Acyl-CoA Binding Domain-Containing Protein 3 Functions as a Scaffold to Organize the Golgi Stacking Proteins and a Rab33b-GAP

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Abstract

Golgin45 plays important roles in Golgi stack assembly and is known to bind both the Golgi stacking protein GRASP55 and Rab2 in the medial-Golgi cisternae. In this study, we sought to further characterize the cisternal adhesion complex using a proteomics approach. We report here that Acyl-CoA Binding Domain Containing 3 (ACBD3) is likely to be a novel binding partner of Golgin45. ACBD3 interacts with Golgin45 via its GOLD domain, while its co-expression significantly increases Golgin45 targeting to the Golgi. Further, ACBD3 recruits TBC1D22, a Rab33b GTPase Activating Protein (GAP), to a large multi-protein complex, containing Golgin45 and GRASP55. These results suggest that ACBD3 may provide a scaffolding to organize the Golgi stacking proteins and a Rab33b-GAP at the medial-Golgi.

Keywords

ACBD3, Golgin45, Golgi, Rab-GTPase, membrane trafficking

Introduction

The Golgi apparatus plays pivotal roles in post-translational modification of glycoproteins and various lipid species, such as glycosphingolipids and glucosylceramides[1]. It is composed of stacks of flattened cisternae that are laterally linked to form a perinuclear ribbon-like structure in mammalian cells[2, 3]. Although considerable advances have been made over the last decades in our understanding of Golgi biogenesis and its cell-cycle and traffic-dependent regulation, the complexities of its structural features and mechanism of domain organization for cargo sorting/processing remain poorly understood [4]. Recently, we proposed that Golgi stack assembly may be dictated by the total amount of cisternal adhesive energy, collectively contributed by at least two Golgin tethers (GM130/Golgin45) and two GRASP proteins, GRASP65/55. Furthermore, we found that GRASP65/55 may be completely substituted with exogenously expressed GM130 or Golgin45 to maintain morphologically and functionally normal Golgi stacks[5-10]. In addition to its role in Golgi stacking, GM130 has been shown to be involved in vesicle tethering, cell cycle control, centrosome organization, cell signaling, growth control, cell polarization and directed cell migration. [11-16]. Golgin45 (also called Basic Leucine Zipper Factor 1 or BLZF1) was initially identified as a nuclear co-factor [17], and was later found to function in Golgi stacking as a GRASP55-binding, Rab2 effector protein [9]. However, relatively little is known about an exact role of Golgin45 in Golgi biogenesis or in membrane trafficking through the Golgi.

To this end, we used a proximity-based proteomic profiling (BioID) to more thoroughly investigate Golgin45 interacting proteins. The results from this screening identified Acyl-CoA Binding Domain Containing 3 (ACBD3) as a major binding partner of Golgin45. ACBD3 (also called PAP7 or GCP60) is a member of a large family of Acyl-CoA binding proteins[18]. It had been shown to directly bind palmitoyl-CoA using its N-terminal Acyl-CoA Binding Protein (ACBP) domain and interact with a Golgin tether Giantin via its Golgi Dynamics (GOLD) domain, suggesting its role in Golgi structure and function [19, 20].

We show here that ACBD3 binds Golgin45 via its GOLD domain and forms a multi-protein cisternal adhesion complex with the two Golgi stacking proteins, Golgin45 and GRASP55 as well as a Rab33b GAP.

Materials and Methods

Cell culture, antibodies and siRNAs

HeLa cells were maintained at 37°C in 5% CO_2 in DMEM (Corning) supplemented with 10% FBS (Gibco). All general chemical reagents were purchased from Sigma Aldrich, unless otherwise stated. Following antibodies were used in this study; anti-ACBD3 (Sigma), anti-myc-tag(Clontech), anti-GFP (Roche), anti-RFP (Abcam), anti-Golgin97 (Cell Signaling Technology), anti-GM130 (BD Bioscience). For gene expression experiments in HeLa cells, pC4-mCherry-Golgin45, pEGFP-C1-ACBD3, pCMV-FLAG-ACBD3, pEGFP-N1-GRASP55, pCMV-myc-TBC1D22A/B, GT-GFP were transfected by lipofectamine 3000 (Life Technologies), and cells were used for experiments after 24 hrs post-transfection. For siRNA Transfection, HeLa cells were seeded onto 6-well plates at a density of 2.0×10^5/well. After 24 hrs, cells were transfected with 50 nM final concentration of siRNAs with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's instructions. The following sequences of siRNAs were used in the study; the control siRNA, UUCUCCGAACGUGUCACGU; ACBD3 siRNA-1, GCAUUAGAGUCACAGUUUA; ACBD3 siRNA-2: GCUGAAGUUACAUGAGCUA. Giantin siRNA-1 : GCUAAAGAGUGUAUGGAAA, Giantin siRNA-2: GCUGCUGCAGAGAAUAAUA

Immunofluorescence staining and Confocal Microscopy

HeLa cells were grown to subconfluent density on glass coverslips for 24 hrs. The cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma Aldrich) for 20 min at room temperature. Those cells were then permeabilized with PBS, containing 0.3% Triton X-100 (Sigma Aldrich) and 0.1% BSA for 10 min and blocked with blocking buffer (Thermo Pierce) for 30 min at room temperature, followed by 2-3

hrs incubation with specified primary antibodies. The cells were subsequently washed in PBS three times and incubated with Alexa Fluor 647-conjugated anti-rabbit/mouse, Alexa Fluor 568-conjugated anti-rabbit/mouse or Alexa Fluor 488-conjugated anti-rabbit/mouse antibody (Life Technologies) for 1 hr at room temperature. The cells were washed with PBS three times and stained with Hoechst 33342 (Santa Cruz Biotechnology) for 10 min at room temperature. The stained cells were examined using Zeiss LSM710 with a 63 × oil immersion objective.

Co-immunoprecipitation and Immunoblotting

Co-IP experiments were performed as follows: cells were lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl2·6H2O, 0.1 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40, 1× protease inhibitor cocktail (Roche)) for 30 min at 4 °C. Crude lysates were subjected to centrifugation at 15, 000 x g for 15 min at 4 °C. Supernatants were incubated with anti-RFP agarose beads (MBL life science) with rocking for 4 h at 4 °C. The beads were washed three times with lysis buffer and one time with PBS. Proteins were eluted by boiling in 1× SDS running buffer and subjected to SDS-PAGE for immunoblotting. For immunoblotting, membranes were probed with specific primary antibodies and then with peroxidase-conjugated secondary antibodies. The bands were visualized with chemiluminescence (ECL, Bio-Rad). Representative blots are shown from several experiments.

BioID

HeLa cells (three 15-cm dishes of cells for each experimental condition) were transfected with pcDNA3.1-myc-BirA*-Golgin45. In a control experiment for Golgi localization specificity, HeLa cells were transfected with pcDNA3.1-myc-BirA*-Rab6 with Lipofectamine® 3000 (Invitrogen), according to manufacturer's instructions. As a control for biotinylation specificity, HeLa cells were mock treated and subjected to the same subsequent treatment. After 24 hrs transfection, cells were incubated for 6 hrs in complete media supplemented with 50 μM biotin. After three PBS washes, cells were lysed at 25°C in 1 mL lysis buffer (50

mM Tris, pH 7.4, 500 mM NaCl, 0.4% SDS, 2% Triton X-100, 5 mM EDTA, 1 mM DTT, and 1x Complete protease inhibitor [Roche]) and sonicated to disrupt any visible aggregates. After the first sonication, an equal volume of 50 mM Tris (pH 7.4) was added before additional sonication and then centrifuged at 16,000 xg for 30 minutes at 4°C. Supernatants were incubated with 600 µl Streptavidin-agarose (Invitrogen) for 1 hr at room temperature. Beads were collected and washed five times for 5 min at 25°C in 1 mL 1 x lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 0.2% SDS, 1% Triton X-100, 2.5 mM EDTA, 0.5 mM DTT, and 1x Complete protease inhibitor [Roche]). This was repeated five times with PBS. 25% of the sample was reserved for Western blot analysis, for which the bound proteins were removed from the agarose beads by boiling in 50 µL of Laemmli SDS-sample buffer at 98°C for 10 min. The remaining beads (75%) were reserved for analysis by mass spectrometry.

MS Sample Preparation, Analysis and Data Analysis

Independent triplicates of mock treated, Myc-BirA-Rab6 and Myc-BirA-Golgin45 BioID experiments (75% of the beads) were prepared and analyzed by MS independently in a label free format. For each BioID experiment, two replicates were digested with LysC/trypsin and one replicate with GluC to increase proteome coverage. Samples were prepared by on-beads digestion as follows. Briefly, biotinylated proteins were denaturated on beads with 25 µl of denaturation buffer (8 M urea, 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol) and incubated for 30 min. Proteins were alkylated by the addition of 5.5 mM iodoacetamide for 20 min in the dark and digested with the endoproteinase LysC for 3 hours at 37°C followed by overnight digestion with trypsin or were digested with GluC. Unbound peptides were then desalted following the protocol for StageTip purification [21]. Peptides were subjected to reversed-phase chromatography on a Thermo Easy nLC system connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) through a nano-electrospray ion source, as described previously [22]. The resulting mass spectroscopy (MS) and MS/MS spectra were analyzed using MaxQuant (version

1.4.1.4) as described previously [22]. All calculations and plots were performed with the R software package (www.r-project.org/) and analysis of label free BioID experiment were carried out following the quantitative interactomics approach as previously described (**supplementary Fig.1**) [23].

Results and Discussion

BioID identifies ACBD3 as a novel interactor of Golgin45.

BioID utilizes a promiscuous biotin ligase (BirA*) fused to a protein of interest to identify functionally relevant proteins which are proximate to the target protein and label them with biotin in living cells [24, 25]. We transiently over-expressed myc-BirA-Golgin45 in HeLa cells and pulled down biotinylated proteins by streptavidin beads, followed by identification of interacting proteins by mass spectrometry (**Fig.1A**). As a preliminary experiment, we performed confocal experiments to check whether myc-BirA-Golgin45 is correctly targeted to the Golgi. As shown in **supplementary Fig.2**, we found that myc-BirA-Golgin45 indeed co-localizes with endogenous GRASP55 at the Golgi, while some were found in the nucleus as well. Upon incubation with biotin for 6 hours, biotinylation of Golgi-associated proteins could be detected by staining with streptavidin-Alexa488, suggesting that both Golgi targeting and *in vivo* biotinylation work, as intended.

Myc-BirA-Rab6 was used as a control experiment for BioID, since Rab6 is a Rab-GTPase mainly found in the *trans*-Golgi and the TGN, therefore anatomically separated from medial-Golgi localized Golgin45 [26, 27]. Combining three independent BioID experiment results, we found three proteins that are highly enriched in Golgin45 BioID, but not in Rab6 BioID, with high scores for interaction specificity. Presence of both GORASP2 (Golgi reassembly-stacking protein 2; GRASP55) and TNKS1 (tankyrase-1) were expected because they are known as Golgin45-associated proteins [9, 28]. The only new Golgin45 interactor in the mass spectrometry results was ACBD3 with specificity, comparable to GRASP55 and TNKS1 (**Fig.1B-D**). GRASP55 is the Golgi stacking protein and a known binding partner of Golgin45[9], whereas TNKS1 was recently identified as a Golgin45 interacting protein, but its role in Golgi structure and function remains unknown [28]. ACBD3 is a 60-kD Golgi-associated protein with a number of suggested functions, ranging from steroidogenesis to membrane trafficking[19, 29, 30]. ACBD3 had been

shown to be targeted to the Golgi apparatus through its C-terminal GOLD domain binding to a Golgin tether, Giantin [19, 31]. Overexpression of the GOLD domain disrupts protein trafficking from the endoplasmic reticulum (ER) to the Golgi[19]. Since Golgin45 is a Golgi matrix protein and required for Golgi structure maintenance and membrane trafficking, interaction of ACBD3 with Golgin45 merited further investigation.

The GOLD domain of ACBD3 specifically binds the coiled-coil domain of Golgin45.

We used immunoprecipitation experiments to see whether full length ACBD3 could be pulled down with Golgin45. We transfected mCherry-Golgin45[10] or co-transfected mCherry-Golgin45 and GFP-ACBD3 into HeLa cells for 18 hours, followed by cell lysis and immunoprecipitation using anti-RFP agarose. The results (**Fig.2A**) showed that ACBD3 was readily detected using anti-GFP antibody. Interestingly, we were not able to detect Giantin in the pull-down fraction, which contradicts the notion that Giantin is required for ACBD3 recruitment to the Golgi [19]. In order to investigate whether ACBD3-Golgin45 interaction on the Golgi membranes might require Giantin, we knocked down Giantin using RNAi for 48 hours and checked if ACBD3 dissociates from the Golgi membranes. Surprisingly, Giantin depletion did not significantly influence ACBD3 targeting to the Golgi, suggesting that ACBD3 might be recruited to the Golgi via more than one docking protein/site (**Supplementary Fig.3**), which requires future study for clarification.

We then further dissected the molecular interaction between ACBD3 and Golgin45 by co-immunoprecipitation experiments. ACBD3 is known to contain at least three functional domains, the N-terminal Acyl-CoA binding domain (ACBP), the coiled coil domain in the middle, and the C-terminal GOLD domain (**Fig.2B;** domain map). EGFP-ACBD3 or its fragments were co-transfected with mCherry-Golgin45 in HeLa cells, followed by immunoprecipitation using anti-RFP-agarose. EGFP-ACBD3 full

length and EGFP-GOLD were successfully co-immunoprecipited with mCherry-Golgin45, but not EGFP-ACBP and EGFP-coiled coil (CC), suggesting the GOLD domain of ACBD3 as the binding site for Golgin45 (**Fig.2B**).

We also looked for the domain on Golgin45 that mediates its binding to ACBD3. When the amino acid sequence of Golgin45 was blasted, the only predicted sequence feature of Golgin45 is the coiled-coil region in the middle[9]. Therefore, we divided Golgin45 into three fragments and repeated the immunoprecipitation experiments with EGFP-ACBD3 full length. As shown in **Fig.2C**, ACBD3 co-immunoprecipitated with the coiled-coil fragment of Golgin45 only, but not with the other two fragments. Finally, we confirmed the specificity of this domain interaction by immunoprecipitation experiments between the coiled-coil domain of Golgin45 and the GOLD domain of ACBD3, and found that these two domains can be co-immunoprecipitated specifically, whereas neither the N-terminal (1-122) nor the C-terminal (301-400) fragments of Golgin45 showed any detectible interaction with the GOLD domain in the same experiments (**Fig.2D**). These results suggest that the GOLD domain of ACBD3 binds the coiled-coil domain of Golgin45.

Co-expression of GFP-ACBD3 increases localization of mCherry-Golgin45 to the Golgi.

To further test ACBD3 and Golgin45 interaction *in vivo* and explore the functional consequences of this interaction, we examined the localization of these two proteins by confocal microscopy. In HeLa cells co-transfected with mCherry-Golgin45 and Golgi resident Galactosyltransferase fused to GFP (GT-GFP), over-expressed Golgin45 proteins were mostly found in nucleus, and only ~15% of the cells examined showed significant localization of Golgin45 at both the Golgi and the nucleus (**Fig.3A**), suggesting that there are only limited number of Golgin45 docking sites on the Golgi membranes. When GFP-ACBD3 was co-transfected, nearly 100% of the cells examined showed significant Golgin45 localization to the Golgi, while ~23% of the co-transfected cells showed exclusive Golgi localization of Golgin45. Similar results

were obtained, when GRASP55-EGFP was co-transfected with mCherry-Golgin45, strongly suggesting that both GRASP55 and ACBD3 are likely to provide Golgi docking sites for Golgin45.

Since the GOLD domain of ACBD3 was co-immunoprecipitated with Golgin45, we then tested whether the GOLD domain alone can enhance Golgin45 targeting to the Golgi (**Fig.3B**). Consistent with the co-immunoprecipitation experiments, co-expression of the GOLD domain of ACBD3 significantly increased targeting of mCherry-Golgin45 to the Golgi, while co-expression of the ACBP or the CC domain showed only a negligible effect.

Lastly, when the coiled-coil domain of Golgin45 was over-expressed with GRASP55-EGFP, this fragment of Golgin45 was almost exclusively found in the cytoplasm. However, co-expression of GFP-ACBD3 increased Golgi localization of Golgin45 CC fragment over 80% of the cells examined by confocal (**Fig.3C**), whereas co-expression of GRASP55-EGFP or GT-GFP failed to increase recruitment of mCherry-Golgin45 CC domain to the Golgi. Taken together, our data suggest that ACBD3 serves as a scaffold for Golgin45 targeting to the Golgi membranes through their interaction between its GOLD domain and the CC domain of Golgin45.

ACBD3 recruits a Rab33b GAP to the Golgi and forms a multi-protein complex with GRASP55 and Golgin45.

ACBD3 has recently been shown to directly interact with TBC1D22, a GTPase activating protein (GAP) specific for the GTPase Rab33b during viral replication [32-34]. If this binding occurs *in vivo*, it would suggest that any region of Golgi membrane to which ACBD3 is targeted may also become depleted of Rab33b. We therefore tested whether the proposed ACBD3-TBC1D22 interaction [32] occurs physiologically and whether this binary complex may further interact in a larger complex containing Golgin45 and its other known ligand, GRASP55 [9].

First, HeLa cells were co-transfected with HA tagged-ACBD3, GRASP55-EGFP and mCherry-Golgin45. Cells were lysed after 24 hrs post-transfection and immunoprecipitated with anti-ACBD3 antibody, followed by western analysis. The results showed that both GRASP55 and Golgin45 were co-immunoprecipitated well with ACBD3 (**Fig.4A**), suggesting that ACBD3 is likely to be a part of the cisternal adhesion complex, although its exact role in Golgi stacking is unclear at present. Next, we tested if TBC1D22 can be co-immunoprecipitated with this complex. We co-transfected myc-tagged TBC1D22 with EGFP-ACBD3 and mCherry-Golgin45 for 24 hrs. After cell lysis, we used anti-RFP-agarose beads to pull down Golgin45 to see whether ACBD3 and TBC1D22 can be co-immunoprecipitated with Golgin45. The results clearly showed that both ACBD3 and TBC1D22, but not endogenous Golgin97, were pulled down with Golgin45 (**Fig.4B**). These results suggest that both ACBD3 and TBC1D22 are a part of GRASP55-Golgin45 cisternal adhesion complex.

During the course of this study, we also found that targeting of TBC1D22 to the Golgi is most likely to be dependent on ACBD3, as efficient Golgi localization of myc-TBC1D22 was observed only when EGFP-ACBD3 was co-expressed (**Fig.4C**), while co-expression of GRASP55-GFP failed to enhance Golgi targeting of TBC1D22, further supporting the specificity of this effect.

Combining with the previously reported studies, we propose the binding interactions among these proteins, as illustrated in **Fig.4D**. Golgin45 forms a multi-protein complex with GRASP55 via the known PDZ domain interaction [9], while the GOLD domain of ACBD3 is likely to bind Golgin45-GRASP55 complex via the coiled-coil domain of Golgin45. Finally, TBC1D22 is recruited to ACBD3-Golgin45-GRASP55 complex by binding to the coiled coil domain of ACBD3, as reported by Greninger et al [32].

Golgin45 was originally identified in a yeast two-hybrid assay for GRASP55 interacting proteins as well as an effector protein of Rab2-GTP. The novel interaction with ACBD3 was an entirely unanticipated outcome of a BioID survey making no prior assumptions, but has been well validated by testing a

number of predictions *in vivo* concerning localization and immunoprecipitation from extracts. ACBD3 had been known to have several binding partners at the Golgi, including Giantin [19], Golgin160 [31], PI4KIIIβ [35], TBC1D22 [32], suggesting that it may play an important role as a scaffolding protein for formation of various membrane micro-domains at the Golgi.

Consistent with this hypothesis, our results indicate that ACBD3 might have additional Golgi docking sites, other than Giantin. This makes sense in that Giantin is typically found at the Golgi rim, whereas GRASP55 and Golgin45 function as Golgi stacking proteins between apposed cisternae. So it is only logical to consider that ACBD3 might form at least two distinct protein complexes at the Golgi, including Giantin-ACBD3 and GRASP55-Golgin45-ACBD3-TBC1D22, respectively.

In summary, we suggest that, unlike the previously accepted notion, the Golgi stacking proteins, Golgin45 and GRASP55, are likely to be a part of a large multi-protein complex, which may also regulate membrane dynamics of Rab-GTPases at the medial-Golgi.

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Author contributions

XY, MB, RC, SL, JM, LZ, FM, PZ, SJ and YQ performed the experiments. RC, JR, YQ and IL designed the experiments. JR, YQ and IL wrote the manuscript.

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Figure legends

Figure 1. Proximity-based proteomic profiling identifies novel interactors of Golgin45

(**A**) Schematic illustration for BioID experiments. Golgin45 cDNA was fused with a mutant *E. coli* biotin ligase (BirA*) and transfected into HeLa cells. Proteins proximate to this fusion protein was selectively biotinylated in living cells. After cell lysis and protein denaturation, biotinylated proteins are affinity purified using Streptavidin-agarose. Tagged proteins were processed for analysis by mass spectrometry. (**B-D**) Candidate proteins identified in Golgin45 (BLZF1) BioID and Rab6 BioID were presented in the volcano plots. The experiments were done in triplicates. Representative results are presented here in volcano plots.

Figure 2. The GOLD domain of ACBD3 binds the coiled-coil domain of Golgin45

(**A**) ACBD3, but not Giantin, co-immunoprecipitates with Golgin45. HeLa cells were transfected with either mCherry-Golgin45 alone or mCherry-Golgin45 and GFP-ACBD3, followed by immunoprecipitation using anti-RFP antibody. Following SDS-PAGE of the lysates, presence of ACBD3 and Giantin in the pull-down fraction were tested by western analysis. (**B**) Domain structure of ACBD3 showing its three domains. Green, purple and blue boxes indicate the ACBP domain, the coiled-coil domain and the GOLD domain, respectively. Full-length ACBD3 and its three individual domains were tested for their interactions with Golgin45. HeLa cells were co-transfected with plasmids encoding mCherry-Golgin45and each of the following proteins EGFP-ACBD3, EGFP-ACBP, EGFP-coiled coil, and EGFP-GOLD. After 24 hours, cells lysates were incubated with anti-RFP beads at 4^{o}C overnight. Bound proteins were analyzed by SDS-PAGE, followed by western blotting using anti-RFP and anti-GFP antibodies. (**C**)

Schematic representation of Golgin45 domain structure. The only predicted domain is the coiled-coil domain represented by a grey box. mCherry tagged full-length Golgin45 and its fragments (amino acids 1-122, 123-300, 301-400) were co-transfected with EGFP-ACBD3 in HeLa cells. Cell were lysed 24 hours after transfection and then incubated with anti-RFP beads at 4^{o}C overnight. Bound proteins were separated by SDS-PAGE and detected with anti-RFP and anti-GFP antibodies. (**D**) EGFP-ACBD3 GOLD(376-528) can be specifically co-immunoprecipitated with the coiled-coil domain of Golgin45 (123-300). EGFP-ACBD3 plasmid was co-transfected with mCherry-tagged Golgin45 fragments (amino acids 1-122, 123-300, 301-400) for 24 hours, followed by cell lysis and incubation with anti-RFP beads at 4^{o}C overnight. Bound proteins were separated by SDS-PAGE and detected with anti-RFP and anti-GFP antibodies.

Figure 3. ACBD3 recruits Golgin45 full length or Golgin45 CC domain to the Golgi

Colocalization of ACBD3 and Golgin45 was tested using confocal microscopy. (**A, B, and C**) HeLa cells were transfected with indicated plasmids for 24 hours. Cells were then fixed, permeabilized and stained with anti-GM130 antibody and DAPI (nucleus). The subcellular localization of Golgin45 was measured and quantified with data collected in three individual experiments, as described in the methods (n=20-25 cells). Line quantification graphs for intensity of Golgi co-localization with GM130 are shown to the right. Scale bars: 10 μm.

Figure 4. ACBD3 recruits TBC1D22, a Rab33b GAP, to Golgin45/GRASP55 complex

Golgin45 forms a multi-protein cisternal adhesion complex with GRASP55, ACBD3 and TBC1D22 in the medial Golgi cisternae. Note that Golgin45-GRASP55 and ACBD3-TBC1D22 domain interaction had previously been investigated and reported by others [9, 32, 36]. These multi-protein interactions were investigated in HeLa cells by co-immunoprecipitation. Cells were collected 48 hours after HA-ACBD3, GRASP55-EGFP and mCherry-Golgin45 (**A**) or EGFP-ACBD3, myc-TBC1D22 and mCherry-Golgin45 (**B**)

were co-transfected and cell lysates were immunoprecipitated with anti-ACBD3 antibody and protein G beads (A) or anti-RFP beads (B) at 4^{o}C overnight. Bound proteins were separated by SDS-PAGE and western blotting. (**C**) HeLa cells were co-transfected with plasmids encoding EGFP-ACBD3 or GRASP55-EGFP along with myc-TBC1D22 and mCherry-Golgin45, respectively. After 24 hours, cells were fixed, permeabilized, and stained with anti-myc tag antibody and DAPI for DNA. Note that we tested both TBC1D22A and TBC1D22B during the course of these experiments and obtained similar results for the two isoforms. For simplicity, we used TBC1D22 to denote both isoforms (n=25). Line quantification graphs for co-localization of Golgin45 with TBC1D22 and ACBD3 (GRASP55 for bottom panel) are shown to the right. (**D**) Scheme illustrating the proposed protein-protein interactions for TBC1D22-ACBD3-Golgin45-GRASP55 complex at the Golgi. Scale bar: 10 μm.

Supplementary Fig.1 Pairwise correlations between all BioID experiments for Figure 1 and the combined ratios

Supplementary Fig.2 myc-BirA-Golgin45 is correctly targeted to the Golgi. HeLa cells were transfected with myc-BirA-Golgin45 for 18 hours, followed by incubation with 50 μM biotin for 6 hours. Cells were then fixed and stained with streptavidin-Alexa488 for biotinylated proteins, anti-myc antibody/Alexa568 for myc-BirA-Golgin45 and anti-GRASP55/Alexa647 as a Golgi marker. We then examined the cells using LSM710 confocal microscope. Scale bar = 5 μm.

Supplementary Fig.3 Giantin depletion does not significantly influence ACBD3 targeting to the Golgi

Giantin was knocked down in HeLa cells by transfecting with Giantin siRNA for 48 hours, as described in the methods. Cells were then stained with anti-Giantin/alexa488 and anti-ACBD3/alexa568 antibodies, followed by examination under confocal microscope. Giantin knockdown efficiency was tested by western blot, as shown on the left side. Scale bar = 10 μm.

Fig.1

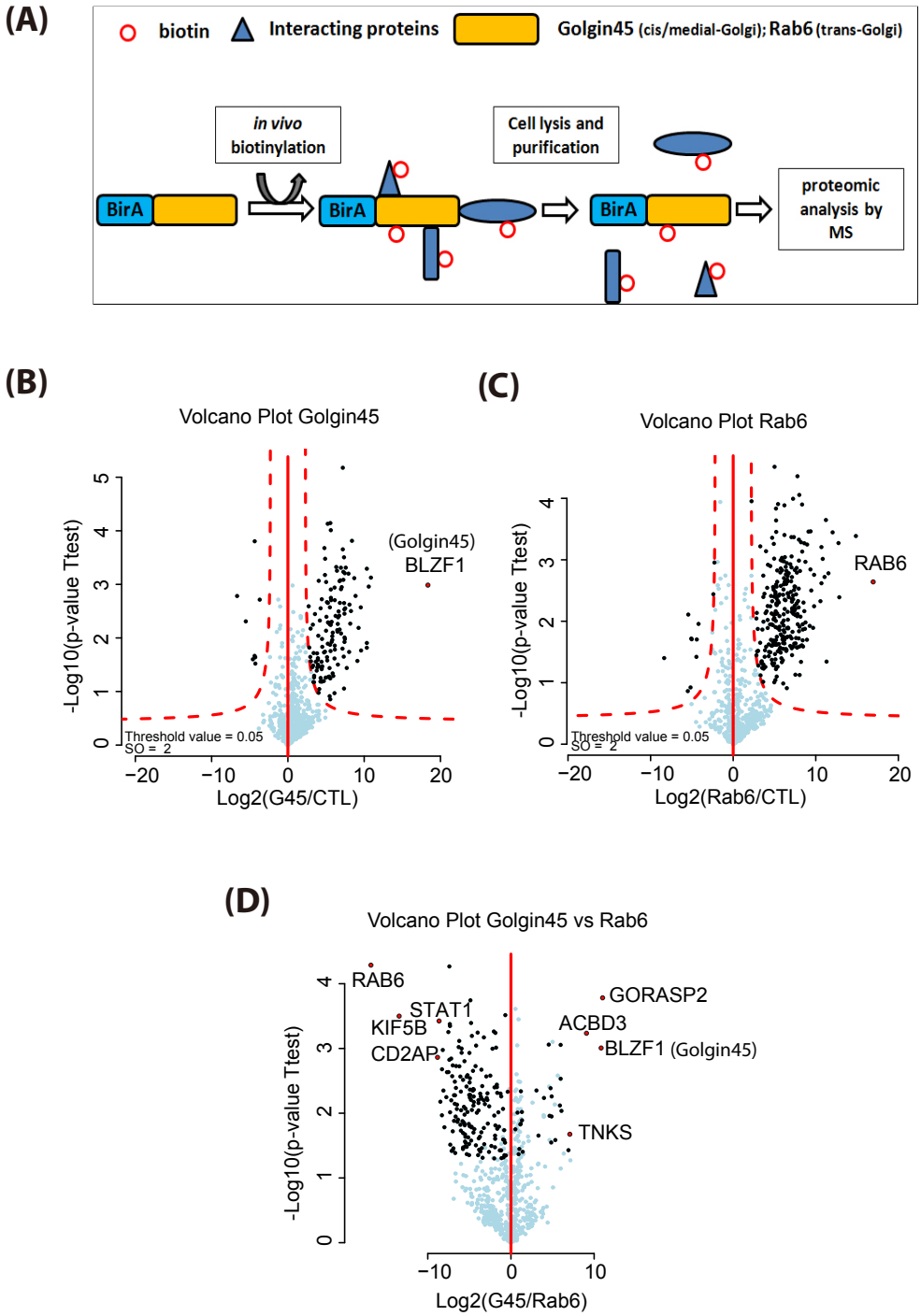

(A)
biotin
Interacting proteins
Golgin45 (cis/medial-Golgi); Rab6 (trans-Golgi)
in vivo biotinylation
Cell lysis and purification
proteomic analysis by MS
BirA
BirA
BirA
(B)
Volcano Plot Golgin45
(Golgin45) BLZF1
-Log10(p-value Ttest)
Log2(G45/CTL)
Threshold value = 0.05
SO = 2
(C)
Volcano Plot Rab6
RAB6
-Log10(p-value Ttest)
Log2(Rab6/CTL)
Threshold value = 0.05
SO = 2
(D)
Volcano Plot Golgin45 vs Rab6
RAB6
STAT1
KIF5B
CD2AP
GORASP2
ACBD3
BLZF1 (Golgin45)
TNKS
-Log10(p-value Ttest)
Log2(G45/Rab6)

Fig.2

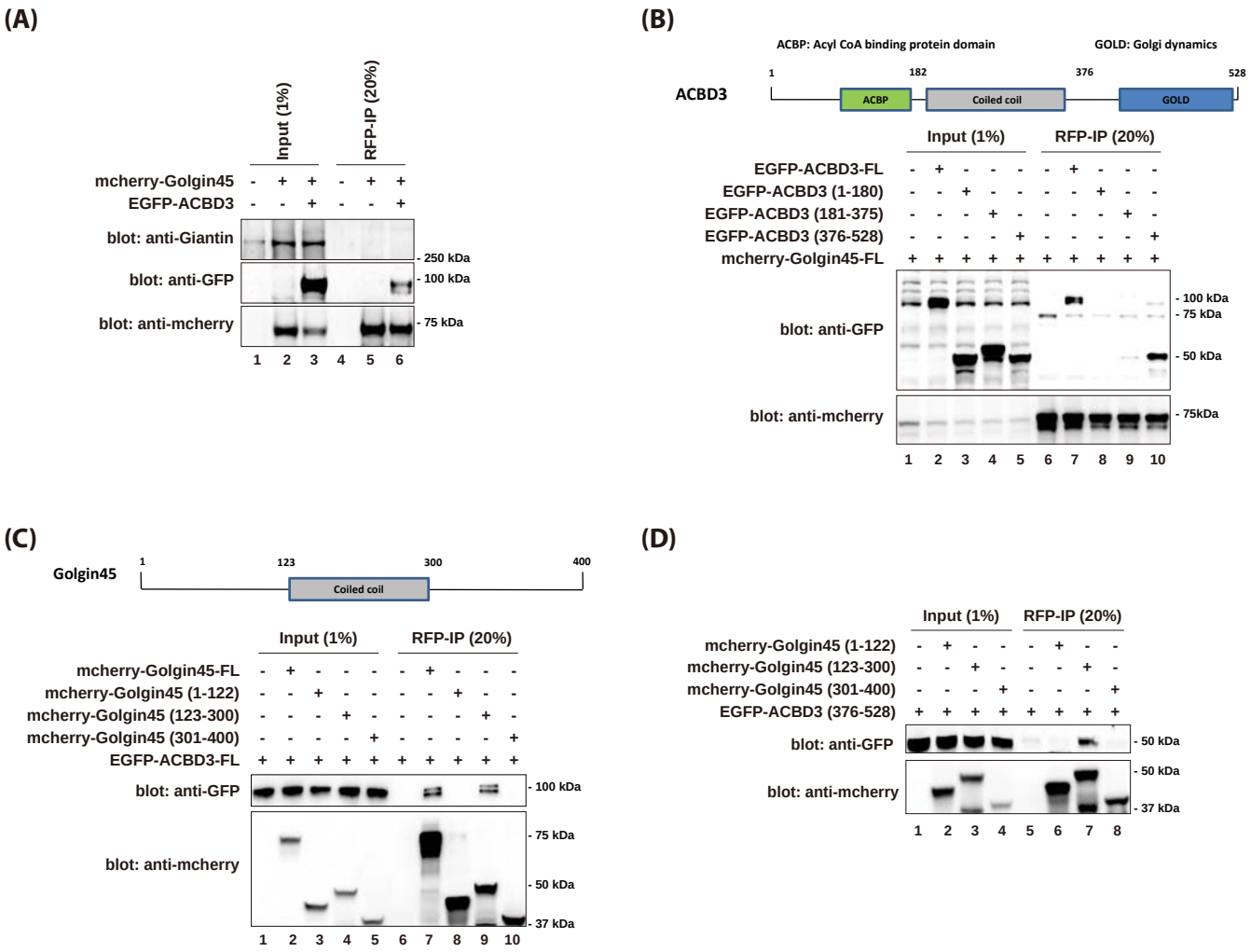
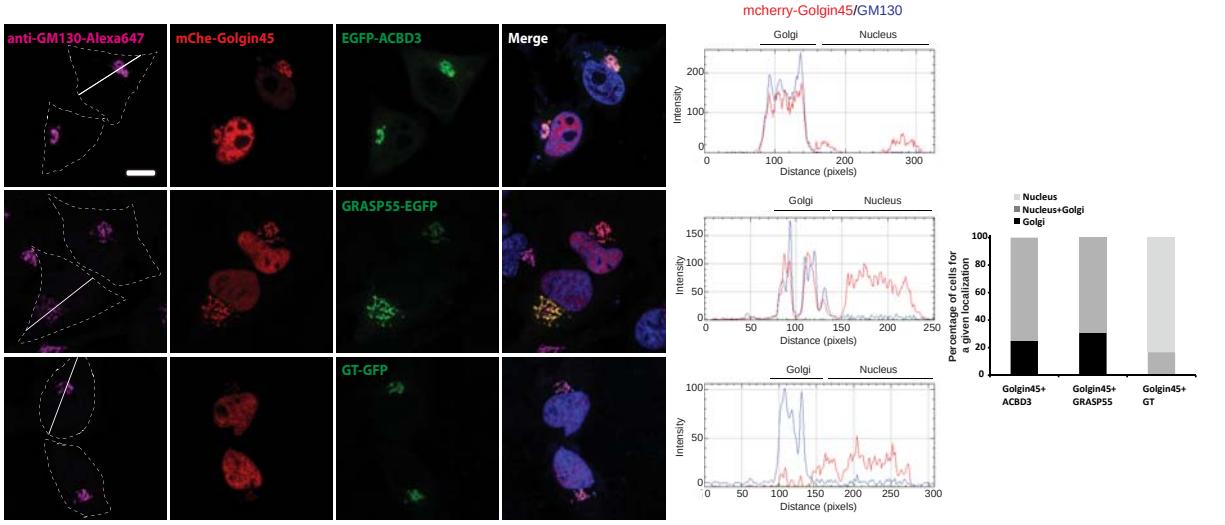
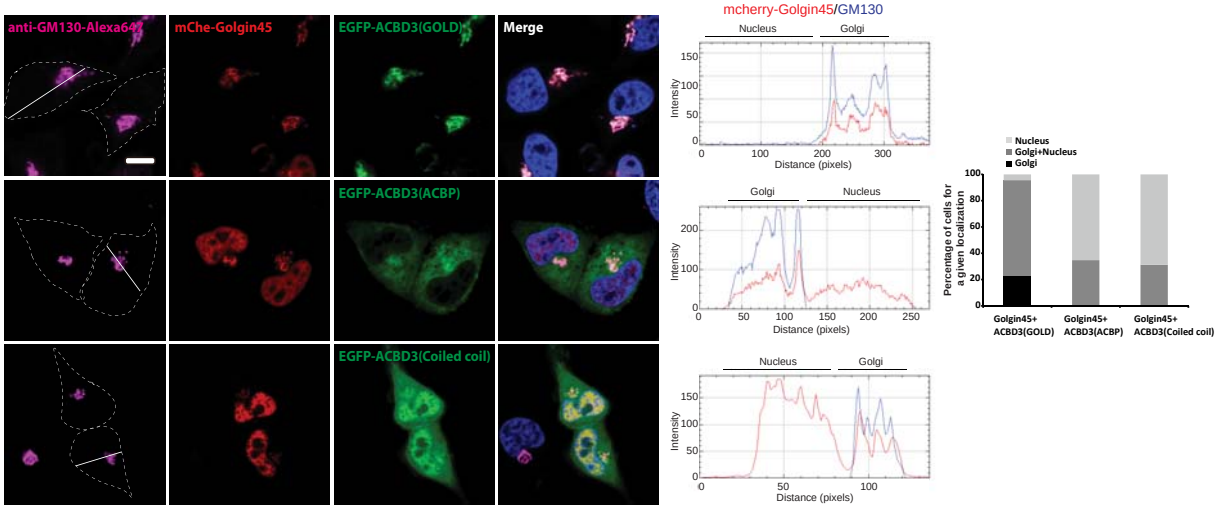

(A)
Input (1%)
RFP-IP (20%)
mcherry-Golgin45 - + + - + +
EGFP-ACBD3 - - + - - +
blot: anti-Giantin
blot: anti-GFP
blot: anti-mcherry
- 250 kDa
- 100 kDa
- 75 kDa
1 2 3 4 5 6
(B)
ACBP: Acyl CoA binding protein domain
GOLD: Golgi dynamics
ACBD3
1
182
376
528
ACBP
Coiled coil
GOLD
Input (1%)
RFP-IP (20%)
EGFP-ACBD3-FL - + - - - - + - - -
EGFP-ACBD3 (1-180) - - + - - - - + - -
EGFP-ACBD3 (181-375) - - - + - - - - + -
EGFP-ACBD3 (376-528) - - - - + - - - - +
mcherry-Golgin45-FL + + + + + + + + + +
blot: anti-GFP
blot: anti-mcherry
- 100 kDa
- 75 kDa
- 50 kDa
- 75kDa
1 2 3 4 5 6 7 8 9 10
(C)
Golgin45
1
123
300
400
Coiled coil
Input (1%)
RFP-IP (20%)
mcherry-Golgin45-FL - + - - - - + - - -
mcherry-Golgin45 (1-122) - - + - - - - + - -
mcherry-Golgin45 (123-300) - - - + - - - - + -
mcherry-Golgin45 (301-400) - - - - + - - - - +
EGFP-ACBD3-FL + + + + + + + + + +
blot: anti-GFP
blot: anti-mcherry
- 100 kDa
- 75 kDa
- 50 kDa
- 37 kDa
1 2 3 4 5 6 7 8 9 10
(D)
Input (1%)
RFP-IP (20%)
mcherry-Golgin45 (1-122) - + - - - + - -
mcherry-Golgin45 (123-300) - - + - - - + -
mcherry-Golgin45 (301-400) - - - + - - - +
EGFP-ACBD3 (376-528) + + + + + + + +
blot: anti-GFP
blot: anti-mcherry
- 50 kDa
- 50 kDa
- 37 kDa
1 2 3 4 5 6 7 8

Fig.3

(A)


anti-GM130-Alexa647
mChe-Golgin45
EGFP-ACBD3
Merge
GRASP55-EGFP
GT-GFP
mcherry-Golgin45/GM130
Golgi
Nucleus
Intensity
Distance (pixels)
Nucleus
Nucleus+Golgi
Golgi
Percentage of cells for a given localization
Golgin45+ ACBD3
Golgin45+ GRASP55
Golgin45+ GT

(B)


anti-GM130-Alexa647
mChe-Golgin45
EGFP-ACBD3(GOLD)
Merge
EGFP-ACBD3(ACBP)
EGFP-ACBD3(Coiled coil)
mcherry-Golgin45/GM130
Nucleus
Golgi
Intensity
Distance (pixels)
Nucleus
Golgi+Nucleus
Golgi
Percentage of cells for a given localization
Golgin45+ ACBD3(GOLD)
Golgin45+ ACBD3(ACBP)
Golgin45+ ACBD3(Coiled coil)

(C)

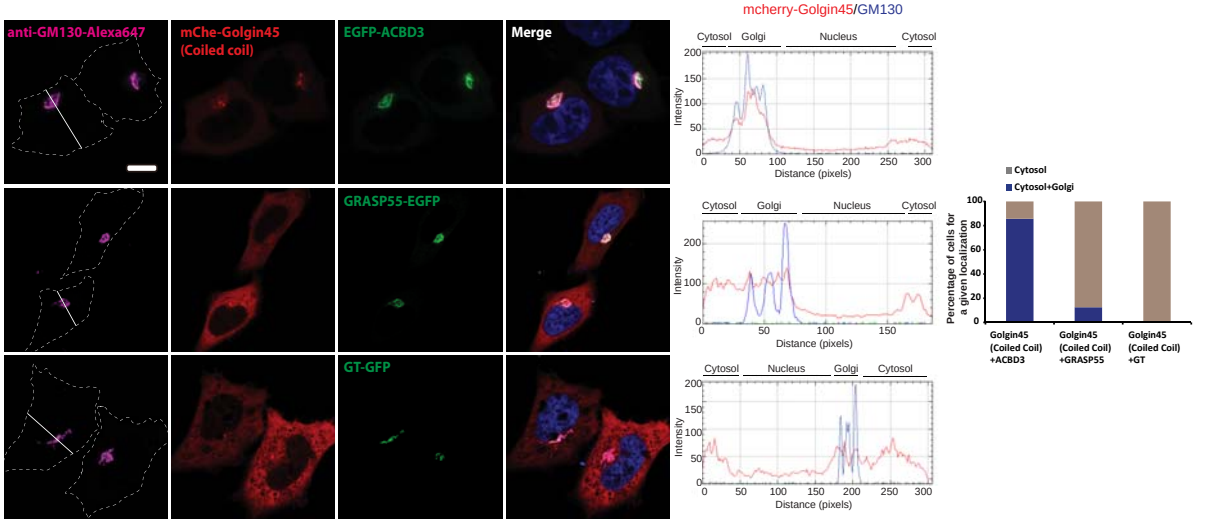
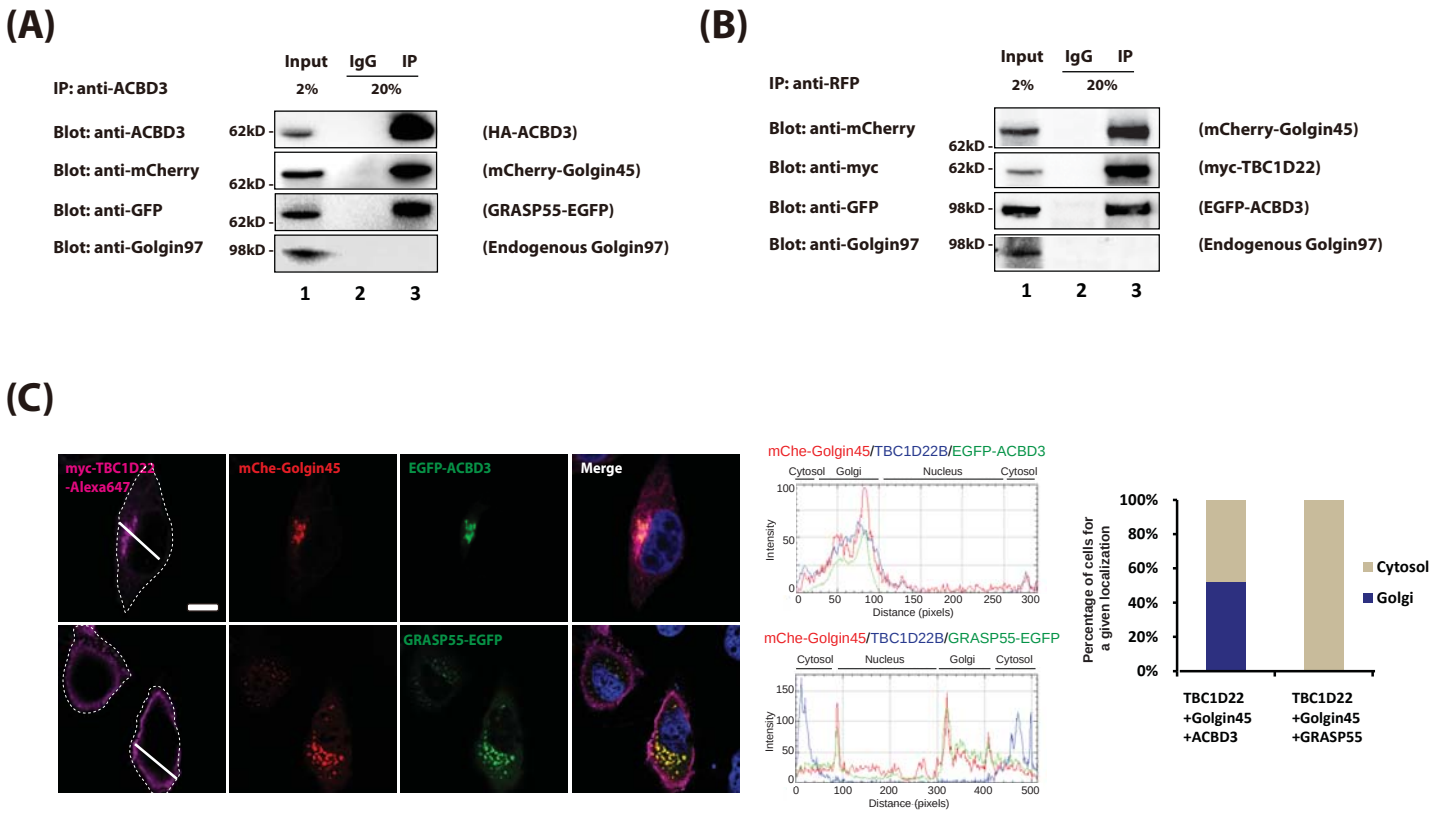

anti-GM130-Alexa647
mChe-Golgin45 (Coiled coil)
EGFP-ACBD3
Merge
GRASP55-EGFP
GT-GFP
mcherry-Golgin45/GM130
Cytosol
Golgi
Nucleus
Intensity
Distance (pixels)
Cytosol
Cytosol+Golgi
Percentage of cells for a given localization
Golgin45 (Coiled Coil) +ACBD3
Golgin45 (Coiled Coil) +GRASP55
Golgin45 (Coiled Coil) +GT

Fig.4


(A)
Input
IgG
IP
IP: anti-ACBD3
2%
20%
Blot: anti-ACBD3
62kD
(HA-ACBD3)
Blot: anti-mCherry
62kD
(mCherry-Golgin45)
Blot: anti-GFP
62kD
(GRASP55-EGFP)
Blot: anti-Golgin97
98kD
(Endogenous Golgin97)
1 2 3
(B)
Input
IgG
IP
IP: anti-RFP
2%
20%
Blot: anti-mCherry
62kD
(mCherry-Golgin45)
Blot: anti-myc
62kD
(myc-TBC1D22)
Blot: anti-GFP
98kD
(EGFP-ACBD3)
Blot: anti-Golgin97
98kD
(Endogenous Golgin97)
1 2 3
(C)
myc-TBC1D22
-Alexa647
mChe-Golgin45
EGFP-ACBD3
Merge
GRASP55-EGFP
mChe-Golgin45/TBC1D22B/EGFP-ACBD3
Cytosol
Golgi
Nucleus
Cytosol
Intensity
Distance (pixels)
mChe-Golgin45/TBC1D22B/GRASP55-EGFP
Cytosol
Nucleus
Golgi
Cytosol
Intensity
Distance (pixels)
Percentage of cells for a given localization
100%
80%
60%
40%
20%
0%
Cytosol
Golgi
TBC1D22 +Golgin45 +ACBD3
TBC1D22 +Golgin45 +GRASP55
(D)
Rab33b-GAP TBC1D22
ACBD3
ACBP
Coiled coil
GOLD
Golgin45
Coiled coil
GRASP55
PDZ

supplementary Fig.1

ANALYSIS

Extract intensities

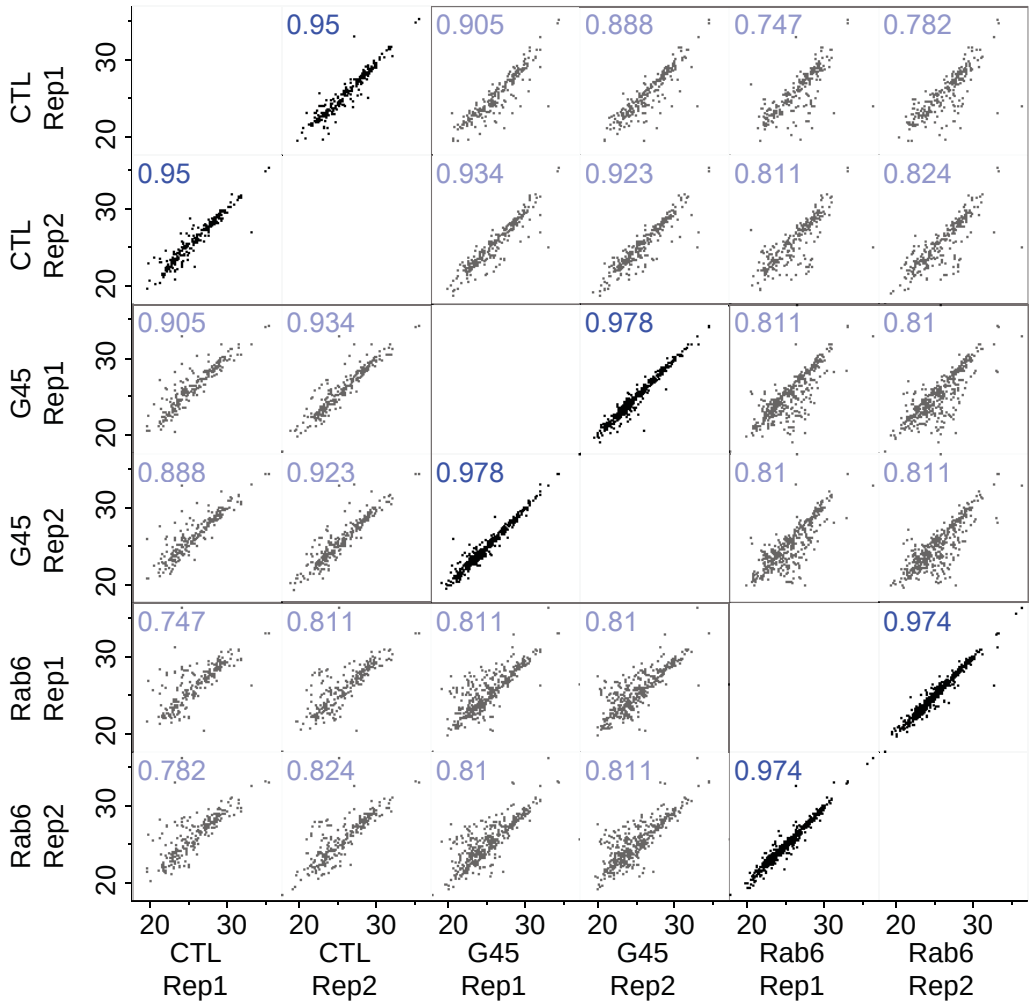
↓

Log2 transformed (raw data Excel sheet) → Impute missing values (TTest Excel sheet) → Pairwise Ttest

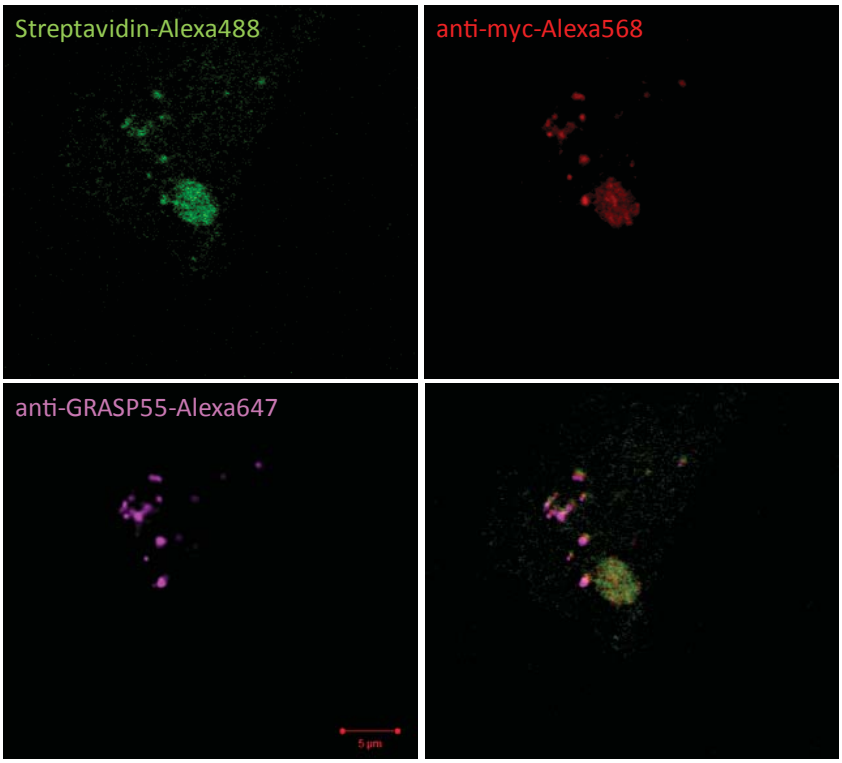
GLOBAL PARAMETERS

	Log2(intensity Control)		Log2(intensity G45)		Log2(intensity Rab6)	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
Mean	25.4	25.0	24.8	24.8	24.8	24.5
Standard deviation	3.0	3.1	2.9	2.9	2.6	2.7
Valid values	246	293	469	473	599	638

CORRELATION OF REPLICATES



Supplementary Fig.2



Supplementary Fig.3