats. Western blots were quantified by densitometry using the LI-COR Image Studio software. Error bars for all graphs represent standard deviation. Statistical analysis was done using one-way ANOVA, two-way ANOVA or paired t test using GraphPad Prism software.

Table 1. List of plasmids used in the study

Plasmid Name	**Source**	**Citation**
AAVS1-T2-CRISPR in pX330	Addgene #72833	[37]
AAVS1-CMV-OsTIR1F74G	Addgene #140536	[37]
AAVS1-Tet-OsTIR1(F74G)-V5	Addgene #158664	[37]
LAMP2-GFP	Santiago Di Pietro	[41]
Lenti-Cas-9 blast	Addgene #52962	[42]
M6PRmNG-P2A-mScarlet3-GS15	This study	

in pLenti-COG4$_{pr}$- Neo DEST		
MK289 (mAID-mClover-NeoR)	Addgene #72827	[43]
mVps54-13myc in pLenti-COG4$_{pr}$ - Neo DEST	This study	
mVPS54-13myc-mAID-mClover in pLenti-COG4$_{pr}$-Neo DEST	This study	
OsTIR1 (F74G)-V5 pLenti CMV Neo DEST (705-1)	Farhana Sumya	This study
pCl-neo-VPS54-13myc	Juan Bonifacino	[31]
pENTR1A no ccDB (w48-1)	Addgene #17398	[44]
PLenti-CMV-Neo-DEST (705-1)	Addgene #17392	[44]
PLenti-COG4$_{pr}$-Neo DEST (705-1)	Irina Pokrovskaya	
pMD2.G	Addgene #12259	[45]

pMDLg/pRRE	Addgene #12251	[45]
pRSV-Rev	Addgene #12253	[45]

Table 2. List of primary and secondary antibodies

Antibody	**Source/Catalog #**	**Species**	**WB dilution**	**IF dilution**
ATP7A	Santa Cruz #Sc-376467	Mouse	1:500	1:300
β-actin	Sigma, #A5441	Mouse	1:1000	-
B4GALT1	R&D Systems, AF-3609	Goat	1:500	1:300
BET1L/GS15	BD Biosciences #610961	Mouse	-	1:500
BET1L/GS15	This lab	Rabbit	-	1:500
BIG1	Santa Cruz #sc-50391	Rabbit	-	1:300
C1GALT1	Santa Cruz	Mouse	1:500	1:300

	# SC100745			
Carboxypeptidase D/CPD	Kerafast # EB5001	Rabbit	1:1000	1:500
Cathepsin D/CATD	Sigma, #C0715	Mouse	1:500	-
CD-M6PR/MPR	Santa Cruz # sc365196	Mouse	1:1000	-
CD-M6PR/MPR	Abcam # AB134153	Rabbit	-	1:1000
CI-M6PR/IGF2R	Abcam # AB124767	Rabbit	1:2000	1:1000
COPB2	ABclonal, # A7036	Rabbit	-	1:400
GALNT2	Thermo Fisher # PA521541	Rabbit	1:1000	-
GBF1	BD transduction laboratories #612116	Mouse	-	1:500
GM130/GOLGA2	BD Biosciences, # 610823	Mouse	-	1:500
GM130/GOLGA2	CalBiochem, # CB1008	Rabbit	-	1:300
GOSR1/GS28	BD Biosciences # 611184	Mouse	1:500	1:500
MGAT1	Abcam, # ab180578	Rabbit	1:500	-

MYC	Bethyl #A190-105A	Rabbit	1:2000	1:1000
MYC-Tag	Cell signaling #2276	Mouse	1:1000	1:500
STX5	Santa Cruz # sc-365124	Mouse	-	1:100
STX6	R&D Systems, #AF5664-SP	Sheep	1:1000	1:400
STX10	ProteinTech #11036-I-AP	Rabbit	1:1000	-
STX16	Abcam #AB134945	Rabbit	1:2000	1:1000
TGN46/TGOLN2	Bio-Rad, #AHP500G	Sheep	1:2000	-
TMEM87A	Novus #NBP1-90532	Rabbit	1:1000	1:400
VAMP4	Synaptic Sys # 136-002	Rabbit	1:500	-
VPS51	Sigma # HPA061447	Rabbit	1:1000	-
VPS52	Juan Bonifacino	Rabbit	1:1000	-
VPS53	Thermo Fisher, #PA520548	Rabbit	1:1000	-
VPS53	Santa Cruz	Mouse	1:500	-

	# sc514920			
VPS54	St John's Lab, # STJ115181	Rabbit	1:1000	-
VTI1A	BD # 611220	Mouse	1:500	1:300
IRDye 680 anti-Mouse	LiCOR/926-68170	Goat	1:40000	-
IRDye 800 anti-Rabbit	LiCOR/926-32211	Goat	1:40000	-
IRDye 800 anti-Goat	LiCOR/926-32214	Donkey	1:40000	-
Alexa Fluor 647 anti-Rabbit	Jackson Immuno Research/711-605-152	Donkey	1:500	1:1000
Alexa Fluor 647 anti-Mouse	Jackson Immuno Research/715-605-151	Donkey	1:500	1:1000
Alexa Fluor 647 anti-Goat	Jackson Immuno Research/ 705-605-147	Donkey	-	1:1000
DyLight 647 anti-Sheep	Jackson Immuno Research/713-605-147	Donkey	-	1:1000
Cy3-anti-Rabbit	Jackson Immuno Research/711-165-152	Donkey	-	1:1000
Cy3-anti-Mouse	Jackson Immuno Research/715-165-151	Donkey	1:500	1:1000
Alexa Fluor 488	Jackson Immuno	Donkey	1:500	1:1000

anti-Rabbit	Research/711-545-152			
Alexa Fluor 488 anti-Mouse	Jackson Immuno Research/715-545-151	Donkey	1:500	1:1000

Results

Development and validation of rapid GARP inactivation system

Previous investigation of hTERT-RPE1 GARP-KO cells [36] [46] [35] revealed that VPS54-KO specifically inactivates GARP complex resulting in dramatic changes in Golgi structure and physiology. To uncover primary defects associated with GARP dysfunction, an auxin-inducible degron version 2 (AID2) system [37] was utilized. VPS54, the unique subunit of the GARP complex, was tagged with plant degron mAID and stably expressed under the control of COG4 promoter region [47] in the VPS54-KO cells to achieve near-endogenous level of expression. The constructed cellular system also expressed auxin receptor OsTIR1 (F74G) mutant that in the presence of auxin homolog 5-phenyl-indole-3-acetic acid (AA) should form a complex with mAID, directing hybrid protein for poly-ubiquitination and proteasomal degradation. First, we tested the functionality of VPS54-mAID protein by western blot (WB) (Figure 1 A, B) and immunofluorescence microscopy (IF) analysis (Figure 1C). We found that a decrease in total protein abundance of TGN46/TGOLN2, B4GALT1, and GS15/BET1L observed in VPS54-KO cells was restored upon the expression of VPS54-mAID (Figure 1C). Furthermore, a decrease in colocalization of TGN46 (Figure 1D) and GS15 (Figure 1E) with the *trans*-Golgi marker P230/GOLGA4 in VPS54-KO cells was rescued in VPS54-KO cells expressing VPS54-mAID. A similar functionality test of VPS54-mAID in HeLa

VPS54-KO cells revealed that proper Golgi localization of TGN46, GBF1, and COPB2 was restored upon expression of VPS54-mAID (Figure S1A-C). Hence, the VPS54-mAID construct is functional.

Acute depletion of VPS54 does not affect the protein abundance of its protein partners.

Once we confirmed that the cells expressing VPS54-mAID could rescue the VPS54-KO defects, we next aimed to induce the rapid depletion of VPS54 by treating the cells with 5-Ph-IAA (AA, Figure 2A). We tested the efficiency of VPS54 depletion by treating the cells with AA for 0, 0.5, 1, 2, and 3 h, respectively. WB and IF analysis demonstrated that approximately 70 % of the VPS54 was depleted in 30 minutes, and in 3 hours, almost all VPS54 was degraded (Figure 2B-C). Prolonged (24-48 h) treatment with AA resulted in a continuous degradation/depletion of VPS54-mAID (data not shown). A similar rapid depletion of VPS54 was observed in HeLa VPS54-KO cells expressing VPS54-mAID (Figure S2A). IF analysis confirmed a complete depletion of VPS54-mAID in the Golgi in AA-treated cells (Figure S2B). We next examined if the depletion of VPS54 can lead to the degradation of other GARP subunits. In agreement with the data obtained with VPS54-KO cells (unpublished data), the total protein abundance of VPS51, VPS52, and VPS53 remains mostly unchanged in cells acutely depleted for VPS54 (Figure 2D-F). VPS51, VPS52, and VPS53 are the shared subunits of GARP and EARP complex. Their unchanged expression indicates that rapid VPS54 depletion has not resulted in destabilization and degradation of the EARP complex.

Acute depletion of VPS54 alters the protein abundance and localization of a subset of TGN proteins.

The GARP is believed to tether the endosome-derived vesicles at the TGN. Several TGN resident proteins, including TGN46/TGOLN2, ATP7A, TMEM87A, CPD, and mannose-6-phosphate receptors, are known to cycle between the endosomes and Golgi [48] [32].

TGN46 is a single-pass type I transmembrane protein believed to function as a receptor for secretory cargoes [49]. TGN46 is localized to the TGN in a steady state, it cycles between the TGN, endosomes, and the PM [50] [51] [52] [53] [54]. Since the TGN46 was significantly depleted in GARP-KO cells [36], we reasoned that TGN46 instability could be the primary defect of GARP dysfunction. Indeed, we found that TGN46 was significantly depleted within 3 hours of the induction of VPS54 degradation (Figure 3A). In addition, in VPS54 depleted cells, TGN46 was significantly mislocalized from the Golgi to punctate peripheral structures. TGN46 mislocalization was specific since the localization of golgins GM130/GOLGA2 and P230/GOLGA4 was unaffected by GARP dysfunction (Figure 3B, E). Indeed, VPS54 depletion resulted in a significant decrease in colocalization of TGN46 with P230 (Figure 3C). We reasoned that following the rapid degradation of VPS54, the TGN46 rerouted to endolysosomes and is subjected to lysosomal degradation. In support of this model, treatment of VPS54-depleted cells with lysosomal protease inhibitor (PI) resulted in partial restoration of TGN46 expression (Figure S3A). Furthermore, co-transfection of VPS54 depleted cells with rat homolog of TGN46, TGN38-GFP, and endolysosomal marker Lamp2-mCherry resulted in partial colocalization of TGN38 with lysosomes (Figure S3B).

Menkes proteins (also known as ATP7A/B) are integral to the mammalian copper transport system, cycling continuously between the Golgi complex and the plasma membrane [55] [56]. VPS54-KO resulted in mislocalization of ATP7A that was reversed by expression of VPS54-mAID, indicating that ATP7A cycling is GARP-dependent (Figure S3C). Indeed, the total protein abundance of ATP7A was significantly decreased within 3 hours of the induction of VPS54 degradation (Figure 3D). Consistent with ATP7A mislocalization in GARP-KO cells, acute depletion of VPS54 also altered Golgi localization of ATP7A (Figure 3E, F). The internal environment of the Golgi is slightly acidic at pH 6.0-6.7 and is maintained by ion channels such as Golgi-pH-regulating cation channel GolpHCat/TMEM87A [57]. We have discovered a significant decrease in the total protein level of TMEM87A in VPS54-depleted cells, indicating that TMEM87A recycling depends on GARP function (Figure 3G). In agreement to TMEM87A sensitivity in GARP-KO cells, the Golgi localization of TMEM87A and protein stability was significantly decreased in cells acutely depleted for VPS54 (Figure 3H-I).

Carboxypeptidase D/CPD, a transmembrane TGN enzyme is known to recycle through endosomes and the plasma membrane [58]. IF analysis of CPD localization in wild-type and VPS54-KO RPE1 cells confirmed its Golgi localization and revealed a decrease in Golgi staining in GARP-KO cells (Figure S3D). CPD stability and localization were significantly affected in cells acutely depleted for VPS54 (Figure 3J-L). Hence, the expression and localization of four TGN transmembrane proteins was specifically altered upon rapid GARP inactivation.

Rapid VPS54 depletion causes Cathepsin D sorting defects and enhances fibronectin secretion, without significant alterations in stability or localization of mannose-6-phosphate receptors.

MPRs are crucial for transporting lysosomal enzymes, like Cathepsin D, from the Golgi to the endosomes and then to the lysosomes [59]. There are two types of MPRs: cation-dependent MPR (CD-MPR/MPR) and cation-independent MPR (CI-MPR/IGF2R) [60] [61] [48]. After delivering their cargo, MPRs are recycled back to the Golgi for subsequent rounds of enzyme transport, and GARP is expected to be a part of the recycling machinery for MPRs [11]. Our previous work on GARP-KO cells showed an increase in the secretion of pro-Cathepsin D [36]. In agreement with the data in VPS54-KO cells, we observed a significant increase in the secretion of pro-Cathepsin D from cells acutely depleted for VPS54 (Figure 4A). At the same time, no changes in intracellular mature Cathepsin D or its precursor were observed (Figure 4B). Interestingly, pro-Cathepsin D secretion was accompanied by the increased secretion of Fibronectin/FN1 (Figure 4C), indicating a general dysfunction of TGN protein sorting machinery in cells acutely depleted for VPS54. We further investigated if GARP dysfunction stimulates the fibronectin release or if this is a result of protein overproduction and found that the intracellular fibronectin level remains unchanged (Figure 4D). These results collectively indicate that VPS54 acute depletion leads to TGN sorting defects.

Previous investigation of MPRs localization in HeLa cells suggested that siRNA depletion of VPS52 resulted in “accumulation of recycling MPRs in a population of light, small vesicles downstream of endosomes” [11]. To test if this is the case in cells rapidly

depleted for VPS54, the stability and localization of CD-MPR and CI-MPR was tested. Total protein level of CI-MPR and its Golgi localization were not significantly changed between control and VPS54 depleted cells (Figure 4E-G), indicating that the trafficking pathway and/or machinery of CI-MPR are different from other TGN transmembrane proteins. However, the protein level of CD-MPR was significantly decreased after acute VPS54 depletion (Figure 4H), and a visible increase in vesicle-like haze around the Golgi was observed within 3 h of the induction of VPS54 degradation (Figure 4I). Surprisingly, the colocalization of CD-MPR to TGN golgin P230 in VPS54 depleted cells was increased (Figure 4J), indicating that CD-MPR react to GARP depletion differently than other TGN resident proteins. The data suggests that the missorting of cathepsin D in GARP-depleted cells is mostly unrelated to the mistargeting of MPRs.

Acute GARP dysfunction affects a subset of Golgi enzymes and results in O-glycosylation defects.

Each Golgi cisterna houses a specific set of different Golgi enzymes, ion channels, pH sensors, and transporters [62] [63] [64] [65] [66]. The Golgi enzymes catalyze the addition or removal of sugars to/from cargo glycoproteins and the addition of sulfate and phosphate groups [67]. Our previous study revealed that several tested Golgi enzymes, including B4GALT1, MGAT1, and GALNT2 were significantly depleted in GARP-KO cells [36]. Since these enzymes localize in different Golgi sub-compartments, we aim to determine if the decrease in their expression is the primary or secondary defect associated with VPS54 depletion. We observed that the reduction in protein level of B4GALT1 occurs only after a prolonged VPS54 depletion, indicating that this is not the

immediate effect of GARP dysfunction (Figure 5A). Likewise, we observed no change in Golgi localization of B4GALT1 in cells acutely depleted for VPS54 (Figure 5B-C). Similar results were obtained with MGAT1 and GALNT2 (Figure S5A-D), suggesting that reduced expression of Golgi enzymes is an indirect consequence of GARP depletion. However, GARP acute depletion affected B4GALT1 localization to some extent since the localization of this enzyme became more sensitive to changes in Golgi pH induced by the chloroquine treatment (Figure S6), indicating that GARP activity is needed for proper *trans*-Golgi homeostasis.

In agreement with the proposed GARP-related trans-Golgi dysfunction, we observed a significant decrease in the expression of another enzyme, C1GALT1, within 3 h of the induction of VPS54 degradation (Figure 5D). Consistent with this, there was a decrease in colocalization of C1GALT1 with GM130 (Figure 5E-F). We reasoned that C1GALT1 misocalization/degradation could potentially lead to a specific defect in O-glycosylation. C1GALT1 transfers galactose from UDP-galactose to Tn antigen (GalNAcα1-O-Ser/Thr) to form core 1 *O*-glycan structure, T antigen. This step is critical for the biosynthesis of complex *O*-glycans [68]. *Helix pomatia* agglutin (HPA) binds to Tn antigen. Testing total cellular lysates in cells acutely depleted for VPS54 with HPA-647 lectin detected a significant increase in HPA-647 binding to several protein bands as early as 6 h after the induction of VPS54 degradation (Figure 5G), confirming C1GALT1 partial dysfunction. *O*-glycosylation abnormalities progressively increased upon a prolonged (16-48 hours) depletion of VPS54. GARP-associated *O*-glycosylation defect was specific since GNL-647 blot did not detect any abnormalities in proteins extracted from

VPS54 acutely depleted cells even after a prolong AA treatment (Figure 5H), indicating that N-glycosylation defects are not a primary manifestation of GARP dysfunction.

GS15 is the Golgi SNARE that depends on GARP activity.

SNAREs promote the fusion of vesicles containing cargo to their target membrane compartment. Once the TGN-derived transport vesicles get fused to the endosomal compartment, the SNAREs must return back to the TGN as a normal process of recycling. Qc SNARE GS15/BET1L is shown to have increasing concentrations across the cisternae toward the *trans*-Golgi [69]. GS15 is believed to cycle via the endosomes, as it was found to be trapped in endosomes when endosome to Golgi recycling is disrupted [70]. In our study of VPS54-KO cells, we observed a significant decrease in total protein level and Golgi localization of GS15 [36]. We wondered if GS15 is sensitive to the rapid VPS54 degradation. Indeed, after 3 hours of VPS54 degradation induction, we observed that GS15 is mislocalized from the Golgi (Figure 6B-C). GS15 mislocalization led to significant depletion of GS15 protein (Figure 6A), indicating that Qc SNARE mislocalization and consequent degradation is a primary defect of GARP dysfunction. GS28/GOSR1 is a partner of GS15 in the STX5/GOSR1/BET1L/YKT6 SNARE complex and GS28 depletion led to GS15 instability [71]. Interestingly, we observed that GS28 protein stability or localization was not significantly altered upon VPS54 acute depletion, and its expression was decreased only after 48 h of GARP malfunction (Figure 6D and data not shown), indicating that GS15 relies on GARP function independently of its SNARE partner. Moreover, the stability of another GS15 SNARE partner, Qa SNARE STX5, was insensitive to GARP dysfunction (Figure 6F).

GARP was shown to regulate the formation and/or stability of TGN STX16/STX6/VTI1A/VAMP4 SNARE complex [22]. Surprisingly, we found that the expression of STX6, VTI1A, VAMP4, and STX10 remained unaffected by VPS54 degradation (Figure 6F). This data indicates that GS15 is the unique Golgi SNARE protein, which relies on GARP for its localization and stability.

Acute VPS54 depletion mislocalizes vesicular adaptor proteins and COPI coats

Previous investigation of GARP-KO cells revealed that several Golgi-located vesicular coats, including AP1, GGA, and COPI, were mislocalized to the cytosol and peripheral membranes. Coat binding to the Golgi membrane requires activation of ARF GTPases, facilitated by ARFGEF proteins GBF1, BIG1/ARFGEF1, and BIG2/ARFGEF2 [72]. In GARP-KO cells, ARFGEFs were mislocated from the Golgi ribbon to the cytosol and endolysosomal compartment [46]. To test if the Golgi coat localization defect is a primary manifestation of GARP malfunction, localization of β-adaptin (Figure 7A), GGA2 (Figure 7B), COPB2 (Figure 7C), and GBF1 (Figure 7D) was determined in cells acutely depleted for VPS54. Co-localization analysis revealed that 3 h of VPS54 depletion was sufficient for significant alterations in localization of all three coats and GBF1. In contrast, the localization of BIG1 was unchanged (Figure S7), suggesting that GBF1 mislocalization could be a primary defect that led to the malfunction of Golgi vesicular coats.

Rapid VPS54 depletion causes accumulation of GARP-dependent vesicles

Previous electron microscopy analysis of VPS54-KO cells revealed significant structural alterations in the Golgi complex, including swollen and partially fragmented cisternae.

Notably, there was no substantial accumulation of small trafficking intermediates in GARP-KO cells, raising questions about GARP's role as a vesicle tether [46]. To further investigate, we employed high-pressure freezing (HPF) and freeze substitution (FS) sample preparation for transmission electron microscopy (TEM) to identify early morphological changes in VPS54-depleted RPE1 cells. Our analysis revealed a significant increase in small vesicle-like structures near the Golgi (Figure 8A, B), supporting the role of GARP in vesicle tethering and suggesting that vesicle accumulation is a transient phenotype of GARP complex dysfunction. Stalled GARP-dependent vesicles are likely to be cleared by autophagy, since we observed a number of autophagosomes in the Golgi area of VPS54 depleted cells. Some of these autophagosomes were filled with vesicle-like structures (Figure 8A, right panel shown by asterisk). Additionally, analysis of TEM images revealed the presence of large, round structures (0.2–0.6 microns in diameter) in the Golgi area. Electron-dense material frequently accumulated on one side of this organelle, possibly indicating the aggregation of lumenal cargo. The remainder of the Golgi stack appeared intact and not fragmented, indicating that the swollen Golgi in GARP-KO cells is likely a secondary manifestation of GARP dysfunction (Figure 8A). We hypothesized that the round structures represent altered TGN or enlarged late endosomal compartments resulting from depletion of TGN46 or other components of endosome-to-TGN recycling machinery. If this is the case, the enlarged structures must carry endosome-to-TGN receptors, like MPRs, which are known to recycle through this pathway [11].

To investigate whether GARP dysfunction leads to the accumulation of MPR in enlarged structures, we used VPS54-mAID cells stably expressing MPR-NeonGreen. Live-cell

imaging of VPS54-depleted cells revealed the presence of MPR-NeonGreen signal in Golgi membranes, small vesicles, and large round organelles, similar to round structures observed by EM (Movie 1). We concluded that the accumulation of enlarged structures and small vesicles are primary defects associated with GARP dysfunction. To better understand the nature of GARP-dependent vesicles, RPE1 VPS54-mAID cells were mechanically disrupted and fractionated through differential centrifugation to separate Golgi membranes (P30) and vesicles (P100) (Figure 8C). We analyzed the distribution of three categories of proteins: endosome-TGN cycling receptors, Golgi enzymes, and Golgi SNAREs. Western blot analysis revealed that acute GARP inactivation led to the redistribution of several trans-Golgi/TGN proteins, including CD-MPR (Figure 8D), CPD (Figure 8E), GS15 (Figure 8F), STX10 (Figure 8F), and STX6 (Figure 8F), into the vesicular fraction. Notably, the v-SNARE GS15 showed a significant increase in the vesicular pool following rapid GARP depletion (Figure 8F; Figure S8A), prompting us to use it to isolate the vesicles containing GS15 by native immunoprecipitation (Figure S8B). Western blot analysis demonstrated a 2.5-fold increase in GS15 protein in pull-down vesicles after acute GARP depletion (Figure S8C), while TGN46 levels decreased significantly (Figure S8D). A significant decrease in TGN46 signal in GS15 vesicles isolated from GARP-depleted cells likely indicates that the recycling pathways of TGN46 and GS15 are differently affected by VPS54 depletion (Figure S8D).

WB analysis of several Golgi enzymes, including B4GALT1, MGAT1, and GALNT2, did not revealed any significant changes in their abundance in GS15-positive vesicles isolated before and after VPS54 acute depletion (data not shown), but another Golgi

enzyme, C1GALT1 was notably enriched in GARP-dependent vesicles, suggesting that its mislocalization contributes to the *O*-glycosylation defects observed in VPS54-depleted cells (Figure S8E). Additionally, the endosome-TGN SNAREs STX6 (Figure S8F) also showed a significant increase in GARP-dependent GS15-positive vesicles, despite no overall change in its total protein levels (Figure S8F).

Overall, the analysis of human cells acutely depleted for VPS54 revealed a marked increase in GS15-positive vesicles containing a subset of Golgi recycling proteins, highlighting a specific role for the GARP complex in the Golgi-endosomal trafficking cycle.

Discussion

In this study, we have uncovered the immediate defects associated with GARP dysfunction and differentiated between the primary and secondary defects observed in previous studies of GARP knock-out and knock-down mammalian cells [36] [46] [3] [11] [22]. We discovered that the mislocalization of vesicle coat proteins, increase in number of GARP-dependent vesicles, alteration of *trans*-Golgi morphology, decrease in stability and mislocalization of a subset of endosome-to-TGN cycling proteins, and *O*-glycosylation as primary defects of GARP dysfunction (Figure 9).

While the degron activation resulted in a rapid depletion of VPS54 it did not change the total protein abundance of other subunits of GARP, supporting the notion that the EARP complex that is sharing VPS51, VPS52, and VPS53 with GARP is significantly more abundant in mammalian cells [73]. In the future, it will be important to construct and test

cells acutely depleted of both shared and unique EARP subunits to determine the specific roles of each complex in the TGN/endolysosomal trafficking cycle.

Mislocalization of three distinct protein coat complexes (COPI, AP-1, and GGA) was observed as one of the earliest phenotypes following VPS54 depletion. The dissociation of these coat complexes from the Golgi membrane is likely due to a reduction in ARF1-GTP levels, which are essential for the membrane association of all three vesicular coats [74]. This decrease in ARF1-GTP is likely caused by the mislocalization of the ARFGEF protein GBF1. It is intriguing that a malfunction in a TGN-localized trafficking factor primarily affects cis-medial GBF1 before influencing the *trans*-Golgi ARFGEF BIG1. GARP has been shown to be critical for cellular sphingolipid homeostasis [75], which may, in turn, influence phosphoinositide turnover through lipid exchange mechanisms at endoplasmic reticulum/TGN contact sites [76]. Since GBF1 binds to phosphoinositides, particularly PI3P, PI4P, and PI(4,5)P2, for membrane association [77], one possibility is that GARP depletion acutely disrupts the balance of Golgi phosphoinositides, thereby affecting GBF1 membrane binding. Another potential explanation for coat mislocalization could be the transient accumulation of non-tethered GARP-dependent vesicles.

Previous analysis of HeLa cells detected accumulation of CI-MPR in vesicle-like cytoplasmic staining and in a light membrane fraction in VPS52-RNAi-depleted cells [11], suggesting the buildup of small trafficking carriers. However, more recent EM studies of GARP knockout cells did not detect vesicle accumulation [46], raising questions about the role of GARP in vesicle tethering. Both RNAi and CRISPR/Cas9 KO techniques require several days for protein depletion, which could introduce artifacts

or allow cellular adaptation to the loss of the target protein. The detection of GARP-dependent vesicles by EM in cells acutely depleted for VSP54, along with the identification of specific cargo proteins associated with these trafficking carriers, provides the first direct experimental evidence supporting GARP's role as a vesicular tether. EM data also suggested that accumulated GARP-dependent vesicles are getting removed by autophagy, resolving the discrepancy between phenotypes in acutely depleted and GARP KO cells.

What is the protein cargo of GARP-dependent transport carriers? Our analysis identified several transmembrane Golgi resident proteins whose abundance and/or localization were significantly affected by acute VPS54 depletion. The list includes TGN46, ATP7A, TMEM87A, CPD, CD-MPR, C1GALT1, GS15, STX6 and STX10. Consistent with previous reports in VPS54-KO cells, the TGN46 protein was highly sensitive to VPS54 depletion. We found that TGN46 began to mislocalize from the Golgi into punctate structures within one hour of inducing VPS54 degradation (unpublished data), making TGN46 the fastest responder to VPS54 loss. This suggests that TGN46 may cycle between the TGN and endolysosomal compartments at a rapid rate, and GARP dysfunction quickly leads to its degradation in lysosomes. Supporting this hypothesis, pretreatment of GARP deficient cells with protease inhibitors partially rescued TGN46 expression. Although TGN46 was detected in immunoprecipitated GS15 vesicles, GARP malfunction did not lead to accumulation of TGN46 in GS15 carriers, indicating that the trafficking itinerary of this putative cargo receptor is distinct from that of GS15. Future investigations using fluorescently tagged TGN46 in cells acutely depleted for

GARP and monitoring transport carriers via live microscopy, should help clarify this issue.

ATP7A, also known as Menke's protein is predominantly localized at the TGN and is responsible for regulation of copper homeostasis in the cell [6] [78] [79] [80] [81]. During steady state, ATP7A is in the Golgi but when the cellular copper level is high, ATP7A regulates the intracellular copper levels ATP7A's localization from the TGN to the plasma membrane of cells [82]. A study by Heather et al., showed that ATP7A interacts with the COG complex [83] and the ablation of the COG complex downregulated ATP7A in mammalian cells [84], indicating rapid intra-Golgi recycling of copper transporter. ATP7A has also been shown to physically interact with AP-1 coat complex and that AP-1 regulates ATP7A localization under basal copper concentrations [85]. AP-1 is preferentially regulating endosome to Golgi retrograde trafficking [86] and it is likely that GARP depletion blocks constitutive endosome/Golgi recycling of ATP7A leading to its mistargeting and degradation. Another GARP sensitive protein, TMEM87A appears to play a critical role in maintaining Golgi pH, and its knockout in mice lead to Golgi fragmentation and altered protein glycosylation [57] [87]. Furthermore, overexpression of TMEM87A in VPS54-KO cells partially restored retrograde transport from endosomes to the TGN [3]. Notably, GARP-deficient cells became hypersensitive to chloroquine treatment that elevated Golgi pH. The specific mislocalization of C1GALT1 enzyme, along with TMEM87A depletion-related changes in Golgi pH, may contribute to *O*-glycosylation defects in VPS54 depleted cells.

Interestingly, in addition to GARP, TMEM87A localization depends on *trans*-Golgi coiled-coil tethers (golgins) Golgin-97/GOLGA1, Golgin-245/GOLGA4, and

GCC88/GCC1 and TMEM87A-containing vesicles can be relocalized to mitochondria coated with one of these tethers [20]. It remains currently unclear whether TGN golgins and GARP regulate distinct or overlapping trafficking pathways. On one hand, golgin-decorated mitochondria can attract trafficking intermediates carrying CI-MPR [20], a receptor unaffected by acute VPS54 depletion, suggesting that GARP and golgins may be involved in tethering different membrane carriers. On the other hand, both GARP and TGN golgin membrane recruitment is regulated by the same small GTPase, ARFRP1 [31], and our preliminary data show a very close spatial proximity between golgins and GARP (data not shown), pointing to a possible coordination between the two. Future investigations into membrane trafficking in cells deficient in both golgins and GARP will help clarify whether there is redundancy within the TGN tethering machinery [88].

Our data indicated that acute depletion of VPS54 specifically affected the sorting of the lysosomal enzyme cathepsin D, resulting in increased secretion of its precursor. However, we observed that the localization of CI-MPR remained unchanged, while CD-MPR mostly stayed in the Golgi proper, challenging the notion that GARP is directly involved in the trafficking of MPR proteins in human cells. One explanation of the microscopy data is that the tethering of MPR-carrying intermediates is primarily mediated by TGN golgins [20], but biochemical data suggesting a more complex scenario. Cellular fractionation of VPS54-depleted cells showed that some CD-MPR, but not CI-MPR, was redistributed to the GARP-dependent vesicle fraction, indicating that the two mannose-6-phosphate receptors may follow different TGN-endosome pathways. It is plausable that the partial mislocalization of CD-MPR, along with potential

changes in TGN acidity due to the mislocalization of TMEM87A, could be a primary cause of the missorting of pro-cathepsin D.

Interestingly, we found VPS54 acute depletion also affected the secretion of fibronectin (FN1). The increase in secretion of FN1 was not due to the increase in its expression. One of the reasons for the increase secretion of FN1 could be related to the altered pH and/or morphology of the TGN. A study by Mayuko *et. al.* showed that cells with altered Golgi morphology stimulate the transport of secretory alkaline phosphatase [89], suggesting the importance of Golgi morphology and GARP machinery in controlling the rate and quality of protein secretion. This potential GARP function is in agreement with the recently discovered AP-1 driven cycling of secretory cargo in yeast cells [90].

How many types of trafficking intermediates are regulated by GARP complex? Initial characterization of GS15-containing vesicles revealed accumulation of three GARP-dependent proteins, Golgi enzyme C1GALT1 and two SNAREs GS15 and STX6. However, the abundance of TGN46 was significantly decreased in GS15-containing carriers isolated from VPS54 deprived cells, indicating that at least some of TGN46 is returned from post-Golgi compartments by different carriers.

The exact composition and distribution of vesicle fusion machinery regulated by GARP is another question. Qc SNARE GS15 is accumulated in GARP-dependent trafficking intermediates, but GS15-KO results in phenotypes much milder than VPS54-KO [46] [71] and GARP is not known to regulate SNARE GS15-containing complexes. Instead, GARP is predicted to regulate STX16/STX6/VTI1A/VAMP4 SNARE complex [22], but, intriguingly, STX16 SNARE assembly is also regulated by COG vesicle tethering complex [91]. Moreover, VPS54 acutely depleted cells did not show any mislocalization

or degradation of R-SNARE VAMP4, while Qa–SNARE STX16 is accumulated in GS15-independent vesicle carriers (data not shown). Future proteomic analysis of GARP-dependent trafficking intermediates should clarify these important questions.

In summary, analysis of human cells acutely depleted for VPS54 revealed key cellular defects linked to GARP dysfunction (Table 3). Future proteomic studies on GARP-dependent trafficking intermediates, combined with proximity labeling and in vitro methods, will enhance our understanding of GARP's role in membrane trafficking.

Table 3. Golgi proteins affected by the acute and prolong GARP depletion

	Early defects (up to 6 h)	Late defects (after 6 h of depletion and KO)
TGN proteins	TGN46 ATP7A TMEM87A CPD	
Golgi SNAREs	GS15/BET1L	GS28/GOSR1
Golgi enzymes	C1GALT1	B4GALT1 MGAT1 GALNT2
Calcium homeostasis maintenance proteins		SDF4 ATP2C1
ARFGEFs	GBF1	BIG1
Coats/adaptors/accessory proteins	β-adaptin GGA2 COPB2	

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Figure legends

Figure 1: Expression of VPS54-mAID rescues VPS54-KO defects. (A) Western blot (WB) analysis of RPE1 cell lysates from wild-type (WT), VPS54 knock out (VPS54-KO), and VPS54-KO cells rescued with VPS54-mAID. Blots were probed with anti-myc (to detect VPS54-myc-mAID) and anti-β-actin antibodies. (B) WB analysis of RPE1 cell lysates from WT, VPS54-KO, and VPS54-mAID, probed with anti-TGN46, anti-B4GALT1, and anti-GS15 antibodies. β-actin was used as a loading control. (C) Confocal microscopy images of WT, VPS54-KO, and VPS54-mAID RPE1 probed for TGN46, GS15, and P230. (D) Quantification of IF images in (C). Pearson's correlation coefficient was used to assess the colocalization of TGN46 and P230. (E) Pearson's correlation coefficient was used to assess the colocalization of GS15 and P230. At least

50 cells were analyzed per sample for the quantification. Statistical significance was determined using one-way ANOVA. ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2: VPS54-mAID rapid depletion does not affect the stability of other GARP subunits. (A) Diagram illustrating the cellular setup for the rapid depletion of VPS54 subunit of GARP complex using 5-Ph-IAA (Auxin Analogue or AA). (B) RPE1 cells expressing VPS54-mAID were treated with AA for 0, 0.5, 1, 2, and 3 h respectively to deplete VPS54. (Top panel) WB with anti-myc antibody. (Bottom Panel) Quantification of the blots from three independent experiments. (C) RPE1 cells expressing VPS54-mAID were treated with AA for 3 h and co-stained for myc (red) and P230 (green). (D) WB of RPE1 cells expressing VPS54-mAID, treated with AA for 0, 2, 3, 6, and 48 h was performed using anti-VPS51 antibody. (E) WB of RPE1 cells expressing VPS54-mAID, treated with AA for 0, 2, 3, 6, and 48 h was performed using anti-VPS52 antibody. (F) WB of RPE1 cells expressing VPS54-mAID, treated with AA for 0, 2, 3, 6, and 48 h was performed using anti-VPS53 antibody. The bottom panels in (D), (E), and (F) show quantification of the blots from three independent experiments.

Figure 3: Acute depletion of VPS54 alters the abundance and localization of TGN proteins. (A), (D), (G), and (J) RPE1 cells expressing VPS54-mAID were treated with AA for 0, 2, 3, 6, and 48 h respectively to deplete VPS54. (Top panels) WB analysis of cell lysates was performed and probed with (A) anti-TGN46, (D) anti-ATP7A, (G) anti-TMEM87A, and (J) anti-CPD antibody. (Bottom panels) Quantification of the blots from three independent experiments. (B), (E), (H), (K) RPE1 cells expressing VPS54-mAID were treated with AA for 3 h and co-stained for (B) TGN46 and P230, (E) ATP7A and

GM130, (H) TMEM87A and P230, (K) CPD and P230. (C), (F), (I), and (L) Colocalization analysis was performed by calculating the Pearson's correlation coefficient for (C) TGN46 and P230, (F) ATP7A and GM130, (I) TMEM87A and P230, and (L) CPD and P230. At least 50 cells were imaged per sample for the quantification. Statistical significance was assessed using one-way ANOVA. ** $p \leq 0.01$, * $p \leq 0.05$.

Figure 4: Acute VPS54 depletion causes secretory defects and relocalization of CD-MPR to vesicle. (A) (Top panel) WB analysis of secreted Cathepsin D from RPE1 VPS54-mAID cells treated with AA for 0 h and 6 h respectively. (Bottom panel) Quantification of secreted Cathepsin D from three independent experiments. (B) WB analysis of the whole cell lysates from (A), probed with anti-Cathepsin D antibody. (Bottom panel) Quantification of intracellular Cathepsin D from three independent experiments. (C) (Top panel) WB analysis of the secreted Fibronectin from RPE1 VPS54-mAID cells treated with AA for 0 h and 6 h respectively. (Bottom panel) Quantification of secreted Fibronectin from three independent experiments. (D) WB analysis of the whole cell lysates from (C), probed with anti-Fibronectin antibody. (Bottom panel) Quantification of intracellular Fibronectin from three independent experiments. Statistical significance was assessed using paired t-test. ** $p \leq 0.01$. (E) (Top panel) WB analysis of RPE1 cells expressing VPS54-mAID, treated with AA for 0, 2, 3, 6, and 48 h respectively and probed with anti-CI-MPR antibody. (Bottom panel) Quantification of the blots from three independent experiments. (F) RPE1 VPS54-mAID expressing cells were treated with AA for 3 h and co-stained for CI-MPR and P230. (G) Colocalization of CI-MPR to P230 was calculated between control and 3 h AA treatment

groups using Pearson's correlation coefficient and graph was prepared in GraphPad prism. (H) (Top panel) WB analysis of RPE1 cells expressing VPS54-mAID treated with AA for 0, 2, 3, 6, and 48 h respectively and probed with anti-CD-MPR antibody. (Bottom panel) Quantification of the blots from three independent experiments. (I) RPE1 VPS54-mAID expressing cells were treated with AA for 3 h and cells were co-stained for CD-MPR and P230. (J) Colocalization of CD-MPR to P230 was calculated between the control and 3 h AA treatment groups using Pearson's correlation coefficient and graph was prepared in GraphPad prism. Statistical significance was calculated using paired t-test. * $p \leq 0.05$

Figure 5: Acute VPS54 depletion affects a subset of Golgi enzymes and results in O-glycosylation defects. (A) WB analysis of cell lysates of AA treated RPE1 VPS54-mAID cells probed with (top panels) anti-B4GALT1 (A), and anti-C1GALT1 (D). β-actin was used as a loading control. The bottom panels on (A), and (D) are the quantification of the blots from three independent experiments. (B) Airyscan microscopy of RPE1 VPS54-mAID cells untreated (control) or treated with AA for 3 h and co-stained for B4GALT1 and P230. (C) Colocalization of B4GALT1 with P230 was determined by calculation of the Pearson's correlation coefficient. (E) Airyscan microscopy of RPE1 VPS54-mAID cells untreated (control) or treated with AA for 3 h and co-stained for C1GALT1 and P230. (F) Integrated density ratio of C1GALT1 to P230 in control and AA treated group was determined using ImageJ. Statistical significance was calculated using paired t-test. ** $p \leq 0.01$. (G)Total proteins from AA treated RPE1 VPS54-mAID were resolved by SDS-PAGE and probed with (Top panel) HPA-647 (G), and GNL -647

(H). The bottom panels on (G), and (H) are the quantification of the blots from three independent experiments. Statistical significance was calculated using one-way ANOVA. ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001.

Figure 6: v-SNARE GS15 is mislocalized in VPS54 depleted cells. (A) RPE1 VPS54-mAID cells were treated with AA as indicated and cell lysates were probed with (top panel) anti-GS15 (A), and anti-GS28 (D). β-actin was used as a loading control. The bottom panels on (A), and (D) are the quantification of the blots from three independent experiments. (B) Airyscan microscopy of RPE1 VPS54-mAID cells untreated or treated with AA for 3 h and co-stained for GS15 and P230. (C) Colocalization analysis of GS15 and P230 was done by calculation of the Pearson's correlation coefficient. Statistical significance was calculated using paired t-test. ** p≤ 0.01. (F) WB analysis of RPE1 VPS54-mAID cells treated with AA and probed with antibodies to STX5, STX6, STX10, VAMP4, and VTI1A respectively. β-actin was used as a loading control.

Figure 7: Acute VPS54 depletion mislocalizes vesicular adaptor proteins and COPI coats. (Top panel) Airyscan microscopy of RPE1 VPS54-mAID cells untreated and treated with AA for 3 h and co-stained for (A) β-adaptin and GM130 (B) GGA2 and GM130 (C) COPB2 and GM130 (D) GBF1 and GM130. (Bottom panel) Colocalization analysis of (A) β-adaptin and GM130. (B) GGA2 and GM130 (C) COPB2 and GM130 (D) GBF1 and GM130 was done by calculation of the Pearson's correlation coefficient. ** p≤ 0.01.

Figure 8: Rapid VPS54 depletion results in accumulation of GARP-dependent vesicles and alteration of TGN morphology. (A) Transmission Electron Microscopy of high-pressure frozen RPE1 VPS54-mAID cells grown on sapphire discs before and after 3 h of AA treatment. “G” indicates Golgi stacks. Arrowheads pointed to vesicle-like structures. Arrows indicate the enlarged vacuolar structures accumulated near Golgi. Asterisks indicate the autophagosomes. Scale bar, 500 nm. (B) The graph represents the quantification of total number of vesicles around the Golgi before and after 3 h of AA treatment. (C) Schematic of cellular fractionation experiment to prepare P30 (Golgi), and P100 (Vesicle) fractions from control and AA treated groups. (D) WB analysis of TGN localized proteins (TGN46, CI-MPR, and CD-MPR) in Golgi and vesicle fractions. (E) WB analysis of Golgi enzymes (B4GALT1, MGAT1, C1GALT1, GALNT2, and CPD) in Golgi and vesicle fractions. (E) WB analysis of SNAREs (STX5, GS15, STX10, STX6) in Golgi and vesicle fractions.

Figure 9: Cartoon depicting early and late defects associated with GARP dysfunction. (Left) Control cells with normal Golgi morphology, Golgi enzymes, endosome-Golgi trafficking proteins, and component of trafficking machineries such as coats and SNAREs. (Middle) 3 h post-depletion of VPS54, the trans-side of Golgi enlarged, increase in vesicles, endosome-Golgi trafficking proteins are mislocalized and redistributed to vesicles, decrease in Golgi-associated coat proteins, and SNAREs. (Right) Severe alteration of Golgi structure, depletion of Golgi enzymes, coat proteins, and SNAREs as a late response.

Supplementary Figure legends

Figure S1: VPS54-mAID rescue Golgi localization defects in VPS54-KO HeLa cells. HeLa cells expressing VPS54-mAID and VPS54-KO were stained with antibodies to TGN46 (A), GBF1 (B), and COPB2 (C) and analyzed by confocal microscopy.

Figure S2: Efficient depletion of VPS54 in HeLa cells expressing VPS54-mAID following AA treatment. (A) WB analysis of HeLa cells expressing VPS54-mAID following rapid VPS54 depletion for 0, 0.5, 1, 2, 3, 24, and 48 h respectively and probed with anti-myc (VPS54) antibody. The bottom panel shows the quantification of the blot. (B) HeLa cells expressing VPS54-mAID were co-stained with myc (red) and B4GALT1 (green) after 3 h of VPS54 depletion.

Figure S3: Effect of VPS54-KO on ATP7A and CPD localization. (A) (Top panel) WB analysis of RPE1 cells expressing VPS54-mAID before and after the treatment of AA and protease inhibitor (PI) and probed with anti-TGN46. (Bottom panel) Quantification of the blots from three independent experiments. Statistical significance was assessed using one-way ANOVA. **** $p \leq 0.0001$, ** $p \leq 0.01$. (B) RPE1 cells expressing VPS54-mAID were transiently transfected with LAMP2-mCherry and TGN38-GFP for 24 h followed by 3 h AA treatment and cells were imaged using airyscan microscopy. (C) Airyscan microscopy images of RPE1 WT, VPS54-KO, and VPS54-mAID cells stained with anti-B4GALT1, anti-GM130, and anti-ATP7A antibodies. (D) Co-staining of RPE1 WT and VPS54-KO cells for CPD and P230 using anti-CPD and anti-P230 antibodies.

Figure S5: Acute VPS54 depletion does not affect the Golgi enzymes MGAT1 and GALNT2 in RPE1 cells. (A) WB analysis of total lysates of RPE1cells expressing VPS54-mAID were treated with AA and probed with (Top panel) anti-MGAT1 (A), and anti-GALNT2 (D). β-actin was used as a loading control. The bottom panels on (A), and (D) are the quantification of the blots from three independent experiments. (B) Airyscan microscopy of RPE1 VPS54-mAID cells untreated or treated with AA for 3 h and co-stained for MGAT1 and P230. (C) Colocalization analysis of MGAT1 with P230 was done by calculation of the Pearson's correlation coefficient.

Figure S6: Rapid depletion of VPS54 stimulates B4GALT1 relocation to endosomes following CQ treatment. (A) IF images of RPE1 cells expressing VPS54-mAID. Untreated cells (control), or treated with CQ for 3 hours (CQ), or treated with AA for 1 hour followed by CQ treatment for 3 hours (AA+CQ), or treated with AA and CQ and recovered for 3 hours (AA+CQ+W) or treated with CQ and recovered for 3 hours (CQ+W) were stained for GPP130, B4GALT1, and Giantin. (B) Colocalization analysis of B4GALT1 and Giantin was done by calculation of the Pearson's correlation coefficient. Statistical significance was calculated using one-way ANOVA. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. (C) Colocalization analysis of GPP130 and Giantin was also done by calculation of the Pearson's correlation coefficient. Statistical significance was calculated using one-way ANOVA.

Figure S7: Acute VPS54 depletion does not affect BIG1 localization. (A) Airyscan microscopy of RPE1 VPS54-mAID cells untreated or treated with AA for 3 h and co-

stained for BIG1 and P230. (B) Colocalization of BIG1 and P230 was determined by the calculation of the Pearson's correlation coefficient.

Figure S8: Acute VPS54 depletion led to accumulation of C1GALT1 and STX6 in the GS15 positive vesicles. (A) WB analysis of GS15 in Golgi and vesicle fraction before (-) and after (+) acute VPS54 depletion. (B) Schematic of GS15 IP from the vesicular fraction (S30) using GS15 antibody in control and AA treated groups. (C-F) (Top panel) WB analysis of GS15 IP in control and AA treated groups probed with anti-GS15 (C), TGN46 (D), C1GALT1 (E), and STX6 (F). (C-F) (Bottom panel) The graph represents the quantification of three independent blots.

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Figure 1 Expression of VPS54-mAID and rescue of VPS54-KO cells

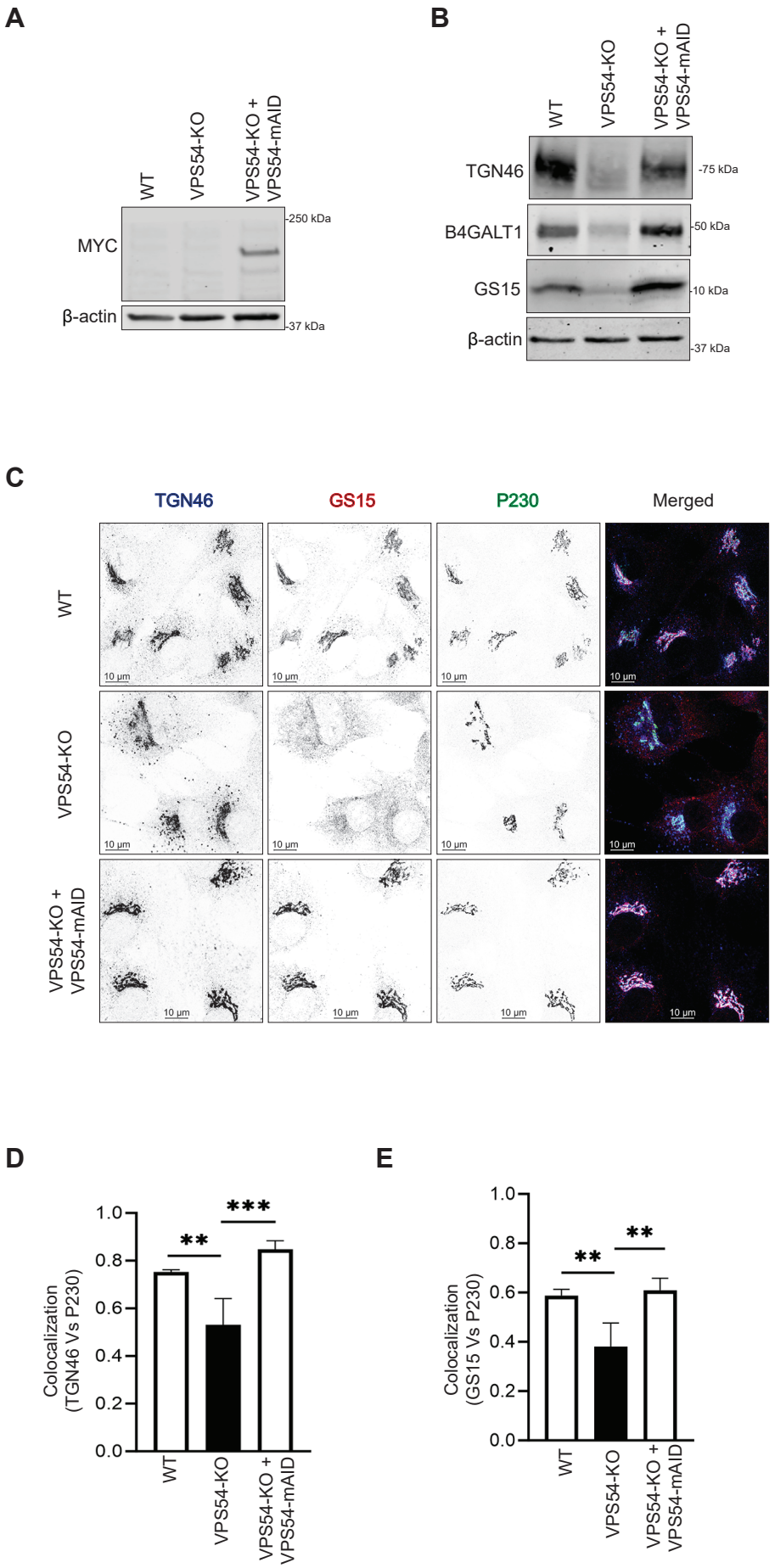


Figure 2 Acute depletion of VPS54 doesnot affect stability of its partner proteins

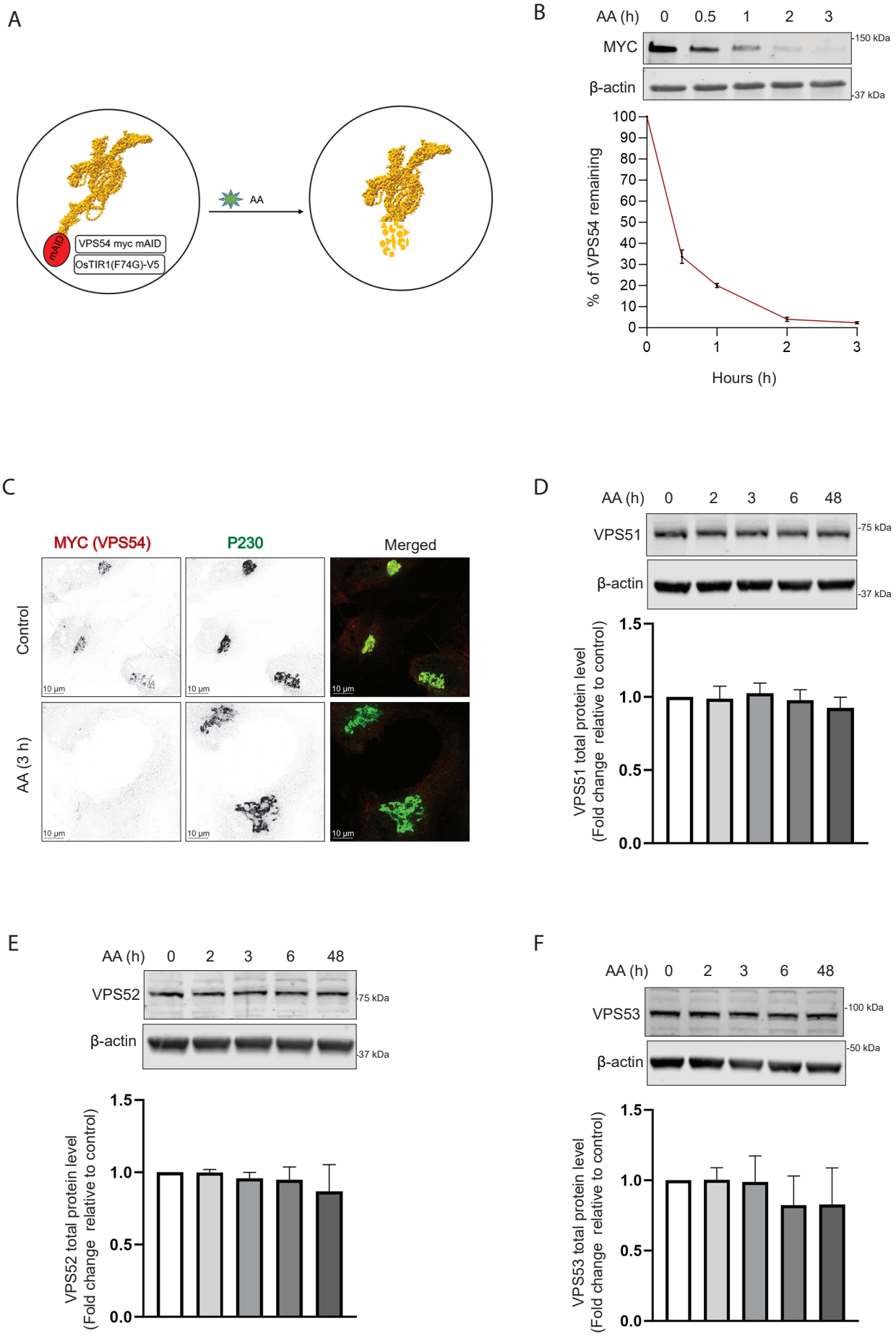


Figure 3 Rapid VPS54 depletion alters the protein abundance and localization of TGN proteins

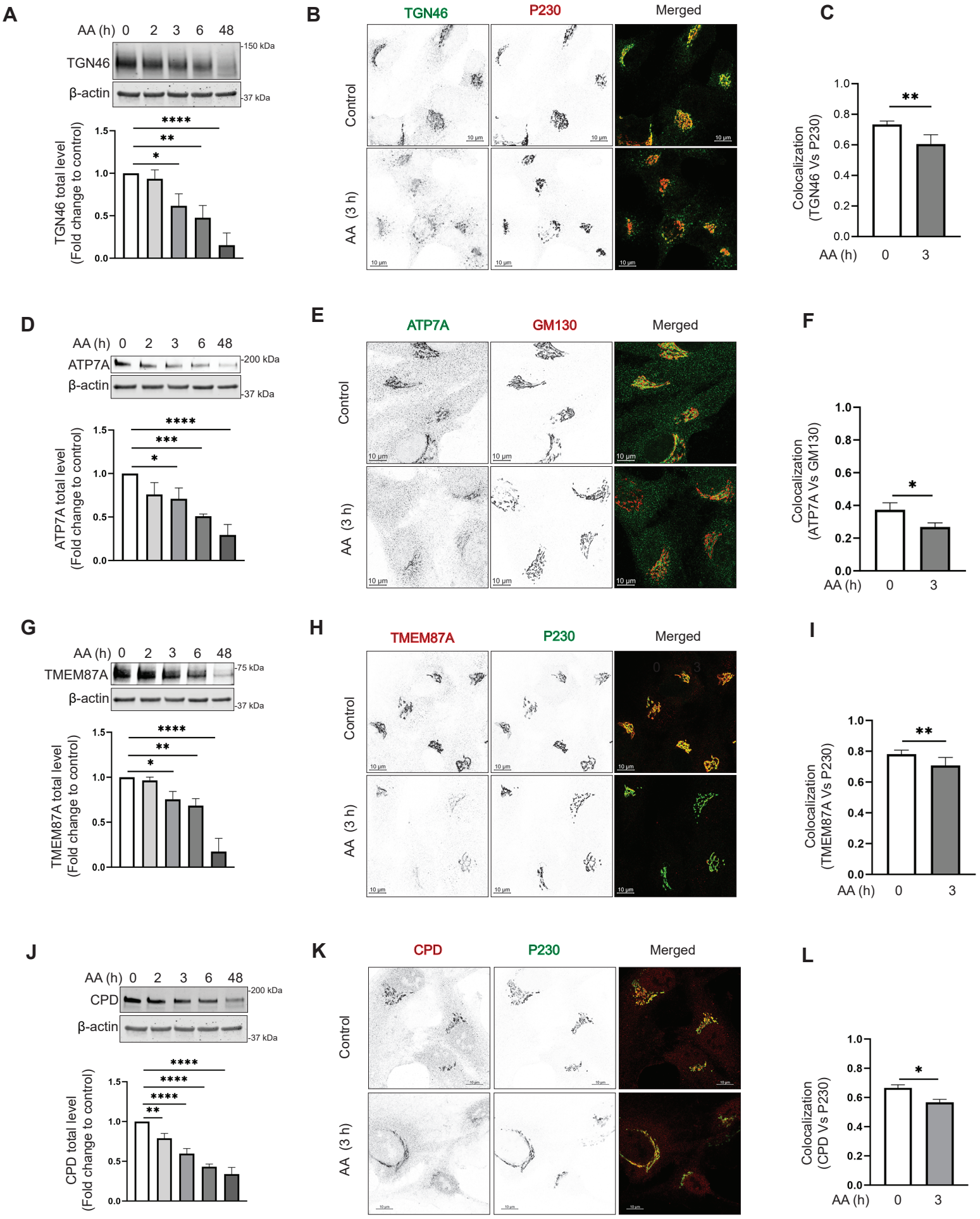


Figure 4 Rapid VPS54 depletion leads to secretory defects and relocalization of CD-MPR to vesicles

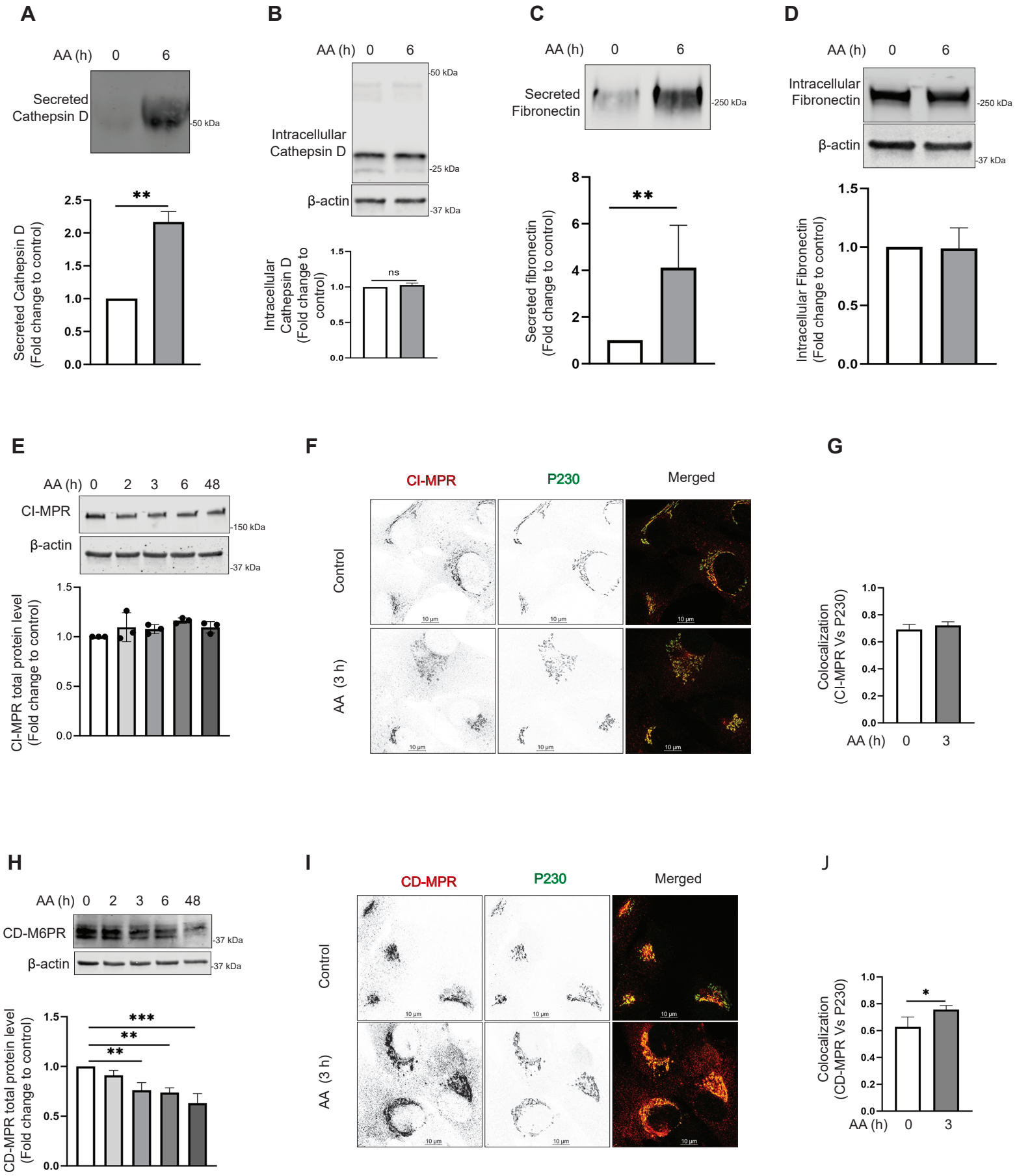


Figure 5 Acute VPS54 depletion affect a subset of Golgi enzymes and results in O-glycosylation defects

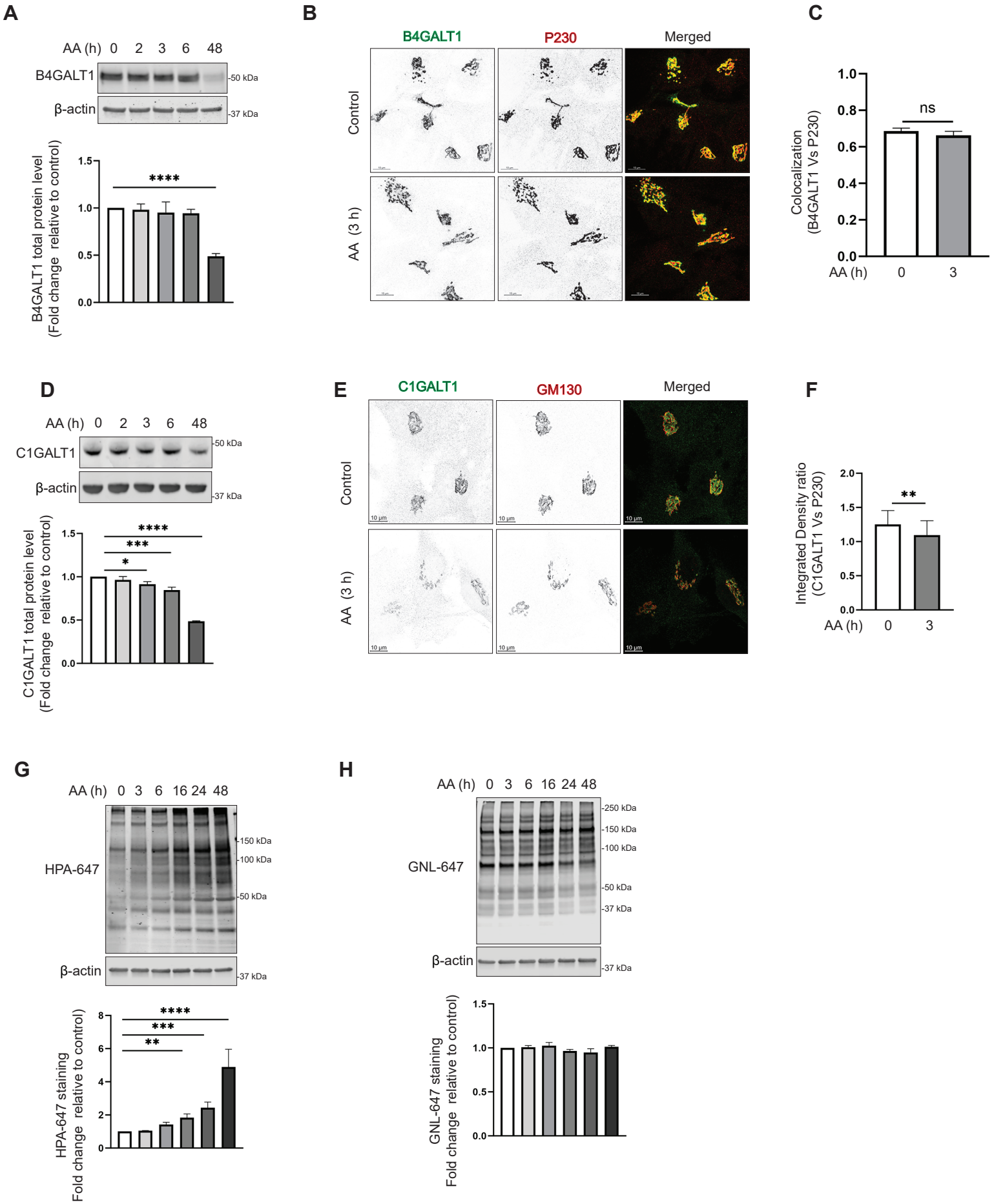


Figure 6 Mislocalization of GS15 in GARP depleted cell is its primary defect

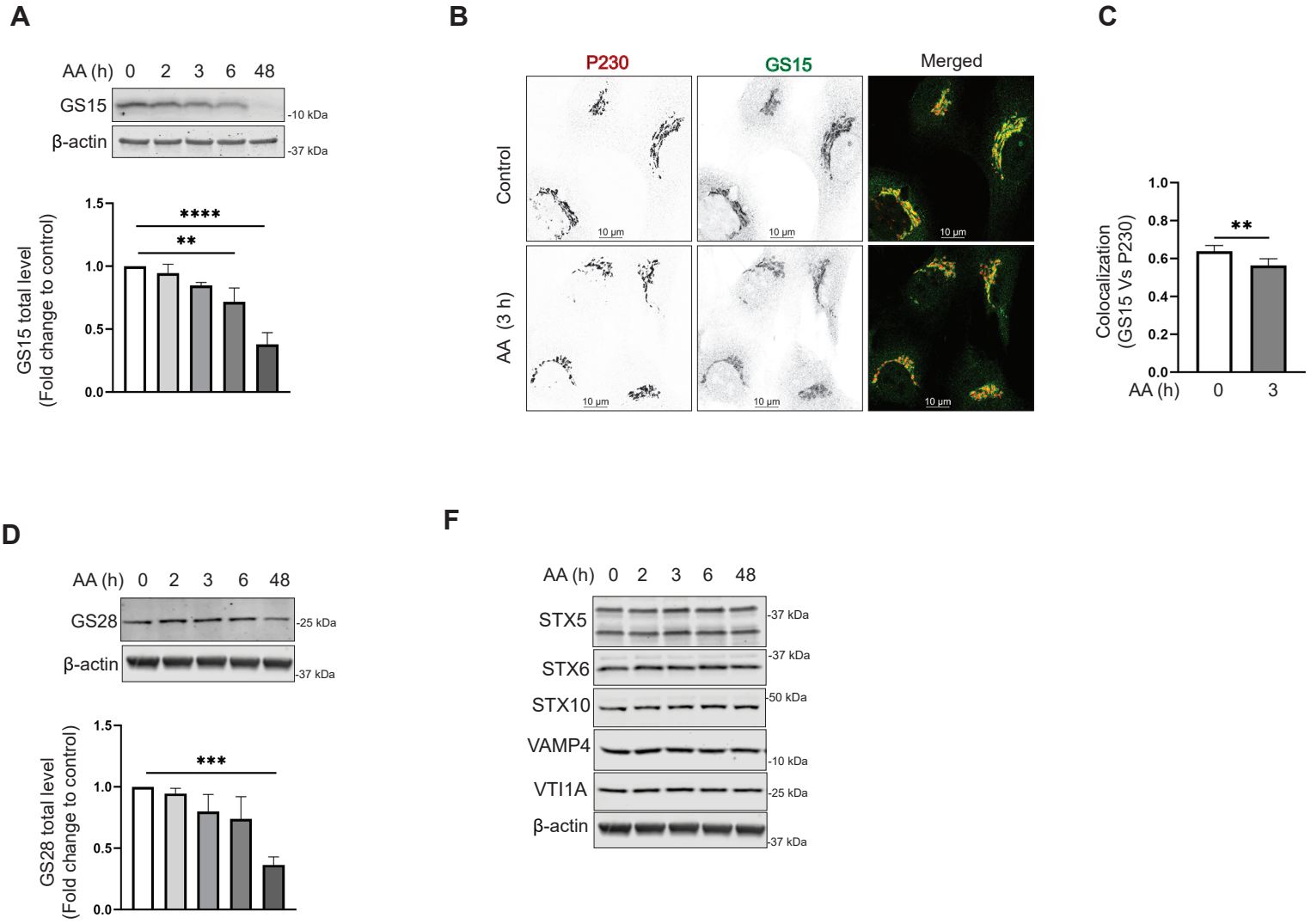


Figure 7 Acute GARP depletion mislocalizes adaptor proteins and COPI coats

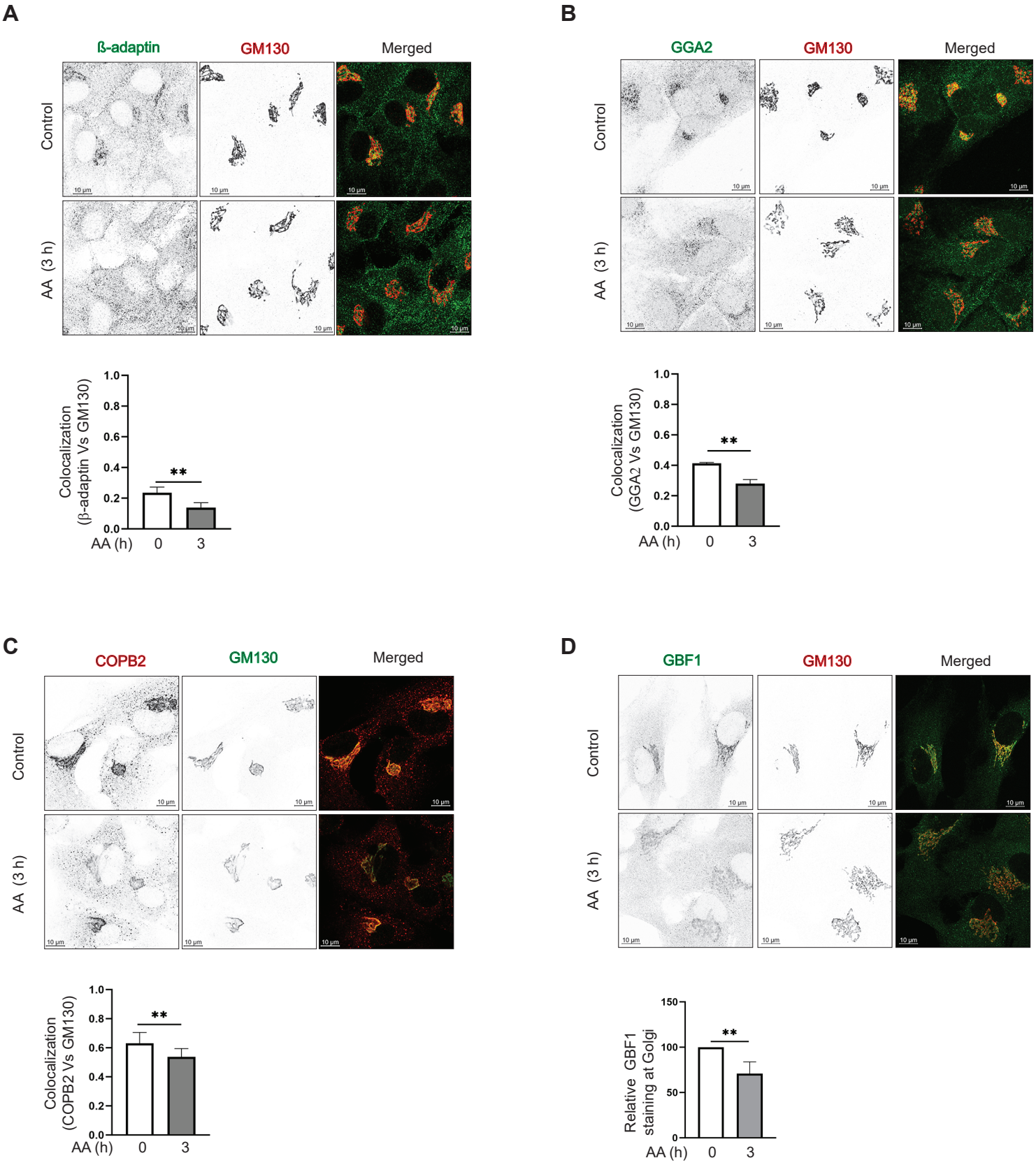
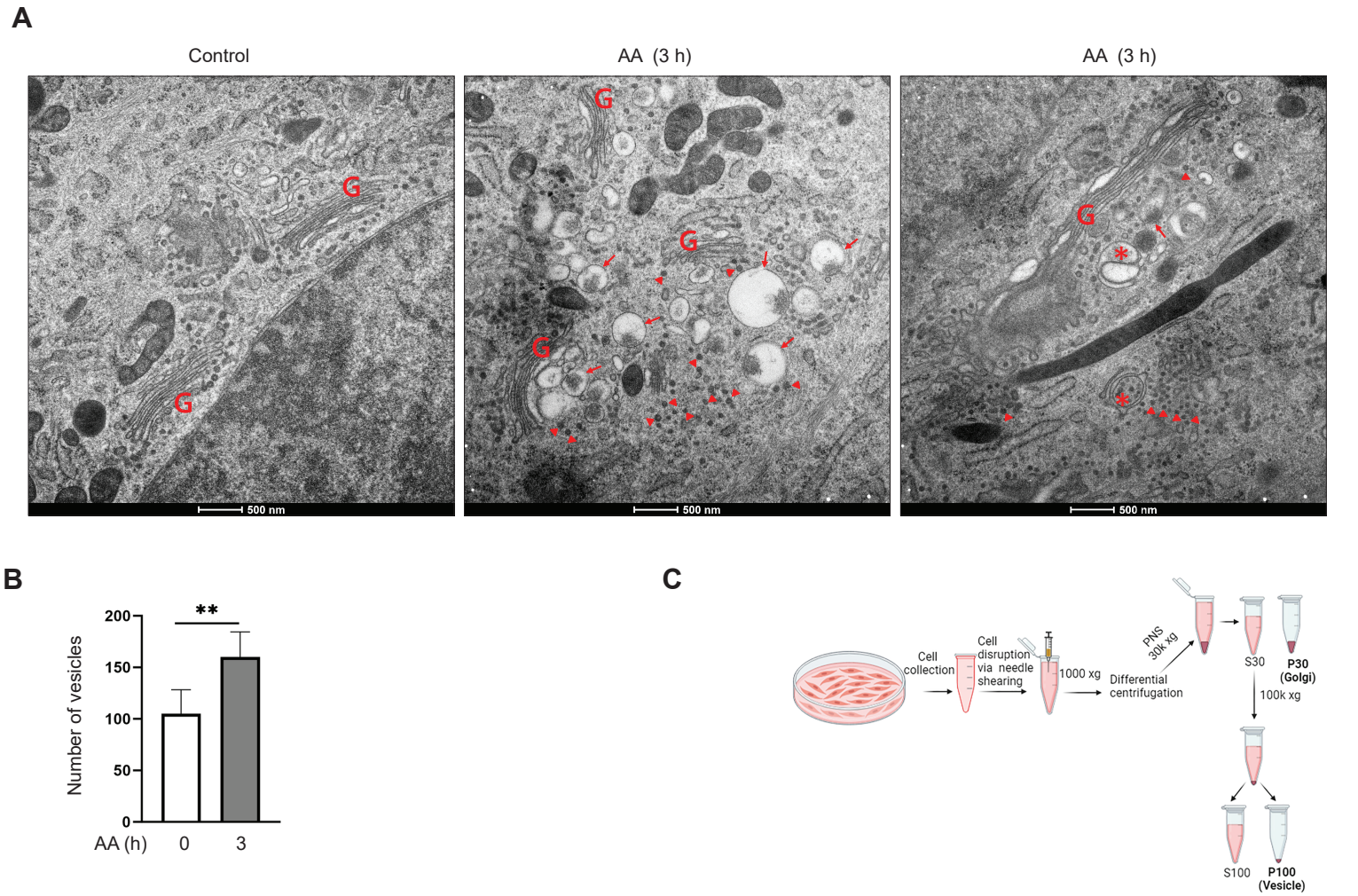


Figure 8 Rapid VPS54 depletion alters TGN morphology and accumulate GARP dependent vesicles



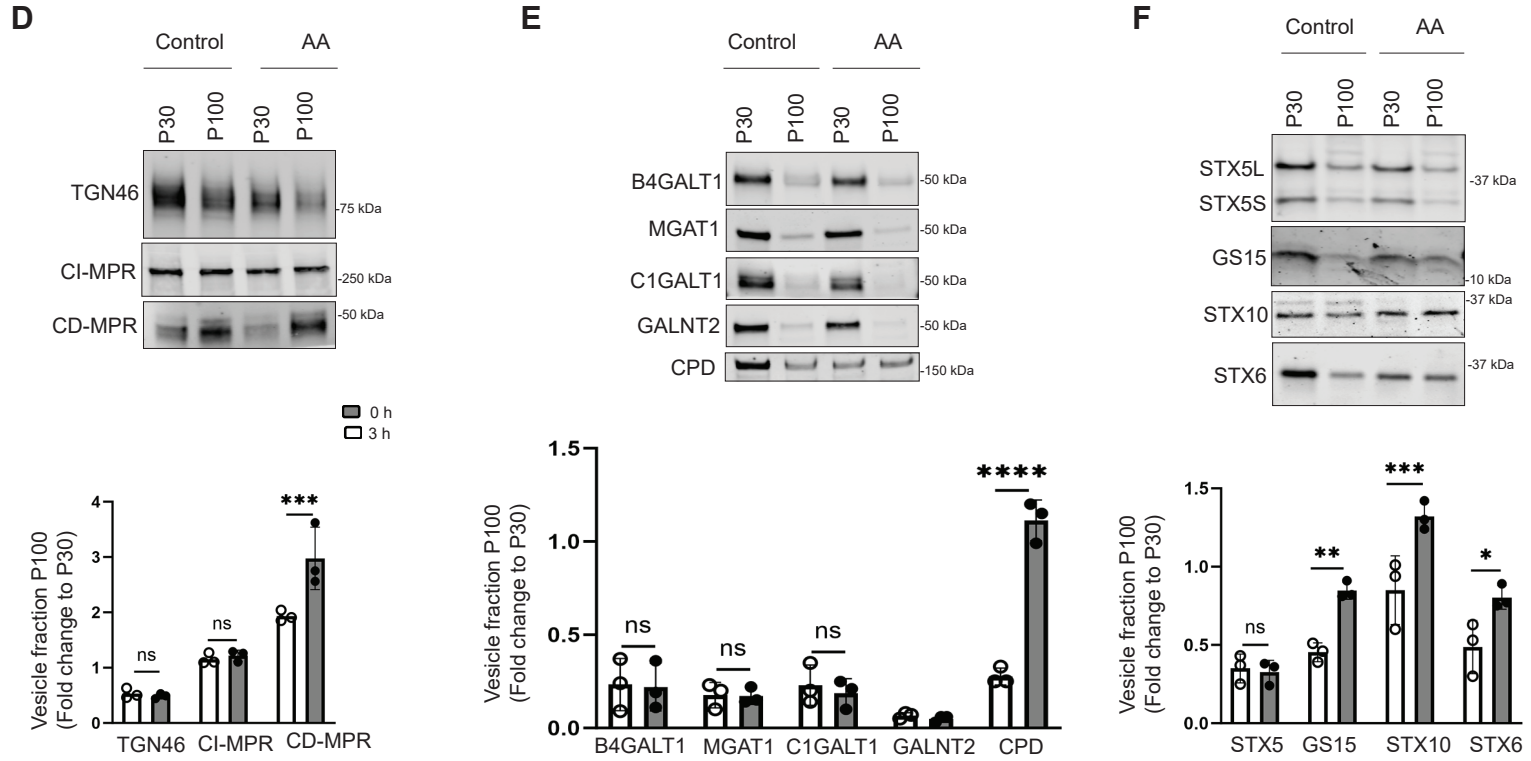
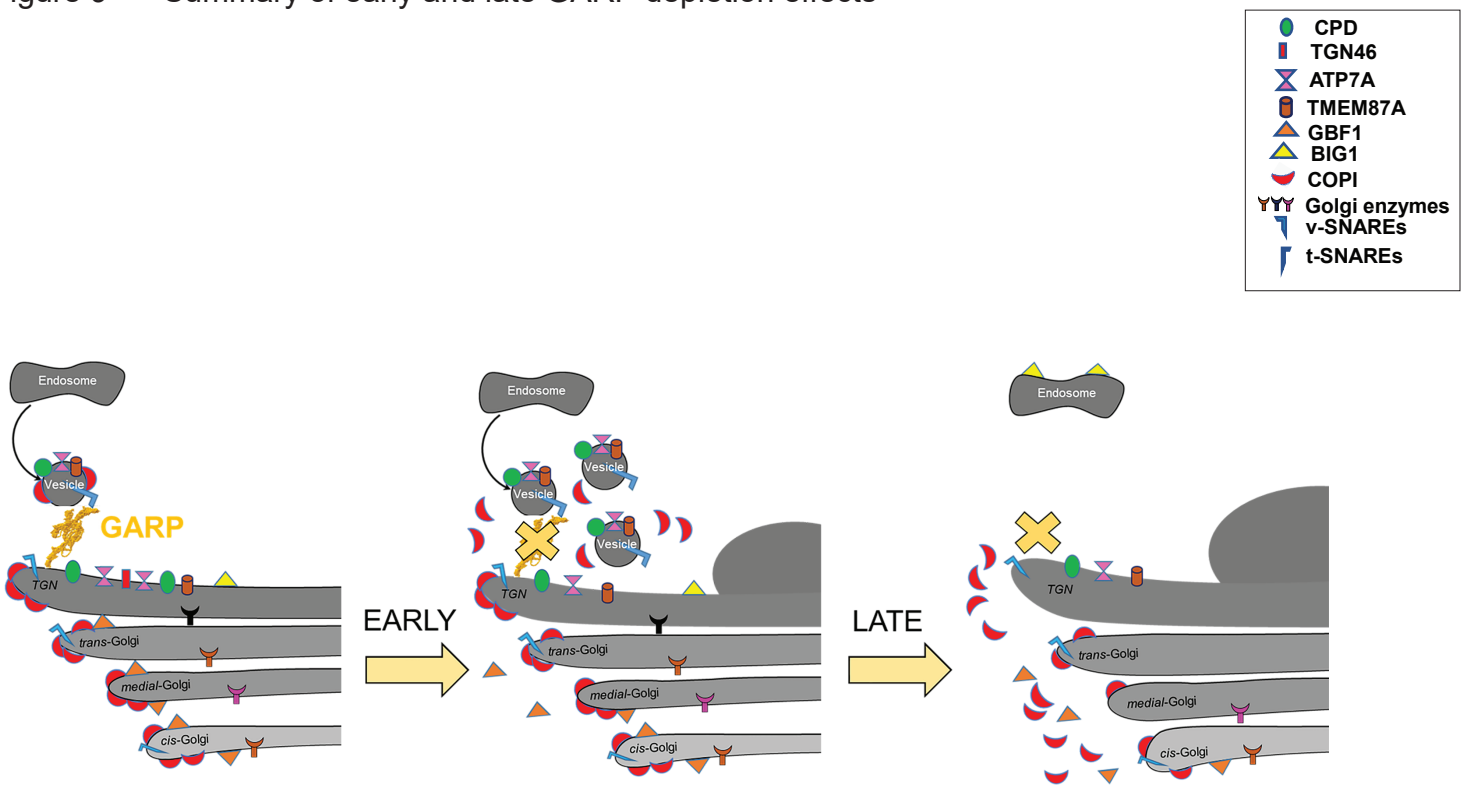



Figure 9 Summary of early and late GARP depletion effects