BioID Performed on Golgi Enriched Fractions Identify C10orf76 as a GBF1 Binding Protein Essential for Golgi Maintenance and Secretion

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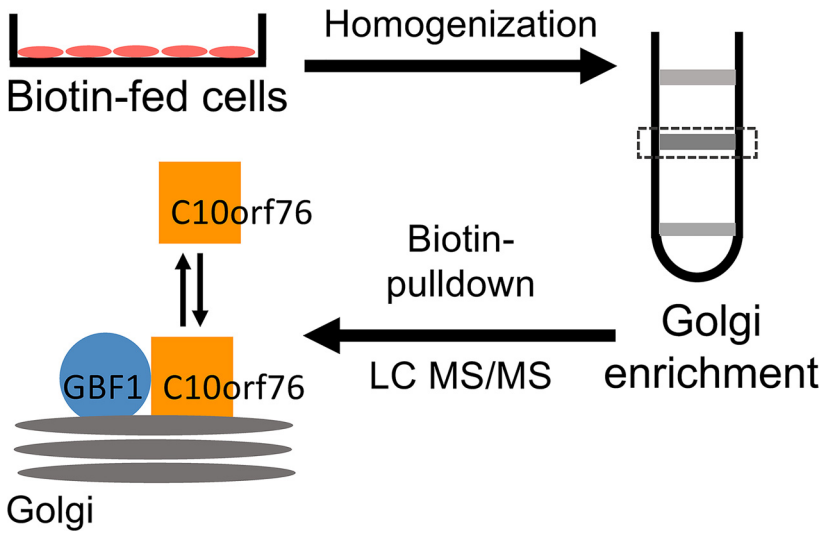
In Brief

The Golgi-specific Brefeldin-A resistance factor 1 (GBF1) regulates Arf activation at the *cis*-Golgi. BioID and mass spectrometry from Golgi-enriched fractions identified C10orf76 as a GBF1 proximal protein that may regulate its recruitment. Knockdown studies revealed that C10orf76 is involved in Golgi maintenance. It interacts with GBF1 and rapidly cycles on and off Golgi structures. Knockdown causes fragmentation, alters GBF1 recruitment, and impairs secretion. Lastly, homologs were identified in most species, suggesting its presence in the last eukaryotic common ancestor.

Graphical Abstract



Highlights

- BioID with Golgi fractions identified C10orf76 as proximal to GBF1.
- Tagged C10orf76 overlaps with Golgi markers.
- C10orf76 binds GBF1 and exchanges rapidly between free and bound forms.
- C10orf76 is essential for maintenance of the Golgi and for secretion.

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BioID Performed on Golgi Enriched Fractions Identify C10orf76 as a GBF1 Binding Protein Essential for Golgi Maintenance and Secretion*[S]

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The Golgi-specific Brefeldin-A resistance factor 1 (GBF1) is the only large GEF that regulates Arf activation at the *cis*-Golgi and is actively recruited to membranes on an increase in Arf-GDP. Recent studies have revealed that GBF1 recruitment requires one or more heat-labile and protease-sensitive protein factor(s) (Quilty *et al.*, 2018, J. Cell Science, 132). Proximity-dependent biotinylation (BioID) and mass spectrometry from enriched Golgi fractions identified GBF1 proximal proteins that may regulate its recruitment. Knockdown studies revealed C10orf76 to be involved in Golgi maintenance. We find that C10orf76 interacts with GBF1 and rapidly cycles on and off GBF1-positive Golgi structures. More importantly, its depletion causes Golgi fragmentation, alters GBF1 recruitment, and impairs secretion. Homologs were identified in most species, suggesting its presence in the last eukaryotic common ancestor. ***Molecular & Cellular Proteomics 18: 2285–2297, 2019. DOI: 10.1074/mcp.RA119.001645.***

The Golgi functions as the central organizing organelle and resides at the center of the secretory pathway (1). The central Golgi stacks receive newly synthesized proteins from the ER-Golgi intermediate compartment (ERGIC)[1] and functions in the modification, sorting, and trafficking of this newly synthesized cargo through to the *trans*-Golgi network (TGN). Once there, proteins can then be secreted or trafficked to other membrane compartments including endosomes, lysosomes, and the plasma membrane. Over one third of the proteins encoded by the human genome are trafficked through the Golgi complex, and consequently, any mutations negatively affecting these processes can impair cellular function and cause disease (2).

Vesicle formation at the Golgi requires the recruitment of coat proteins, a process regulated by ADP-ribosylation factors (Arfs) (3). Arfs belong to a family of small GTPases whose regulatory effects occur through cycling between GTP and GDP bound states. Arf guanine nucleotide exchange factors (GEFs) promote this nucleotide exchange reaction, activating Arfs by exchanging GDP for GTP. Only in their active GTP-bound form are Arfs able to interact with their effectors, including coat proteins, and lipid-modifying enzymes, which are required for vesicle formation, budding, and transport. The time and location of Arf activation is coordinated by their associated ArfGEFs (4).

At the *cis*-Golgi, the only large Arf-GEF able to catalyze Arf nucleotide exchange reactions is the Golgi-specific BFA Resistance Factor 1 (GBF1) (5). The activity of GBF1 must be regulated to ensure proper maintenance of Golgi structure and direct trafficking of cargo between the ERGIC and Golgi stacks. Loss of GBF1 activity leads to Golgi collapse and ultimately, cell death (6). The nucleotide exchange reaction must occur on membranes and requires the recruitment and association of both the ArfGEF and its Arf-GDP substrate. In addition to its role at the Golgi, GBF1 has been reported to function at multiple sites, from endosomes (7) to mitochondria (8).

Recent *in vitro* evidence argues for the existence of protein-based membrane-associated component(s) that facilitate GBF1 binding and recruitment to Golgi membranes (9). However, because of the transient nature of GBF1's interaction with the membrane, the identification of these interacting proteins has proven rather challenging. Genetic screens performed in yeast as well as traditional immunoprecipitation assays have had some success in identifying GBF1 interactors, including GMH1 and p115 (10, 11). However, neither protein was revealed to be involved in regulating GBF1 recruitment. Because of the highly dynamic nature in which GBF1 cycles on and off Golgi membranes, a sensitive technique is required to capture these interactors. Here, we use the proximity-dependent biotinylation method (BioID) on enriched Golgi fractions to identify the GBF1 local interactome, which likely consists of transient, weak and/or poorly soluble GBF1 complexes. The BioID approach relies on the use of an abortive biotin ligase, BirA*, that when appropriately tagged to

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a protein of interest, allows for the irreversible biotinylation of proximal proteins *in vivo* (12–14). When expressed in live cells, supplementation of exogenous biotin will promote the activity of BirA* and the conjugation of biotin to primary amines (*e.g.* lysine side chains) on proteins surrounding the bait (15). These proximal proteins can then be isolated by streptavidin affinity purification and identified by mass spectrometry. The coupling of BioID with Golgi enrichment allowed our focus on the identification of Golgi-localized proteins.

Using this method, we identified a previously uncharacterized peripheral Golgi protein, C10orf76 (also referred to as ARMH3 by NCBI) that interacts with GBF1 and appears to be involved in GBF1 recruitment, Golgi maintenance, and protein secretion.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Cells were maintained in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 5% CO_2 and 37 °C. BFA was purchased from Sigma-Aldrich (St-Louis, MO) and dissolved in DMSO at 1 mg/ml. Doxycycline was purchased from Fisher Scientific (Ottawa, Canada) and dissolved in UltraPure distilled water (Invitrogen) at 1 mg/ml. Puromycin was purchased from Gibco and dissolved in UltraPure distilled water at 10 mg/ml. Sequa-brene was purchased from Sigma-Aldrich and dissolved in UltraPure distilled water (Invitrogen) at 8 mg/ml. Biotin was purchased from Sigma-Aldrich and dissolved in serum-free DMEM at 1 mM. The cell lines used in this study include HeLa cells (ECACC; Sigma-Aldrich, 93031013), HEK293 cells (ATCC, CRL-1573), HeLa cells stably expressing Enhanced GFP (EGFP)-tagged GBF1 (9), and Flp-In T-Rex HeLa cells containing a tetracycline operator regulated BirA*-FLAG-GBF1 or BirA*-FLAG transgene (16).

Isolation of the tetracycline inducible BirA*-FLAG-GBF1 (16) or BirA*-FLAG HeLa cells involved Flip-In T-REx and Gateway cloning systems (Invitrogen). First, PCR amplified full-length GBF1 was introduced into a Gateway pENTRY vector using a TOPO cloning kit by Invitrogen. The GBF1 gene cassette was then transferred from the pENTRY plasmid into the pcDNA5-pcDEST-BirA-FLAG-N-ter vector obtained from Dr. Anne-Claude Gingras (Lunenfeld-Tanenbaum Research Institute, Toronto, Canada) using the LR clonase enzymes. The pcDNA-pcDEST-BirA-FLAG-N-term vector with and without the GBF1 gene cassette was cotransfected with pOG44 into HeLa T-Rex Flp-In cells obtained from Dr. S. Taylor (University of Manchester, Manchester, United Kingdom). Stable cell populations containing BirA*-FLAG-GBF1 or BirA-FLAG were selected for using 150 μg/ml hygromycin over a two-week period. Tetracycline regulated expression of the transgene in hygromycin resistant cells was confirmed by treatment with 0.1 μg/ml doxycycline followed by immunoblotting for the FLAG-tagged proteins (17) (See supplemental Fig. S1). Molecular biology manipulations were performed as per manufacturer's instructions.

The following primary antibodies were used for IF experiments: mouse anti-FLAG (Rockland, Limerick, PA, at 1:100), mouse anti-GBF1 (clone 25) (BD Bioscience; 1:1000), rabbit anti-giantin (1:2500) (From Dr. Edward K.L. Chan; University of Florida Health, Jacksonville; 1:2000), mouse anti-p115 (clone 7D1) (from Dr. Gerry Waters through the late Dr. Dennis Shields; 1:1000), sheep anti-TGN46 (AbD Serotec; 1:1000), mouse anti-β COP (M3A5) (Sigma-Aldrich; 1:250). The following primary antibodies were used for immunoblotting experiments: mouse anti-FLAG (Rockland; 1:10,000), rabbit anti-GBF1 (9D4) (17); 1:500), mouse anti-tubulin (Sigma-Aldrich; 1:1000), mouse anti-GM130 (BD Bioscience; 1:250), mouse anti-VDAC1 (Abcam; 1:5000). Streptavidin-cy3 (Invitrogen; 1:1000) was used to detect biotinylated proteins in IF experiments and Alexa Fluor 690 streptavidin (Invitrogen; 1:10,000) was used for detection in immunoblotting experiments, both without a secondary antibody. Secondary antibodies used for IF were all obtained from Invitrogen, used at 1: 1000, and include: Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 647 donkey anti-rabbit, Alexa Fluor 647 donkey anti-mouse, Alexa Fluor 555 donkey anti-sheep. Secondary antibodies used for immunoblotting were all obtained from Invitrogen, used at 1:10,000, and includes: Alexa Fluor 680 goat anti-rabbit, Alexa Fluor 750 goat anti-mouse.

Proximity-dependent Biotinylation and Enrichment of Golgi Membranes—Proximity-dependent biotinylation occurred under conditions described in Roux *et al.*, 2013. In brief, tetracycline inducible BirA*-FLAG-GBF1 or BirA*-FLAG HeLa cells were grown in five 15 cm cell culture dishes until 80% confluence. Cells were then treated first with 0.1 μg/ml doxycycline for six hours to allow for expression of BirA*-FLAG proteins, followed by further supplementation with 50 μg/ml exogenous biotin for an additional 24-hour incubation. Cells were then collected by treatment with 0.25% trypsin-EDTA (Gibco, Carlsbad, CA) followed by neutralization with DMEM with 10% FBS, and centrifugation at 298.2 $\times$ *g* for 10 min in a Sorvall RT7 benchtop centrifuge with a RTH-750 swinging bucket rotor. Pelleted cells were then washed in PBS to remove residual medium components and then re-pelleted.

For preparation of Golgi-enriched membrane samples pelleted cells were then resuspended in ice-cold homogenization buffer (10 mM Tris-HCl pH 7.6, 0.25 M Sucrose, 150 mM KCl, 1.0 mM $MgCl_2$, 2$\times$ complete EDTA-free protease inhibitor (Roche, Mannheim, Germany). Cells were then homogenized by 20 passages through a cell homogenizer (Isobiotech, Heidelberg, Germany) with a 16 μm clearance. Homogenates were centrifuged at 4 °C and 800 $\times$ *g* for 10 min to pellet nuclei and unbroken cells. The supernatant was then adjusted to 1.2 M sucrose and layered in a sucrose gradient for organelle separation by density centrifugation (gradient from top to bottom: 0.25 M, 1.0 M, 1.2 M, 1.3 M, 2 M). Samples were centrifuged at 4 °C and 234116.4 $\times$ *g* for 3 h in an Optima ultracentrifuge with a SW 41 Ti swinging bucket rotor (Beckman Coulter, Missisauga, Canada). Enriched-Golgi fractions were collected between the 0.25 M and 1.0 M sucrose interface, washed with PBS, and pelleted by centrifugation at 4 °C and 163877.9 $\times$ *g* for 20 min in an Optima TLX Benchtop Ultra centrifuge (Beckman Coulter) with a TLA-100.4 rotor.

Pull-down of Biotinylated Proteins and Identification by Mass Spectrometry—The BirA*FLAG and BirA*FLAG-GBF1 cell pellet and Golgi fractions were resuspended in 10 ml and 5 ml of lysis buffer respectively, (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1:500 protease inhibitor mixture (Sigma-Aldrich), 1:1,000 benzonase nuclease (Novagen) and incubated on an end-over-end rotator at 4 °C for 1 h, briefly sonicated to disrupt any visible aggregates, then centrifuged at 45,000 $\times$ *g* for 30 min at 4 °C. Supernatants were transferred to a fresh 15 ml conical tube. 30 μl of packed, pre-equilibrated Streptavidin-Sepharose beads (GE) were added and the mixture incubated for 3 h at 4 °C with end-over-end rotation. Beads were pelleted by centrifugation at 2,000 rpm for 2 min and transferred with 1 ml of lysis buffer to a fresh Eppendorf tube. Beads were washed once with 1 ml lysis buffer and twice with 1 ml of

[1] The abbreviations used are: ERGIC, ER-Golgi intermediate compartment; TGN, *trans*-Golgi network; Arfs, ADP-ribosylation factors; BFA, Brefeldin A; BioID, Proximity-dependent biotinylation; C10orf76, Chromosome 10 predicted ORF of 76 KDa; DMEM, Dulbeco's modified Eagle's medium; GBF1, Golgi-specific Brefeldin-A resistance factor 1; GEF, Guanine nucleotide Exchange Factor; IF, immunofluorescence; WB, western blotting.

50 mM ammonium bicarbonate (pH = 8.3). Beads were transferred in ammonium bicarbonate to a fresh centrifuge tube and washed two more times with 1 ml ammonium bicarbonate buffer. Tryptic digestion was performed by incubating the beads with 1 μg MS-grade TPCK trypsin (Promega, Madison, WI) dissolved in 200 μl of 50 mM ammonium bicarbonate (pH 8.3) overnight at 37 °C. The following morning, 0.5 μg MS-grade TPCK trypsin was added, and beads were incubated 2 additional hours at 37 °C. Beads were pelleted by centrifugation at 2,000 × *g* for 2 min, and the supernatant was transferred to a fresh Eppendorf tube. Beads were washed twice with 150 μl of 50 mM ammonium bicarbonate, and these washes were pooled with the first eluates. The sample were lyophilized and resuspended in buffer A (0.1% formic acid). One fifth of the sample was analyzed per MS run.

High performance liquid chromatography was conducted using a 2 cm pre-column (Acclaim PepMap 50 mm × 100 μm inner diameter (ID)), and 50 cm analytical column (Acclaim PepMap, 500 mm × 75 μm diameter; C18; 2 μm; 100 Å, Thermo Fisher Scientific, Waltham, MA), running a 120 min reversed-phase buffer gradient at 225 nl/min on a Proxeon EASY-nLC 1000 pump in-line with a Thermo Q-Exactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 60,000, then up to the twenty most intense peaks were selected for MS/MS (minimum ion count of 1000 for activation) using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated such that MS/MS of the same *m/z* (within a range of 10 ppm; exclusion list size = 500) detected twice within 5 s were excluded from analysis for 15 s.

For protein identification, raw files were converted to the .mzXML format using Proteowizard (v3.0.10800; (18), then searched using X!Tandem (v2013.06.15.1; (19) against Human RefSeq Version 45 (containing 36,113 entries). Search parameters specified a parent MS tolerance of 15 ppm and an MS/MS fragment ion tolerance of 0.4 Da, with up to two missed cleavages allowed for trypsin. No fixed modifications were set but oxidation of methionine was allowed as a variable modification. Data were analyzed using the trans-proteomic pipeline (20) via the ProHits 5.0.2 software suite (21). Proteins identified with a iProphet cut-off of 0.9 (corresponding to ≤1% probabilistic FDR (22) and 2+ unique peptides were analyzed with SAINT Express v. 3.6.1 (23, 24) to identify high-confidence interactors. The four controls were collapsed to the highest two spectral counts for each hit. Proteins identified with two or more unique peptides and scoring above a Bayesian False Discovery Rate of 1% (see (24) for details on BFDR calculation) were high-confidence proximity interactors. All data are publicly available and have been uploaded to the MassIVE archive (www.massive.ucsd.edu) with ID: MSV000083866.

Experimental Design and Statistical Rationale—Four BioID runs were conducted on FlagBirA*-GBF1 expressing cells, both on the whole cell lysate and on the Golgi fraction. These four runs consisted of two technical replicates ($n = 2$) from two biological replicates ($n = 2$; total $n = 4$). Four control runs of a BioID analysis conducted on the corresponding fractions on cells expressing the FlagBirA* tag alone were used for comparative purposes.

Generation of mCherry-, EGFP-, and FLAG-tagged WT and Mutant C10orf76 Encoding Plasmids—The codon optimized C10orf76 open reading frame was obtained from Dr. Vincent A. Blomen. mCherry-, EGFP-, and FLAG-tagged versions were created by PCR amplification of the C10orf76 open reading frame and subcloning into a mCherry-C1, EGFP-C1, and FLAG-C1 vector, using restriction enzymes SacII and SbfI (for mCherry and EGFP), and SacII and BamHI (for FLAG). EGFP-tagged truncated C10orf76 plasmids were also generated using PCR amplification with the same restriction enzymes and vectors.

Cell Transfection and Imaging Experiments—Fixed-cell imaging experiments were performed with tissue culture cells grown on #1.5 glass coverslips (Fisher Scientific) in 6-well plates. Coverslips were sterilized in 70% ethanol. Transfection of plasmids for transient expression of tagged-proteins were performed on cells grown to ~60–70% confluency using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and cultured for ~18 h to allow for protein expression. For fixation, cells were washed in PBS warmed to 37 °C and fixed in 4% paraformaldehyde (with 100 μM calcium chloride and 100 μM magnesium chloride in PBS) at 37 °C for 20 min. Cells were then incubated in permeabilization buffer (0.1% Triton X-100 in PBS) to allow for subsequent labeling of intracellular structures by antibodies.

Live-cell imaging experiments were performed with cells grown on #1.5 25 mm round glass coverslips (Fisher Scientific) in 6-well dishes. During imaging, coverslips were transferred onto Attofluor cell chambers (Invitrogen) and maintained in CO_2-independent DMEM (Gibco Laboratories, Grand Island) supplemented with 10% FBS and maintained at 37 °C.

Both live- and fixed-cell imaging were performed using wide-field fluorescence microscopy in a DeltaVision Elite (GE Healthcare, Buckinghamshire, UK) microscope equipped with a front-illuminated sCMOS camera driven by softWoRx 6 (GE Healthcare) using a 60 × 1.4 NA oil objective (Olympus, Richmond Hill, Canada). Before analysis, images were deconvolved in softWoRx6 and processed using FIJI imaging software (National Institutes of Health, Bethesda, MD).

Quantification of fixed and live cells images was carried out using Imaris 8 software (Bitplane Scientific Software, South Windsor, CT), similarly to previously published studies (9, 25). In brief, three-dimensional surfaces were created around areas of interest (such as GBF1 positive structures) in selected cells by using the surfaces feature in Imaris 8. The average pixel intensity (Int) values in the surveyed regions and the volumes (Vol) of these regions were measured. Average Golgi intensity values were corrected by subtracting for the average cytosolic intensity. Whole-cell intensity was further corrected by subtracting the average intensity of the image background. The equation used for determining the fraction of signal at the Golgi = Golgi Vol (Golgi Int-cytosol Int)/cell Vol (cell Int-background Int). Average fold increase of signal at the Golgi is determined as fraction of signal at the Golgi after BFA/fraction of signal at the Golgi before BFA.

Fluorescence Recovery After Photobleaching (FRAP)—For FRAP experiments, images were acquired on a Quorum Technologies WaveFX microscope with a Yokagawa CSU 10 spinning disk confocal scan-head and a 60 × 1.42NA oil objective. Imaging was driven by the Perkin Elmer's Volocity program and photobleaching was performed using the Andor iQ3 live cell imaging software. Image analysis and fraction signal at the Golgi was performed and calculated in the same manner as for the live cell experiments described above. Fraction at the Golgi prior to bleaching for both GBF1 and C10orf76 was normalized to 1 for comparison. The average half-life ($t_{1/2}$) values were determined from the exponential portion of each graph.

Electron Microscopy—Cells were grown to about 80% confluency, fixed in Karnovsky (2% paraformaldehyde and 2% glutaraldehyde) and centrifuged at increasing speeds to produce pellets. Secondary fixation was in 1% osmium tetroxide for one hour, followed by staining with 1% uranyl acetate in water for one hour or O/N (All chemicals are from Ted Pella, Inc.). Pellets were dehydrated in increasing concentrations of ethanol and transferred into Epon 812 (EMS # 14120) beams for polymerization. Sectioning was done with UltracutE (Reichert Jung) followed by imaging with a Jeol JEM-2100 transmission electron microscope.

Immunoblotting—Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare) at 376 mA for two hours in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20%

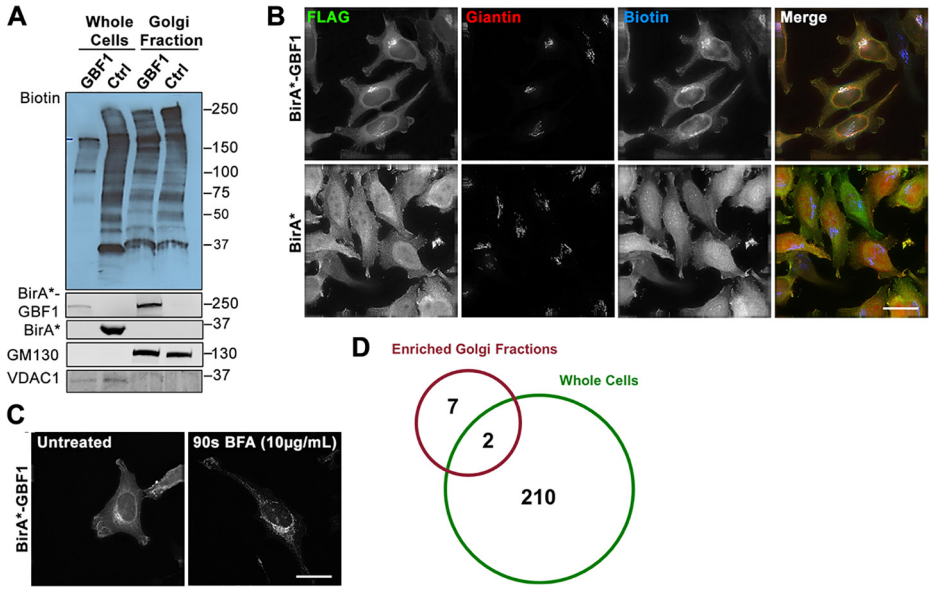

FIG. 1. **BirA*-FLAG-GBF1 localizes to Golgi structures and biotinylates GBF1 proximal proteins at the membrane.** *A* and *B*, HeLa cells stably expressing either BirA*-FLAG-GBF1 or BirA*-FLAG were analyzed 24 h after induction with excess biotin (50 μM). *A*, WB performed using whole cells and sucrose-gradient separated Golgi fractions confirms successful enrichment of GM130 positive Golgi membranes negative for mitochondrial marker VDAC1. In both whole cell and Golgi fractions, BirA*-GBF1 biotinylates proteins different from control. *B*, BirA*-GBF1 localizes to and biotinylates proteins on Giantin positive Golgi membranes, unlike nuclear and cytosolically diffuse BirA*. *C*, BirA*-GBF1 remains sensitive to inhibitory drug BFA (10 μg/ml) and can be actively recruited to juxtanuclear structures on treatment. All images are representative of 3 replicates and are shown as projections of all z-slices. Scale bars = 26 μm. *D*, Venn diagram depicting the 219 protein candidates identified [with BFDR ≤0.01] in the BioID experiment using either whole cell lysates or enriched Golgi fractions.

(v/v) methanol, 2.5% (v/v) isopropanol). Membranes were then blocked in Licor Odyssey Blocking Reagent (Licor Biotechnology, Lincoln, NE) for at least one hour. Blocked membranes were then incubated with primary antibodies then secondary antibodies each in 50% Licor Odyssey Blocking Reagent in PBS. Detection of biotinylated proteins utilized Alexa Fluor 690 streptavidin without a secondary antibody. Membranes were then washed twice in Tris buffered saline with Tween-20 (50 mM NaCl, 0.5% (v/v) Tween-20, 20 mM Tris-HCl pH 7.5) for 10 min each followed by three washes in PBS for 10 min each. Membranes were then scanned on a Licor Odyssey scanner (Licor Biotechnology).

Quantitation of immunoblots was performed using Image Studio version 5.0 (Licor Biotechnology). Band intensities were quantified by manually drawing a rectangle around a region of interest, which was then corrected for background using the average pixel intensity in a 3-pixel region along the edges of the drawn rectangle. Quantified intensity values were then exported with Excel worksheets (Microsoft, Redmond) where mean, standard deviations, and normalization calculations were performed.

shRNA Lentiviral Knockdown—Plasmids encoding shRNAs targeting genes of interest were obtained from the Sigma-Aldrich MISSION shRNA library. Sequences selected for use can be found in supplemental Table S2. Lentiviruses containing the shRNA encoding plasmids were generated by cotransfecting 80% confluent HEK293 cells in 6-well plates with shRNA encoding plasmids and the Sigma Aldrich MISSION Lentiviral Packaging Mix using LipoD293 DNA In Vitro Transfection Reagent (FroggaBio). The secreted viruses were collected over a period of 3 days and filtered with 0.45 μm low protein binding PVDF syringe filter (Millex). Lentiviruses were stored at −80 °C until use.

shRNA knockdowns were performed on tissue culture cells transduced with the appropriate shRNA plasmid containing lentiviruses (multiplicity of infection at two) with 8 μg/ml Sequa-brene (Sigma Aldrich). Cells were incubated with the lentivirus for 24 h prior to replacing the media with DMEM with 10% FBS and 0.5 μg/ml puromycin (Fisher Scientific) to select for transduced cells. All shRNA knockdown time values refer to time post-transduction, and in all cases selective puromycin was added 24 h post-transduction.

Coimmunoprecipitation—HeLa cells at ~60–70% confluency were transfected with the plasmids indicated using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and cultured for ~18 h to allow for protein expression. Cells were then washed twice with PBS and lysed in modified-RIPA lysis buffer (1% NP-40, 150 mM NaCl, 0.35% sodium deoxycholate, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$). Post-nuclear supernatants were collected by spinning the lysates at 4 °C at 800 × *g* for 5 min. The supernatants were then precleared and incubated overnight with 4 μl of rabbit anti-FLAG antibodies (Sigma-Aldrich). 25μl of protein A Sepharose beads were used to precipitate the FLAG-tagged protein complexes.

Peripheral Protein Extraction—Collection of enriched Golgi fractions using a sucrose-gradient was performed as described above (see Proximity Biotinylation Assay and Enrichment of Golgi Membranes under Experimental Procedures section), using nontransgenic HeLa cells. Pelleted membranes were then resuspended in either ice-cold PBS or 0.1 M Na_2CO_3 (pH 11.3) and incubated on ice for 30 min. Lastly, membranes were pelleted by centrifugation at 4 °C and 163877.9 × *g* for 20 min in an Optima TLX Benchtop Ultra centrifuge (Beckman Coulter, Brea, Canada) with a TLA-100.4 rotor. Supernatant and pellets were collected separately and mixed with appropriate quantities of sample buffer prior to gel electrophoresis.

Luciferase Secretion Assay—HeLa cells were transduced with either a nontargeting shRNA encoding plasmid or a GBF1 or C10orf76 shRNA encoding plasmid for 72 h as described above and transfected with a pNL1.3CMV[SecNluc] plasmid (Promega) encoding a NanoLuciferase. Secretion measurements were performed as previously described in (26). In brief, cells were washed with serum-free

medium to remove any already secreted luciferase and placed in fresh medium with or without BFA. After a 2-hour incubation, the medium was assessed for the presence of luciferase using the luciferase substrate coelenterazine (Gold Biotechnology St. Louis, MO) prepared at 1.4 μM in luciferase assay buffer (25 mM glycylglycine pH 7.8, 15 mM K_2PO_4 pH 7.8, 15 mM $MgSO_4$, and 4 mM EGTA). Total cell luciferase was measured similarly after lysis in luciferase lysis buffer (0.1% (v/v) Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol). Signal was quantitated with a fluorescence microplate reader (BioTek). Fraction of secreted luciferase was calculated as (total signal from growth medium)/(total signal from growth medium + total signal from lysed cells).

Homology Searching of C10orf76 Orthologs—C10orf76 orthologs were identified using homology searches performed with BLASTp (Altschul *et al.*, 1997) on a representative sampling of eukaryotic organisms on or before 1/3/19. The human C10orf76 protein sequence was obtained from NCBI (NP_078817.2). See supplemental Table S3 for the complete list of eukaryotic organisms examined and accession numbers for all identified sequences. Searches were performed using a bidirectional strategy. Putative top hits retrieved using the human C10orf76 query were deemed as positive hits only if they also retrieved the human C10orf76 in a reciprocal BLASTp search. Additional BLASTp and tBLASTn searches were performed using the *S. pombe, M. polymorpha,* and *T. spiralis* C10orf76 orthologs to identify potential divergent sequences in *S. cerevisiae*, A, thaliana, and *C. elegans,* respectively, none of which revealed any positive hits. An E-value cutoff of 10^{-12} was applied to all searches.

RESULTS

BirA-FLAG-GBF1 Biotinylates GBF1 Proximal Proteins Present on Golgi Structures*—Previous *in vitro* experiments revealed the presence of a Golgi-localized protease-sensitive component involved in facilitating GBF1 recruitment to membranes (9). Several GBF1 interactors including p115 (10), membrin (10, 27, 28), and GMH1 (11) have been previously identified using classical coimmunoprecipitation and genetic approaches. However, apart from its substrate, Arf-GDP, no known GBF1 interactor has yet been shown to be involved in regulating GBF1 recruitment. Knock-out and/or depletion or overexpression of these GBF1 interactors revealed negligible to minimal impact on GBF1 recruitment. One potential hurdle in identifying components regulating GBF1 recruitment includes GBF1's dynamic shuttling on and off Golgi membranes. Further, very little GBF1 appears required to sustain Golgi function (5, 25). For these reasons, we chose to utilize the proximity-dependent biotinylation approach (BioID) developed and popularized by the Roux laboratory (Sanford Children's Health Research Center, Sioux Falls (SD); (12) to allow detection of transient GBF1 interactors. For these experiments, we generated HeLa cell lines expressing either BirA*-FLAG-GBF1, or BirA*-FLAG alone under the control of a doxycycline-inducible promoter. The BirA*-FLAG expressing cell line acts as a control, for detection of nonspecific background interactions, and the doxycycline inducible system allows for regulated expression of the toxic GBF1 protein. By ensuring the transgene is expressed at moderately low levels we can curb toxicity and other potential nonspecific effects (12). Preliminary experiments first confirmed that doxycycline addition

TABLE I

Spectral counts and Bayesian False Discovery Rates (BFDR) for candidate GBF1 interactors identified in whole cell lysate (WCL) and Golgi-enriched fractions, as indicated (see supplemental Table S1 for coverage and unique peptide information)

Gene Symbol	Gene Name	WCL FlagBirA-only controls					WCL FlagBirA-GBF1						Golgi FlagBirA-only controls					Golgi FlagBirA-GBF1						
		Pool A		Pool B		SUM	Pool A		Pool B		SUM	BFDR	Pool A		Pool B		SUM	Pool A		Pool B		SUM	BFDR
		1	2	1	2		1	2	1	2			1	2	1	2		1	2	1	2		
GBF1	*golgi brefeldin A resistant guanine nucleotide exchange factor 1*	0	0	0	0	0	1412	1283	1299	1332	**5326**	na	0	0	12	9	21	649	619	613	581	**2462**	na
SLC30A6	*solute carrier family 30 member 6*	0	0	0	0	0	43	42	36	37	**158**	**0**	0	0	7	5	12	78	80	89	80	**327**	**0**
SLC30A5	*solute carrier family 30 member 5*	0	0	0	0	0	22	21	26	26	**95**	**0**	0	0	6	4	10	44	52	58	43	**197**	**0**
GOSR1	*golgi SNAP receptor complex member 1*	0	0	0	0	0	2	3	5	0	10	0.05	0	0	4	6	10	31	37	43	24	**135**	**0**
TMEM115	*transmembrane protein 115*	0	0	0	0	0	0	4	3	0	7	0.11	0	0	0	0	0	35	30	28	26	**119**	**0**
TMEM87A	*transmembrane protein 87A*	0	0	0	0	0	2	4	4	0	10	0.05	0	0	7	9	16	21	20	24	25	**90**	**0.01**
C10orf76	*armadillo like helical domain containing 3*	0	0	0	0	0	2	0	0	2	4	0.17	0	0	0	0	0	15	10	15	12	**52**	**0**
CANT1	*calcium activated nucleotidase 1*	0	0	0	0	0	0	0	0	0	0	na	0	0	3	3	6	10	7	13	12	**42**	**0**
RHBDD2	*rhomboid domain containing 2*	0	0	0	0	0	0	0	0	0	0	na	0	0	0	0	0	6	8	6	7	**27**	**0**
ZDHHC17	*zinc finger DHHC-type containing 17*	0	0	0	0	0	0	0	0	0	0	na	0	0	0	0	0	3	5	7	5	**20**	**0**

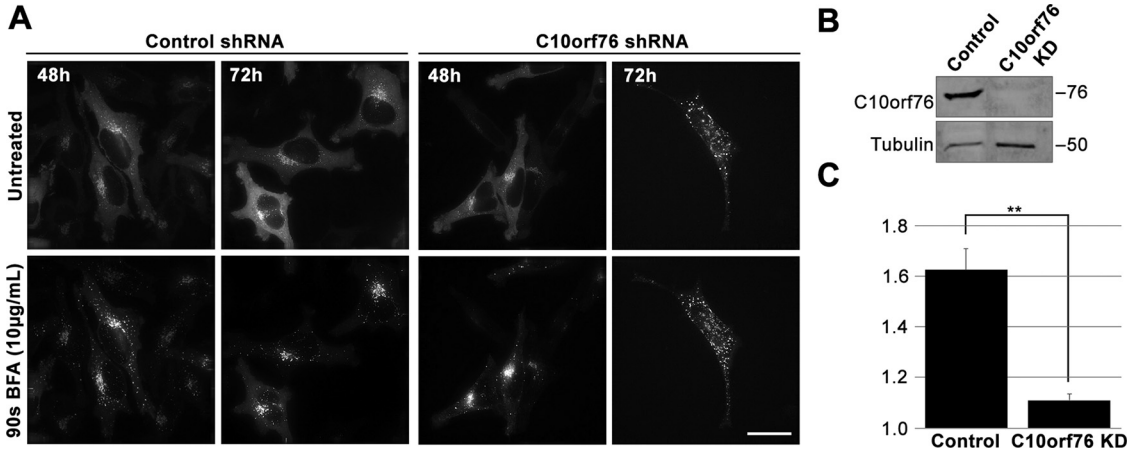

FIG. 2. **C10orf76 knockdown causes redistribution of GBF1 and impairs GBF1 recruitment.** EGFP-GBF1 expressing HeLa cells were transduced using lentiviral vectors containing plasmids encoding nontargeting or C10orf76 shRNAs and successful transductants selected in the presence of puromycin, as described in the Experimental Procedures. *A*, At 72 h, C10orf76-depleted cells show a redistribution of EGFP-GBF1 to disperse punctate structures and causes a poor enrichment of GBF1 to these sites on treatment with BFA (10 μg/ml) for 90 s. All images are representative of 3 replicates and are shown as projections of all z-slices. Scale bars = 26 μm. *B*, Confirmation of C10orf76 knockdown by WB. Images are representative of 3 replicates. *C*, Fold increase of EGFP-GBF1 at existing EGFP-GBF1 positive sites after BFA treatment (10 μg/ml) for 90 s. A value of 1 translates as no impact of BFA and was chosen as the Y-intercept. Cells from the experiment shown in Fig. 2*A* were quantified as described under Experimental Procedures (12 cells per replicate, $n = 3$) (Mean values are 1.62 for control and 1.10 for C10orf76-depleted cells). Bars represent mean values ± standard deviation. A statistically significant difference was observed between the C10orf76-depleted and control cells as measured by a two-tailed unpaired *t* test (**$p = 0.0086$).

(0.1 μg/ml) induced BirA*-FLAG-tagged GBF1 expression at approximately one-third the level of endogenous GBF1 at 24 h post-induction (supplemental Fig. S1). To confirm that the protein localizes to Golgi structures, and that the amount expressed was enough for the labeling of GBF1 proximal proteins at the membrane we performed additional immunofluorescence (IF) and Western blotting (WB) experiments. Such experiments revealed that BirA*-FLAG-GBF1 does biotinylate and modifies a different pool of Golgi localized proteins as compared with BirA*-FLAG control (Fig. 1*A*). This pool of biotinylated proteins along with BirA*-FLAG-GBF1 itself and the Golgi marker, GM130, could be enriched from whole cells using a step sucrose density gradient. This suggests that most proteins biotinylated by BirA*-FLAG-GBF1 indeed localized to the Golgi. The mitochondrial marker VDAC1 was used to ensure that mitochondria, which contain several naturally biotinylated enzymes, were not falsely enriched by the Golgi isolation procedure. IF imaging further confirmed that BirA*-FLAG-GBF1 localized to giantin-positive Golgi structures and that most biotinylated proteins, as detected using streptavidin-Cy3, localized to the Golgi (Fig. 1*B*). In contrast, BirA*-FLAG and its biotinylated products were found dispersed throughout the cytosol and nucleus. To determine whether the BirA*-FLAG tagged GBF1 expressed in these cells was subjected to regulatory signals like that of endogenous GBF1, we examined whether the protein could be recruited to juxta-nuclear Golgi structures on treatment with brefeldin A (BFA). BFA inhibits GBF1 activity by acting as an uncompetitive inhibitor, stabilizing GBF1's interaction with its substrate, Arf-GDP (29, 30). Loss of Arf activation causes an accumulation of inactive Arf-GDP which appears to trigger a feed-forward mechanism driving additional recruitment of GBF1 to Golgi membranes (9, 25). On treatment with 10 μg/ml BFA for 90 s BirA*-FLAG-GBF1 disappeared from the cytoplasm and accumulated at juxta-nuclear structures as well as peripheral puncta, suggesting the protein can be recruited to Golgi membranes like endogenous GBF1 (Fig. 1*C*). Having verified the cell lines for use in BioID experiments, enriched Golgi fractions from biotin treated BirA*-FLAG-GBF1 and BirA*-FLAG expressing HeLa cells were collected and biotinylated proteins were then isolated using streptavidin beads. After extensive washing, streptavidin-bound proteins were treated with trypsin and the eluted peptides identified using nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS), as previously described ((31); supplemental Table S1: all data available at massive.ucsd.edu, accession number MSV000083866). Notably, we found that mass spectrometry analysis conducted on Golgi-enriched fractions identified several unique hits not identified as significant (BFDR ≤0.01) when using whole cell lysates (Fig. 1*D*, Table I, supplemental Table S1).

C10orf76 is a GBF1 Binding Protein Involved in Golgi Maintenance—To identify proteins of interest from the list of 9 biotinylated preys identified by mass spectrometry (Table I), we performed an shRNA screen on EGFP-GBF1 expressing HeLa cells using high-efficiency lentiviral vectors for the delivery of shRNA encoding plasmids, as described under Experimental Procedures (supplemental Table 2). shRNA transductants were then screened at 24, 48, and 72 h post-

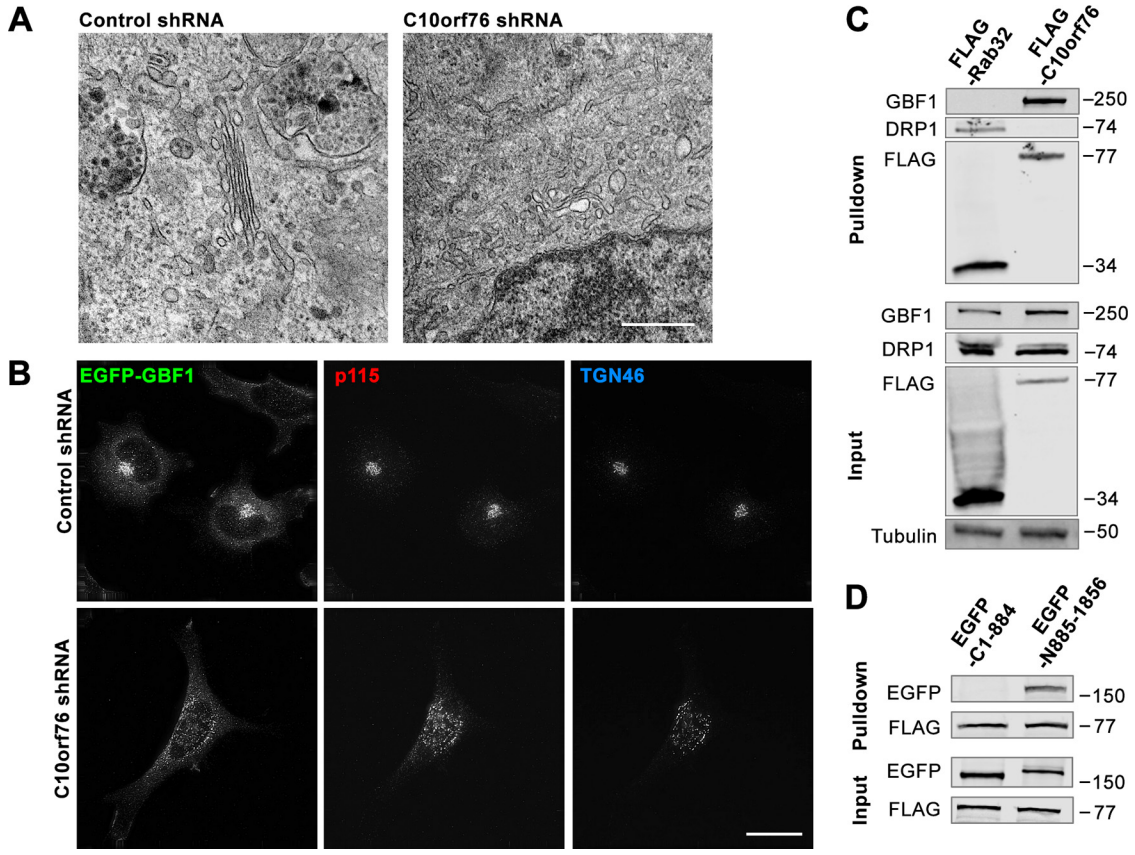

FIG. 3. **C10orf76 is a GBF1 interactor and is essential for Golgi maintenance.** *A* and *B*, EGFP-GBF1 expressing HeLa cells were transduced using lentiviral vectors with nontargeting or C10orf76 shRNAs, selected in the presence of puromycin, and grown for 72 h. *A*, Transmission electron microscopy analysis found that although intact Golgi were observed in all control cells examined, no C10orf76-depleted cells exhibited intact Golgi. 50 cells were examined across 2 replicates. Scale bars = 2 μm. *B*, IF of fixed cells reveal a loss of intact Golgi on C10orf76 depletion. EGFP-GBF1, as well as *cis*- and *trans*- Golgi markers p115 and TGN46, are redistributed to disperse punctate structures in knockdown cells. Images are representative of 3 replicates and are shown as projections of all z-slices. Scale bars = 26 μm. *C* and *D*, Pulldown was performed with either FLAG-C10orf76, FLAG-Rab32, EGFP-C1–884, and/or EGFP-N885–1856. WB show distinct bands corresponding to the pulled-down endogenous GBF1 and EGFP-N885–1856 by FLAG-C10orf76, or Drp1 by FLAG-Rab32. Images are representative of 3 replicates.

transduction by live cell imaging and monitored for abnormal EGFP-GBF1 distribution before and after treatment with BFA.

At 72 h post-transduction, we found that C10orf76 depletion caused a redistribution of EGFP-GBF1 in HeLa cells away from a juxtanuclear-like Golgi localization toward peripheral puncta (Fig. 2*A*). This was not observed in cells treated with nontargeting shRNA or with shRNA targeting the other 8 putative GBF1 interactors (supplemental Fig. S2). Further, addition of BFA for 90 s promoted recruitment in all untreated cells but failed to promote clear recruitment of EGFP-GBF1 to any juxtanuclear site or to existing EGFP-GBF1 positive structures in C10orf76 targeted cells (Fig. 2*A*). Depletion of C10orf76 by shRNA at 72 h post-transduction was confirmed by WB (Fig. 2*B*). To quantitatively confirm the impact of loss of C10orf76 on the recruitment of EGFP-GBF1, we examined the impact of BFA addition (Fig. 2*C*), as before (9). We found that in C10orf76-depleted cells, EGFP-GBF1 was not recruited to membrane sites to the same extent as observed in control cells. These data suggest that C10orf76 is involved in both Golgi maintenance and the regulation of GBF1 recruitment. Apart from C10orf76, the shRNA screen did not identify any other putative interactors whose depletion led to abnormal GBF1 distribution and the failure of BFA to trigger GBF1 recruitment (supplemental Fig. S2).

Because the distribution of EGFP-GBF1 observed in the C10orf76-depleted cells appeared like conditions in which the Golgi may be fragmented, we proceeded to examine the impact of C10orf76 depletion first using electron microscopy and then IF. Transmission electron microscopy confirmed fragmentation (Fig. 3*A*), as no Golgi stacks could be observed (50 cells were examined from each condition) in cells depleted of C10orf76 in two separate biological replicates. Second, IF imaging using antibodies against the cis-Golgi marker p115 and the trans-Golgi marker TGN46 revealed that depletion of C10orf76 did indeed lead to the dispersal of Golgi stacks (Fig. 3*B*). Although GBF1, p115, and TGN46 localized to a juxtanuclear structure in cells treated with a nontargeting shRNA control, depletion of C10orf76 caused a redistribution of these

Golgi markers to peripheral puncta reminiscent of fragmented Golgi. GBF1 and p115 largely redistributed to compartments proximal but distinct from TGN46 suggesting the *cis*- Golgi localization of GBF1 (32, 33) is not affected by partial C10orf76 depletion.

Golgi fragmentation associated with loss of C10orf76 could result from loss of GBF1 recruitment, Arf activation, and COP1 binding on Golgi membranes. We thus examined whether β-COP recruitment is affected in C10orf76-depleted cells. Imaging experiments revealed that β-COP recruitment is maintained on GBF1 positive fragmented Golgi structures in cells depleted of C10orf76 after 72 h (supplemental Fig. S3). Treatment with BFA for 2 min completely abrogated β-COP recruitment to GBF1 positive membrane structures. Together, these data suggest that GBF1 remains functional at fragmented Golgi sites and that Arf activation on Golgi fragments still occurs at 72 h post-transduction.

The possibility that C10orf76 is not only proximal but interacts with GBF1 was investigated using a traditional coimmunoprecipitation approach. Blomen *et al.*, 2014 had previously identified C10orf76 as an interactor of the Golgi phosphatidylinositol 4-kinase beta (PI4KB) and as an essential protein in the replication of coxsackie virus A10 (34). We performed our coimmunoprecipitation using their codon-optimized plasmid encoding a FLAG-tagged C10orf76 (Dr. V. A. Blomen). Controls for the FLAG pulldown were performed using a mitochondria-localized FLAG-tagged Rab32 chimera expressed from a plasmid obtained from Dr. T. Simmen (35). Our results show clear pulldown of GBF1 using FLAG-C10orf76 under conditions where FLAG-Rab32 pulls down its interactor Drp1 but not GBF1 (Fig. 3*C*). Interaction was observed with both full length GBF1, as well as a construct with only the C-terminal half (EGFP N885–1856; Fig. 3*D*). Importantly, no interaction was observed with the N-terminal half lacking the previously identified membrane-association domains (9). These results confirm C10orf76 as a novel GBF1 interactor. C10orf76 is a 78.7 kDa protein (also known as ARMH3) with no known function.

C10orf76 Localizes to Golgi Structures but Is Not Sensitive to BFA—The intracellular localization of C10orf76 was examined using live imaging of cells expressing an mCherry-tagged version. In HeLa cells, the majority of the tagged protein appeared cytosolic, with a portion colocalizing with the previously characterized EGFP-tagged GBF1 at juxtanuclear Golgi sites (Fig. 4*A*). Interestingly, in contrast to GBF1, treatment with BFA (10 μg/ml) for up to 4 min had little impact on the extent of C10orf76 recruitment to Golgi membranes (Fig. 4*B*).

To further examine the interaction with Golgi membranes, we utilized cellular sub-fractionation and Fluorescence Recovery After Photobleaching (FRAP). *In vitro* experiments (Fig. 5*A*) revealed that C10orf76 interacted strongly with membranes, but like GBF1, could be extracted with a high pH carbonate solution. *In vivo* FRAP experiments (Fig. 5*B*) utilizing an EGFP-tagged C10orf76 chimera revealed that Golgi-associated C10orf76 exchanged very rapidly with cytosolic GFP-C10orf76 ($t_{1/2}$ = 6.6 s), faster than that observed for GBF1 ($t_{1/2}$ = 21.2 s). FRAP experiments performed in the presence of BFA after 90 s revealed that the drug did not abrogate but slowed C10orf76 exchange (average $t_{1/2}$ = 12.4 s; two-tailed *t*-Test $p < 0.01$). In sharp contrast, the exchange of GBF1 was largely inhibited by the drug. Together, these results strongly suggest that C10orf76 is not regulated by Arf-GDP in the same manner as GBF1.

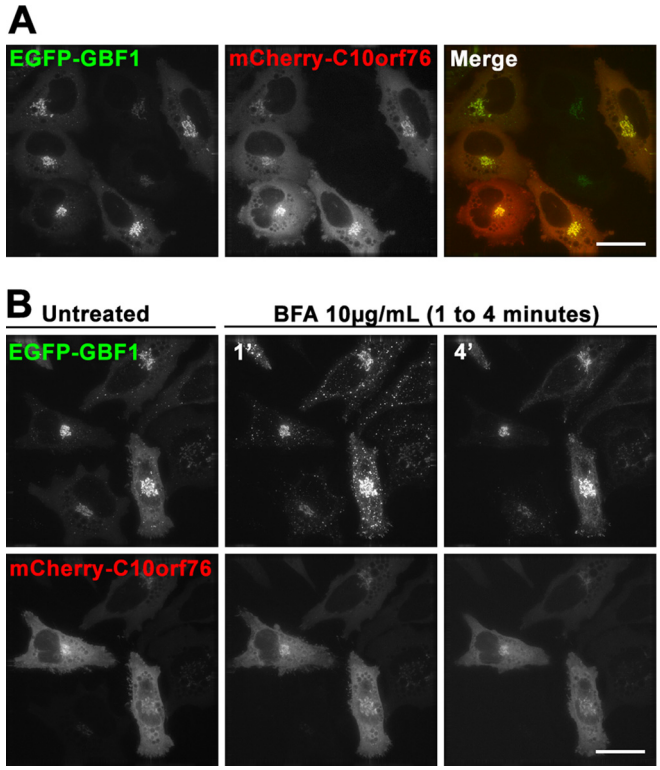

FIG. 4. **C10orf76 localizes to GBF1 positive Golgi structures but is insensitive to BFA.** *A* and *B*, Live cell imaging of EGFP-GBF1 expressing HeLa cells transfected with mCherry- C10orf76. *A*, Overexpressed GBF1 and C10orf76 both occupy cytosolic pools and colocalize at juxtanuclear Golgi structures. *B*, In live cells, treatment with BFA (10 μg/ml) causes loss of GBF1 from cytoplasm, accompanied by recruitment to Golgi and peripheral ERGIC structures but not for C10orf76, which exhibits a similar distribution before and after BFA addition. Images are representative of 3 replicates and are shown as projections of all z-slices. Scale bars = 26 μm.

HeLa cells depleted of C10orf76 exhibited a clear redistribution of p115 and GBF1 (Fig. 3B) and the availability of the codon-optimized and shRNA resistant mCherry-C10orf76 allowed us to confirm the specificity of this effect. To do so, we performed the knockdown experiment again, this time in cells transfected with the codon-optimized and shRNA resistant mCherry-C10orf76. Such images revealed that although the majority of HeLa cells depleted of C10orf76 exhibited a clear redistribution of p115 and GBF1 as compared with control, these markers remained juxtanuclear in all cells expressing ex-

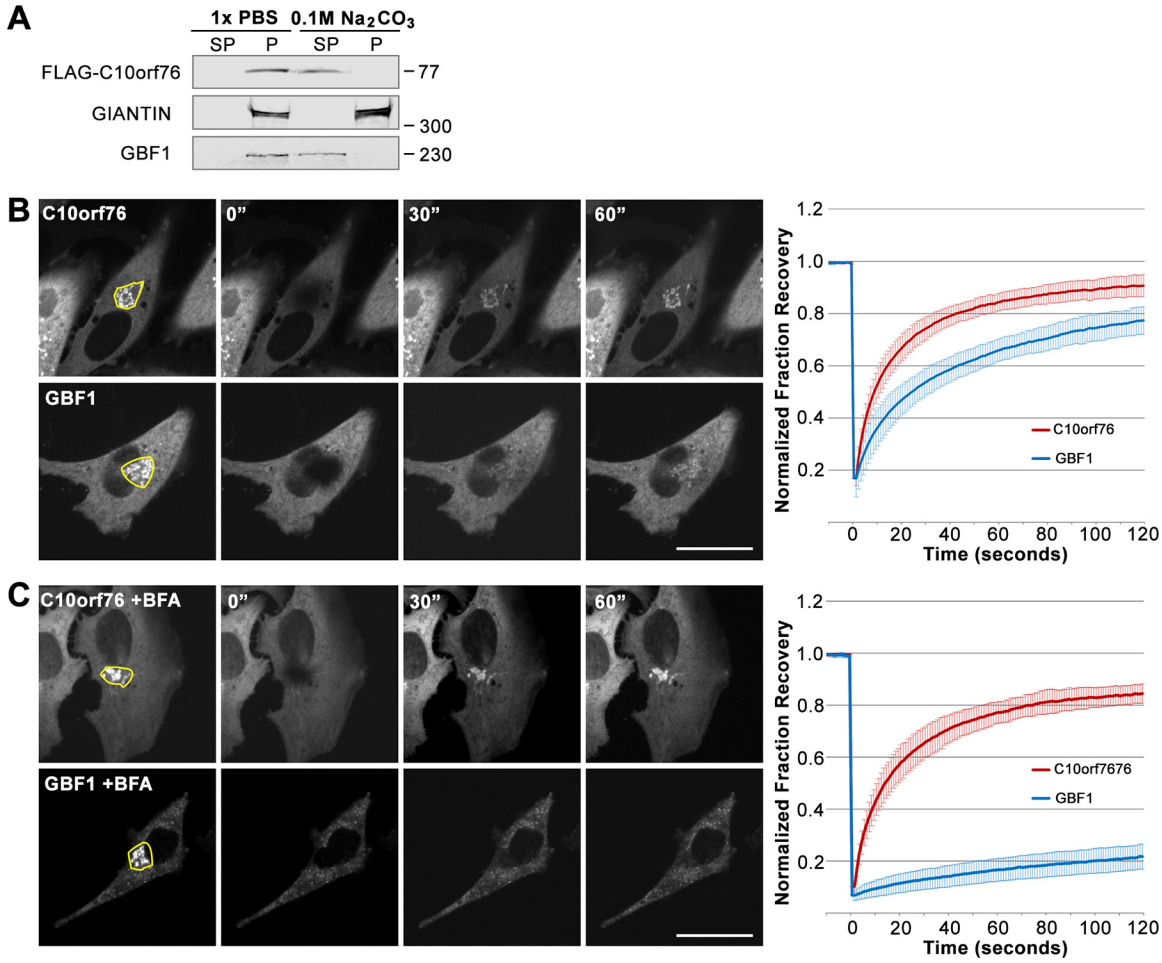

FIG. 5. **C10orf76 is a peripheral membrane protein that cycles on and off Golgi membranes.** *A*, Incubation of sucrose-gradient separated Golgi fractions in 0.1 M Na_2CO_3 (pH 11.3) buffer on ice for 30 min successfully extracted peripheral membrane proteins GBF1 and C10orf76. Integral membrane protein, giantin, remains in the pellet. Images are representative of 3 replicates. *B* and *C*, Fluorescence recovery after photobleaching (FRAP) analyses were performed on EGFP-GBF1 and EGFP- C10orf76 expressing HeLa cells without (*B*) or with (*C*) the addition of BFA (10 μg/ml). EGFP-GBF1 expressing cells were imaged before and after photobleaching of the Golgi region (outlined in yellow) by illumination with a high-intensity laser. After bleaching, images were acquired at a second intervals for 120 s to monitor fluorescence recovery. At least 15 movies were acquired from three separate replicates. Images at 0, 30 and 60 s are shown. The fraction of GFP signal in the Golgi area was normalized to 1 at t = 0 and quantitated as described. Error bars represent ± standard deviation. Scale bars = 26 μm.

ogenous shRNA-resistant C10orf76 (Fig. 6). This suggests that the Golgi fragmentation observed in the C10orf76 shRNA transduced cells was specific and resulted from loss of C10orf76 as opposed to result from an off-target effect. Further observations demonstrated that although C10orf76 depletion led to eventual cell death, cells overexpressing C10orf76 continued to survive at least until 80 h post-transduction.

C10orf76 Appears in A Wide Variety of Organisms—BLASTp searches were performed with human C10orf76 as a query to determine C10orf76's prevalence in other eukaryotic organisms (Fig. 7, supplemental Table S3). Using a reciprocal best hit strategy and an E-value cutoff of 10^{-12}, related sequences were identified in a large variety of clades, but no orthologs were found in the yeast *Saccharomyces cerevisiae*, plant *Arabidopsis thaliana,* or nematode *Caenorhabditis elegans.* However, although C10orf76 is missing in these model systems, it can be found in other plantae, fungi, and nematode species including the common liverwort, *Marchantia polymorpha* (OAE23309.1, PTQ49274.1), fission yeast, *Schizosaccharomyces pombe* (NP_596366.1), and the nematode, *Trichinella spiralis* (KRY28766.1). To ensure that divergent sequences from *S. cerevisiae*, A. thaliana, and *C. elegans* were not missed, additional searches using these other plant, yeast, and nematode C10orf76 orthologs as queries were performed with no success. tBLASTn searches into the nucleotide scaffolds of these organisms also failed to identify any related sequences. This suggests that C10orf76 was likely lost in select yeast, plant, and nematode species. However, C10orf76 appears to be present in the protist *Naegleria gruberi* (XP_002680913.1) from the Excavata supergroup, and the slime mold *Dictyostelium discoideum* (XP_643233.1) from the Amoebozoan super-

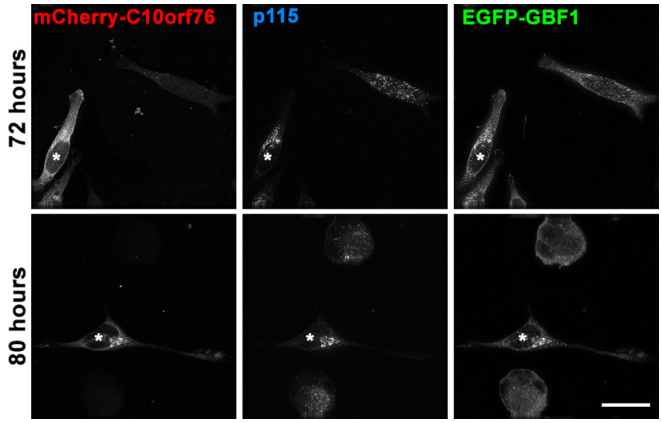

FIG. 6. **C10orf76 overexpression prevents Golgi fragmentation in knockdown cells.** EGFP-GBF1 expressing HeLa cells were transduced using lentiviral vectors with nontargeting or C10orf76 shRNAs for 72 or 80 h. Transfection with an shRNA resistance mCherry-C10orf76 rescues Golgi fragmentation, prevents the redistribution of EGFP-GBF1 and p115 observed at 72 h, and cell death observed at 80 h. All images are representative of 3 replicates and are shown as projections of all z-slices. Scale bars = 26 μm.

group, suggesting it was present in the last eukaryotic common ancestor (LECA).

A relatively conserved domain of unknown function (DUF1741; amino acid 431 to 669) is present in C10orf76 and its orthologs, but is absent from other known proteins. The ability of DUF1741 to target C10orf76 to Golgi membranes was tested using two EGFP-tagged C10orf76 truncation mutants: one containing the N-terminal half (amino acids 1 to 430) and the other, the C-terminal half with DUF1741 (amino acids 431 to 688). Neither exhibited any clear membrane localization in live cells suggesting that neither half of the protein is capable of membrane targeting on its own, or more likely, that the deletion mutations severely compromised protein structure and function (supplemental Fig. S4).

C10orf76 is Required for Secretory Activity—Golgi fragmentation observed on the depletion of C10orf76 suggests a role for the protein in Golgi maintenance. To examine whether secretory activity is impaired on dispersal of Golgi compartments, we performed a luciferase secretion assay to monitor secretory activity in cells depleted of C10orf76. HeLa cells transduced with various shRNA encoding plasmids were transfected with a plasmid encoding a secreted Nano Luciferase 18 h prior to the secretion assay. After replacement with fresh media for collection of secretory products, the transduced cells were then further exposed to either DMSO or BFA, as indicated. As previously observed, inhibition of GBF1 by either shRNA knockdown or treatment with BFA caused a severe reduction in luciferase secretion (Fig. 8*A*, supplemental Fig. S5) (5, 36). Not surprisingly, we also found that depletion of C10orf76 lead to a comparable loss of secretory activity. The requirement of C10orf76 in maintaining cellular secretion suggests that cell death previously observed at 80 h may result, at least in part, from a block in secretory activity.

DISCUSSION

Our work has identified C10orf76, a novel Golgi localized GBF1 interactor involved in Golgi maintenance, secretion, and GBF1 recruitment. C10orf76 cycles rapidly on and off Golgi membranes, and appears BFA insensitive. Further, C10orf76 although likely evolutionarily ancient, appears to have been lost in several model eukaryotic systems including *S. cerevisiae* and *A. thaliana*. We propose that C10orf76 may be a component of a larger protein complex or may facilitate the activities of other membrane proteins involved in regulating GBF1 recruitment to Golgi membranes. A more comprehensive understanding of GBF1 recruitment to various membrane sites, and the regulatory factors that govern GBF1 localization and function, may require identification of novel interacting partners.

Pairing Golgi Enrichment with Proximity Biotinylation—Because its development by Roux and colleagues in 2013, the popularity of BioID has soared. The technique's ability to identify transient, weak, and poorly soluble protein partners makes it a useful complement to traditional protein-protein interaction assays. However, despite the rapid adoption of the BioID method in the molecular biology community, no study has yet been published demonstrating the use of the method on enriched Golgi fractions. Although one would expect proximity partners identified from whole cell lysates to include those identified from enriched Golgi membranes, we instead found that the Golgi enrichment procedure allowed us to identify GBF1 proximal partners that were not significantly enriched using a standard BioID protocol (*i.e.* performed on whole cell lysates); this includes the novel GBF1 interactor, C10orf76. The observed differences in local interactomes could be explained by differences in protein abundance. Although ideally, mass spectrometry would facilitate the identification of entire protein interactomes the range in protein size, sequence, and quantity often results in the oversampling of abundant proteins, with peptides from low abundance proteins being undersampled or undetected. In fact, C10orf76 could only be detected by WB of HeLa cell lysates after first concentrating protein levels by acetone precipitation. This suggests that C10orf 76 is likely a low abundance protein in HeLa cells. By pairing organelle enrichment with proximity biotinylation, one can allow for a more robust assay, improving the probability of detecting low abundance proximal proteins using BioID.

Is C10orf76 a GBF1 Coreceptor?—GBF1 is essential for Golgi maintenance and the loss of a putative GBF1 receptor is expected to lead to Golgi collapse (5). With the depletion of C10orf76 we observed both a fragmentation of the organelle and a reduction in GBF1 recruitment on BFA treatment. The complete KD of GBF1 appears lethal and the reported observations with living cells resulted from partial but enough KD. These data suggest that C10orf76 regulates GBF1 recruitment in mammalian cells. Further, coimmunoprecipitation ex-

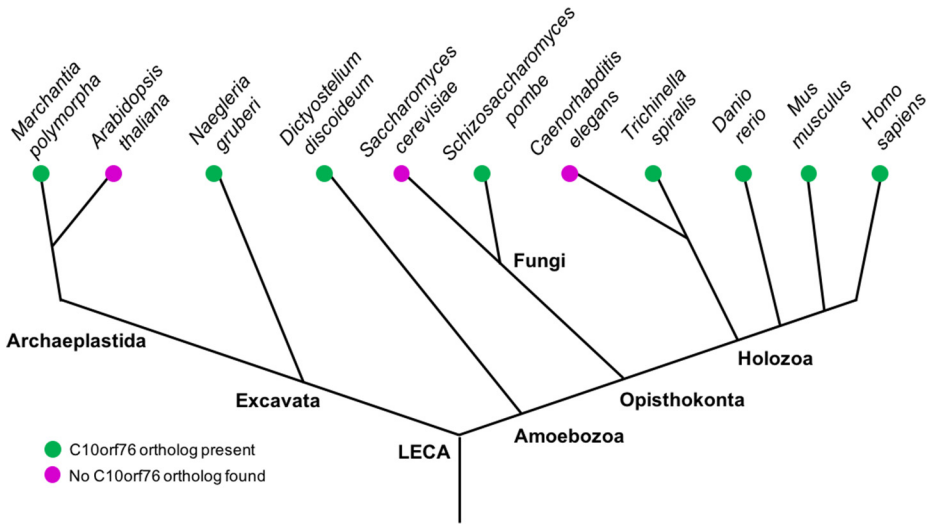

FIG. 7. **C10orf76 is an ancestral protein.** Presence and absence of C10orf76 orthologs in various eukaryotic species as identified using reciprocal best hit BLASTp searches with the human C10orf76 query. Green circle signifies the presence of a C10orf76 ortholog. An E-value cutoff of 10^{-12} was used for all searches.

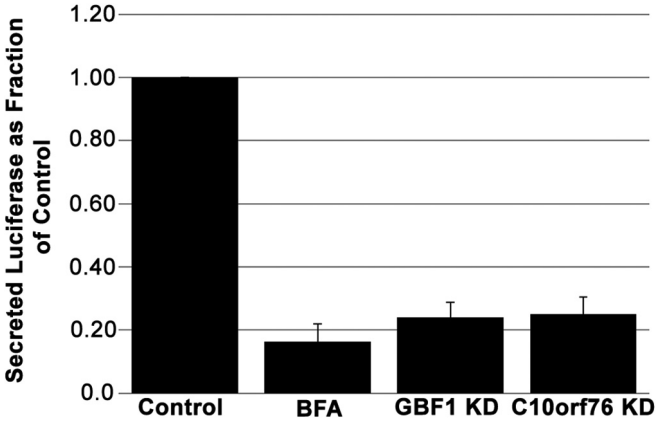

FIG. 8. **C10orf76 is needed to maintain cellular secretion.** HeLa cells were transduced using lentiviral vectors with plasmids encoding nontargeting, C10orf76 or GBF1 shRNAs. Transductants were selected, and after 72 h, transfected with a plasmid encoding a secreted NanuLuc luciferase 18 h prior to the assay. During the assay, growth medium was analyzed for luciferase activity after a 3 h incubation period with or without the addition of BFA (1 μg/ml), as described under Experimental Procedures. Bars represent means ± standard deviation (error bars) ($n = 3$). Depletion of C10orf76 causes a reduction in luciferase secretion similar to the inhibition of GBF1 activity by knockdown or treatment with BFA.

periments revealed that GBF1 interacts with C10orf76 via its C-terminal half. Previous studies from our lab have demonstrated that GBF1 localization to Golgi membranes is dependent on C-terminal domains (9). This agrees with the report of a lipid binding motif in the same region (37). Therefore, C10orf76 may be facilitating GBF1 interaction with Golgi membranes by interacting with its C-terminal half. However, although BFA stimulates the recruitment of GBF1, the distribution of C10orf76 appears to be BFA insensitive. We cannot exclude the possibility that levels of C10orf76 on membranes are saturated or that post-translational modification of membrane bound C10orf76 and/or additional factors may regulate GBF1 recruitment. However, the facts that C10orf76 is a rapidly cycling peripheral membrane protein, and that it is absent in the plant, *A. thaliana,* and yeast, *S. cerevisiae*, suggest that C10orf76 is an unlikely membrane receptor for GBF1. Such conclusion stems from the observation that GBF1 and its orthologs are present in all eukaryotes (38, 39) and one would expect conservation of the recruitment mechanism. GBF1 orthologs have been extensively studied but the mechanisms regulating their recruitment to Golgi membranes in yeast, plant, and worm remains unresolved. The lack of readily recognized C10orf76 sequences in *S. cerevisiae*, *A. thaliana,* and *C. elegans* (40) suggest that although C10orf76 may contribute to the regulation of GBF1 activity in mammalian cells, other more evolutionarily conserved "membrane-associated" components likely govern GBF1 Golgi recruitment. The presence of C10orf76 in multiple clades suggests that C10orf76 may be ancient and was likely subsequently lost in several species over time. But although the loss of C10orf76 in multiple species suggests it is not a true receptor for GBF1, we cannot exclude the possibility that the functional role of C10orf76 may be replaced by other proteins with low sequence similarity. Little is known about the protein structure of C10orf76 apart from the presence of a domain of unknown function, DUF1741, in the C-terminal half of the protein. Truncation mutants with either the N-terminal or C-terminal halves of C10orf76 failed to identify any Golgi targeting regions, suggesting that neither halves can fold and function independently. Further work will be needed to determine the evolutionary history of C10orf76 and to elucidate the function of DUF1741.

Golgi Fragmentation and GBF1 Activity—Knockdown of C10orf76 clearly affected the extent of GBF1 recruitment to Golgi membranes. This was observed in live cells on BFA treatment (Fig. 2*C*); the fact that similar results were observed in fixed cells (supplemental Fig. S3) strongly supports this conclusion. Although Golgi fragmentation was observed on C10orf76 knockdown, β-COP, the downstream effector of

activated Arf, was still recruited to GBF1 positive fragmented membrane sites. Note that such experiments required us to focus on cells with detectable membrane-localized GBF1 3 days post transduction, which suggests that partial C10orf76 knockdown complicates interpretation. However, such results also demonstrate that retention of Golgi remnants need not challenge the requirement for GBF1, Arfs, and COP1 in Golgi maintenance.

The only other known C10orf76 interactor is the phosphatidylinositol-4-kinase β (PI4Kβ) (34). PI4Kβ interacts with the GBF1 substrate, Arf1, and its enzymatic product, PI4P, plays an essential role in maintaining Golgi traffic (41, 42). One potential mechanism explaining C10orf76's role in Golgi maintenance and secretion could be through regulating PI4Kβ function. However, whether the loss of C10orf76 alters PI4Kβ activity has yet to be investigated.

Other Potential GBF1 Regulators—Although C10orf76 may play a role in regulating GBF1 recruitment, our data suggest that there are likely other unidentified protein components involved. Our shRNA screen focused on knocking down single putative interactors and we monitored effects only up to 72 h post-shRNA treatment. Any GBF1 regulators with genetic redundancies or requiring longer than 72 h for effective knockdowns would be missed in our screen. Further, knockdown could not be confirmed for several proteins without validated commercially available antibodies. Double knockdowns and extended shRNA treatment will be needed to further examine the regulatory functions—if any—for these putative GBF1 interactors.

Because previous studies demonstrated the presence of a critical GBF1 recruitment factor on Golgi membranes (9), our study focused primarily on GBF1 proximal partners identified from enriched Golgi membranes. Dozens of proximal partners identified from whole cell lysates were not examined. However, GBF1 has been proposed to function at numerous sites in the cell including: at the plasma membrane where it facilitates glycosylphosphatidylinositol-anchored protein (GPI-AP)-enriched early endosomal compartment (GEEC) endocytosis (7), at lipid droplets where it regulates lipid metabolism and homeostasis (43), and at mitochondrial membranes where it modulates mitochondrial morphology and activity (8). Live cell imaging did not reveal any obvious accumulation of C10orf76 at organelles other than the Golgi; however, it remains possible that C10orf76 may also be present at other membrane sites playing other related roles. The mechanisms targeting GBF1 to various cellular locations, and the regulatory factors that integrate cellular signals governing when and where GBF1 functions, will require further study of these GBF1 proximal proteins and interactors.

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DATA AVAILABILITY

All mass spectrometry data have been uploaded to the public MassIVE archive (www.massive.ucsd.edu) with ID: MSV000083866.

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