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RESEARCH ARTICLE

Interacting partners of Golgi-localized small G protein Arl5b identified by a combination of *in vivo* proximity labelling and GFP-Trap pull down

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The small G protein Arl5b is localised on the *trans*-Golgi network (TGN) and regulates endosomes-to-TGN transport. Here, we combined *in vivo* and *in vitro* techniques to map the interactive partners and near neighbours of Arl5b at the TGN, using constitutively active, membrane-bound Arl5b (Q70L)-GFP in stably expressing HeLa cells, and the proximity labelling techniques BioID and APEX2 in parallel with GFP-Trap pull down. From MS analysis, 22 Golgi proteins were identified; 50% were TGN-localised Rabs, Arfs and Arls. The scaffold/tethering factors ACBD3 (GCP60) and PIST (GOPC) were also identified, and we show that Arl5b is required for TGN recruitment of ACBD3. Overall, the combination of *in vivo* labelling and direct pull downs indicates a highly organised complex of small G proteins on TGN membranes.

Keywords: ACBD3/GCP60; Arl5b; *in vivo* proximity labelling; membrane transport; small G proteins; tethering factors; *trans*-Golgi network

Small GTP-binding proteins (G proteins) of the Rab and Arf families are key regulators of intracellular membrane transport and organelle homeostasis in the endocytic and secretory pathways [1,2]. The Arf family of GTPases constitute the Arf, Sar and Arf-like proteins (Arls) [3-5]. Small G proteins cycle between a GDP-bound inactive conformation and a membrane-associated GTP active conformation [6]. The GTP-bound Rab and Arf family members mediate their suite of functions by the interaction with a variety of binding partners, or effectors, which have a diverse range of activities, and including roles in membrane

Abbreviations

ACBD3, Acyl-CoA-binding domain-containing 3; APEX2, ascorbate peroxidase-based proximity labelling, version 2; Arf, ADP ribosylation factor; Arl, ADP ribosylation factor-like; BioID, proximity-dependent biotin identification; CID, collision-induced activation; CPI, complete protease inhibitors; DMSO, dimethyl sulphoxide; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GAP, GTPase-activating protein; GCP60, Golgi complex-associated protein of 60 kDa; GEF, guanine exchange factor; GFP, green fluorescent protein; GOPC, Golgi-associated PDZ and coiled-coil motif-containing protein; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; HA, haemagglutinin; HRP, horseradish peroxidase; LC–MS, liquid chromatography–mass spectrometry; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI4KB, phosphatidylinositol 4-kinase beta; PIST, protein interacting specifically with Tc10; PVDF, polyvinylidene fluoride; SA, streptavidin; SA-agarose, streptavidin agarose; SA-HRP, streptavidin-horseradish peroxidase; siRNA, small interfering RNA; SRP, signal recognition particle; SSL, solid-state laser; TCL, total cell lysate; TEAB, triethylammonium bicarbonate; TGN, *trans*-Golgi network; WT, wild-type.

tethering, membrane budding, regulation of motor proteins and vesicular fusion. Understanding the biological functions of small G proteins requires knowledge of the identity of their interactive effectors. Although many effectors have been identified for different Rabs [7], effectors have not yet been identified for many of the Arfs/Arls.

The identification of the interactive partners of small G proteins is a challenge in view of the transient and often low-affinity interactions with effectors. A variety of methods had been used in the past to identify the interactive partners of small G proteins, including cross linking, two-hybrid screens and GST pulldowns [7]. Recently, a more promising approach has been the use of *in vivo* proximity biotinylation methods, which have the potential to detect weak and transient interactions *in vivo* and which have been successfully used for a number of the Rab proteins [8,9]. An advantage of this approach is that the labelling is performed *in vivo* with the small G protein associated with its target membrane. A disadvantage of the *in vivo* proximity biotinylation methods is that the proteins labelled include not only interactive partners but also proteins in close proximity to the bait; hence, the interactive partners and close neighbours need to be distinguished. This particularly applies to the application of this method to membrane proteins orientated on the cytosolic side of the membrane, as the biotinylation reaction is not restricted within a closed compartment.

The TGN is a very active cargo sorting and transport hub where transport carriers exit to a variety of destinations and are also received *via* retrograde traffic from endosomes [10-13]. Many (> 20) Rab and Arf family members have been located to the TGN [1,12,14,15]. We have previously identified Arl5b as a TGN small G protein, which regulates endosome to the TGN transport [16]; however, the identity of the interactive partners of Arl5b is unknown. Here, we have incorporated two different proximity-based methods, namely BioID and APEX2. Usually, only one of the two methods has been employed; however, the advantage of including both methods is that each method uses a different chemistry for biotinylation, which potentially broadens the capture of partners, as well as using different fusion proteins with Arl5b, an advantage to minimise inadvertent effects of the fusion partners interfering with the binding of Arl5b to effectors. In addition, we have also incorporated a GFP-Trap pull down in this study to compare the *in vivo* biotinylation methods with an approach which directly identifies interaction partners with sufficient affinity to remain bound after cell extraction. Here, we report the analysis of the three proteomic approaches using constitutively active Arl5b as bait, with the finding that a number of TGN/Golgi-localised small G proteins were detected by all three approaches, suggesting an organised complex of small G proteins, and that the Golgi location of the tethering molecules ACBD3 and PIST is partially regulated by Arl5b.

Materials and methods

DNA constructs

For GFP-Trap, pEGFP-N3 (control vector) and pEGFP-N3-Arl5b(Q70L) were previously described [16]. For BioID, pEGFP-N3-Arl5b(Q70L) was used as a template for PCR to amplify Arl5b(Q70L) DNA fragment and introduce in frame restriction sites EcoR1 and BamHI. This fragment was ligated into *EcoR1*/*BamHI* linearised pcDNA3.1-MCS-BirA(R118G)-HA (#36047; Addgene, Watertown, MA, USA) (control vector) to generate pcDNA3.1BirA(R118G)-Arl5b(Q70L)-HA. Transformed DH5alpha *Escherichia coli* were screened by PCR and positive clones confirmed by sequence analysis. For APEX2, pcDNA3-Connexin43-GFP-APEX2 (#49385; Addgene) was digested with AflIII and BamHI to remove the connexin43 sequence to generate pcDNA3-GFP-APEX2. The Arl5b(Q70L) sequence from pcDNA3.1BirA(R118G)-Arl5b(Q70L)-HA was amplified and ligated into pcDNA3-GFP-APEX2. Clones were identified by colony PCR, and positive clones were confirmed by sequence analysis.

To construct pEGFP-C1-ARF1-myc, full-length cDNA of ARF1 was amplified from human HeLa cells cDNA using the specific primers GGGAACATCTTCGCCAACCTC and CTTCTGGTTCCGGAGCTG. A myc-tag nucleotide sequence (GAACAGAAACTGATCTCTGAAGAAGACC TG) was inserted at the C terminus of ARF-1 in a stepwise PCR process. The final PCR product was digested and cloned into *Nhe*I and *Kpn*I digested pEGFP-C1. The sequence of the construct was confirmed by DNA sequencing.

Antibodies

Purified mouse anti-GM130 clone 35 catalogue # 610822 1 : 300 and mouse antibody to PI4 Kinase β (#611816) were purchased from BD Biosciences, Macquarie Park, NSW, Australia. Mouse monoclonal antibody to the myc epitope (9E10) was obtained from the Walter and Elisa Hall Institute (Melbourne) monoclonal facility. Mouse monoclonal antibody (mAb) to golgin 97 (CDF4) # A21270 used at 1 : 300 was purchased from Thermo Fisher Scientific, Scoresby, VIC, Australia. HA-Tag (C29F4) rabbit mAb #3724 1 : 800 was purchased from Cell Signalling Technologies (CST), Notting Hill, VIC, Australia. Recombinant anti-GM130 rabbit mAb (EP892Y) # ab52649 1 : 1000 was purchased from Abcam (Melbourne, VIC, Australia). Anti-ACBD3 mouse mAb (518) #sc101277 1 : 100 and anti-PIST

mouse mAb (A-7) #sc393026 1 : 100 were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Rabbit polyclonal antibodies to human GCC88 have been previously described [17]. Streptavidin, Alexa Fluor™488 conjugate #S32354 (SA-AF488), Streptavidin, Alexa Fluor™647 conjugate #S32357 (SA-AF647) used at 1 : 1000 were purchased from Thermo Fisher Scientific. Secondary conjugates used for immunofluorescence were goat anti-mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, # A-11001, goat anti-mouse IgG-Alexa Fluor 568 # A11004, goat anti-rabbit IgG-Alexa Fluor 568 # A11011, goat anti-rabbit IgG-Alexa Fluor 647 # A21245, from Thermo Fisher Scientific. All secondary antibodies were used at the dilution 1 : 500. Horseradish peroxidase–conjugated goat anti-rabbit IgG (H + L)-HRP, #656120, and streptavidin–horseradish peroxidase (SA-HRP) #SA10001 were purchased from Thermo Fisher Scientific.

Reagents

GFP-Trap® Agarose (gta-10) and GFP-Trap® Magnetic Agarose (gtma-10) were purchased from ChromoTek (Bionovus, Cherrybrook, NSW, Australia). Dynabeads™ MyOne™ Streptavidin C1 (65001) were from Invitrogen/Thermo Fisher Scientific, cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (CPI) (11836170001) was from Roche/Sigma-Aldrich, Bayswater, VIC, Australia, FuGENE 6 transfection reagent (E2691) was purchased from Promega Alexandria, NSW, Australia, 4% Paraformaldehyde Phosphate Buffer Solution (PFA) (163–20145) was purchased from Wako (Novochem, VIC, Australia), and Dharmacon DharmaFECT1 reagent (T-2001-02) from Millennium Science (Mulgrave, VIC, Australia). Hydrogen peroxide (H_2O_2) 30% (wt/wt) (H1009-100ML), Biotin (cat. no. B4501), Biotin-Phenol (B-P), Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (cat. no. 238813-5G), 4′,6 diamidino-2-pheynylindole (DAPI) (cat no. D5942-5MG), sodium deoxycholate (cat. no. D6750-100G), sodium L-ascorbate (A4034-100G), DMSO (dimethyl sulphoxide) (D2650-100ML) and triethylammonium bicarbonate (TEAB) (cat. no. T7408-500ML) were from Sigma-Aldrich, North Ryde, NSW, Australia. Bond-BreakerTM TCEP (*tris* 2-carboxyethylphosphine) solution, Neutral pH #77720, Pierce™ Formic Acid, LC–MS Grade 99 + % # 28905 were from Thermo Fisher Scientific, Scorsby, Vic, Australia.

Cell culture and transfection

Mycoplasma-free authentic HeLa cells (Curie Institute, Paris), verified by genomic sequencing, were maintained as semiconfluent monolayers in C-DMEM (complete DMEM) (Thermo Fisher Scientific) medium supplemented with 10% (vol/vol) fetal bovine serum (FBS; Sigma-Aldrich, North Ryde, NSW, Australia), 2 mM L-glutamine (Thermo Fisher Scientific/Gibco), 100 $U{\cdot}\mu L^{-1}$ penicillin and 0.1% (wt/vol) streptomycin (Sigma-Aldrich, North Ryde, NSW, Australia) in a humidified 10% CO_2 atmosphere at 37 °C. HeLaArl5b (Q70L) -GFP cell clone [16] was maintained in the presence of 1 $mg{\cdot}mL^{-1}$ G418 (Thermo Fisher Scientific).

Transient transfections of DNA constructs were performed using FuGENE 6 transfection reagent (Promega, Alexandria, NSW, Australia), according to the manufacturer's protocol.

RNA interference

Transfections with siRNA were performed using DharmaFECT1 siRNA transfection reagent (GE Lifesciences/Millennium Science) according to the manufacturer's instructions for 72 h before analysis. Human Arl5b was targeted with the specific siRNA 5′- CAGAGUUCAUCAUUCUUGU -3′ [16] and human ACBD3 siRNA (5′ AAUUGUCUGUGGCAAAUUC -3′). All duplex mission predesigned siRNAs, including a control siRNA, were synthesised by Sigma-Aldrich (North Ryde, NSW, Australia).

Indirect immunofluorescence

Monolayers on coverslips were fixed with 4% (vol/vol) paraformaldehyde (PFA) for 15 min, followed by quenching in 50 mM NH_4Cl/phosphate-buffered saline (PBS) for 10 min. Cells were permeabilised in 0.1% Triton X-100 in PBS for 4 min and incubated in blocking buffer (5% FBS in PBS) for 15 min to reduce nonspecific binding. Monolayers were incubated with primary and secondary conjugates as described [18]. Monolayers were then incubated with primary antibody, diluted in blocking buffer for 1 h, washed in PBS, and incubated with secondary conjugates for 30 min. Monolayers were stained with 4′,6 diamidino-2-pheynylindole (DAPI) for 5 min, washed in PBS and finally rinsed with MilliQ water before being mounted in Mowiol mounting solution (9.6% w/v Mowiol 4-88 polymer, 24% w/v glycerol, 0.1M Tris-HCl pH 8.5).

Confocal microscopy and image analysis

Images were acquired using a laser confocal scanning microscope (Leica TCS SP2 or SP8) using a 63 × 1.4 NA HCX PL APO CS oil immersion objective. GFP and Alexa Fluor 488 were excited with the 488-nm solid-state laser (SSL), Alexa Fluor 568 with a 552-nm SSL, Alexa Fluor 647 with a 638-nm SSL and DAPI with a 405-nm diode laser. Images were collected sequentially for multicolour imaging. Fluorescence images for each experiment were collected using identical settings for quantitative analysis. Quantitation was carried out for the indicated number of cells. The area of the Golgi for each cell was identified using the Golgi marker GCC88. The mean fluorescence intensity (MFI) of ACBD3 or PIST was measured in defined Golgi area using Fiji [19].

Immunoblotting

Cells were lysed in RIPA buffer (1 mM Tris/Cl, pH 7.5, 15 mM NaCl, 0.5 mM EDTA, 0.01% SDS, 0.1% Triton X-100, and 0.1% deoxycholate) containing 1 × cOmplete protease inhibitors (CPI) EDTA-free (Sigma-Aldrich, North Ryde, NSW, Australia). Aliquots of the extracts were added to reducing SDS sample buffer and boiled for 5 min at 100 °C. Proteins were resolved by SDS/PAGE using 4%–12% NuPAGE gels (Thermo Fischer Scientific) and transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bayswater, VIC, Australia) at 10–20 V overnight at 4 °C. The membrane was blocked by drying at 37 °C. The membrane was incubated with antibodies diluted in 5% (wt/vol) skim milk/PBS-0.1% (v/v) Tween 20, or 1% (wt/vol) BSA in PBS-0.1%Tween 20 (1% BSA-PBS/Tween) at room temperature for 1–2 h or overnight at 4 °C and then washed three times, each for 10 min, in 0.1% (vol/vol) PBS/Tween 20. The PVDF membrane was then incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies, diluted in 5% (wt/vol) milk/PBS or 1% BSA-PBS/Tween for 1 h and washed as above.

For streptavidin immunoblots, the protocol was adapted from [20]. PVDF membranes were blocked in BSA blocking buffer [1% w/v bovine serum albumin fraction V, 0.2% (w/v) Triton X-100 in PBS] rocking at RT for 20 min and incubated with SA-HRP, diluted 1 : 20 000 in BSA blocking buffer, with shaking for 1 h, and then washed thrice in PBS. To visualise MagicMark™ XP Western Protein Standard # LC5602 (Invitrogen, Thermo Fisher Scientific), membrane was incubated with anti-mouse-HRP or anti-rabbit-HRP in BSA buffer for 30 min and then washed 3 × in PBS. To reduce background, PVDF was incubated shaking with ABS blocking buffer (10% v/v adult bovine serum, 1% w/v Triton X-100 in PBS) for 5 min, then washed 3 × in PBS and again in PBS for a further 5 min. Bound antibodies were detected by enhanced chemiluminescence (ECL) (RPN2106) (GE Healthcare, Bio-Strategy, Campbellfield, VIC, Australia) and captured using the Chemi-Doc MP imager system (Bio-Rad, South Granville, NSW, Australia).

Immunoprecipitation using GFP-Trap

HeLa WT or HeLaArl5b-GFP cell lines were grown in monolayers in 3 × 100 mm cell culture dishes. One set of HeLa WT dishes was transfected with GFP vector alone for 24 h. At least 5 × 10^7 cells per treatment were harvested and washed in ice-cold PBS. Immunoprecipitation was carried out using either GFP-Trap®_M kit or GFP-Trap®_A kit (ChromoTek, Bionovus Life Sciences, Cherrybrook, NSW, Australia), according to the manufacturer's instructions. Briefly, washed cells were lysed in the provided NP40 Lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40) with CPI (1 × cOmplete Protease inhibitor cocktail) (Sigma-Aldrich) and centrifuged at 20 000 ***g*** at 4 °C to separate insoluble pellet from soluble fraction. GFP-Trap magnetic or agarose beads 20 µl slurry were washed in dilution buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) containing CPI. Soluble lysates were incubated rotating at 4 °C with beads for 2 h. GFP-Trap beads were washed thrice with ice-cold dilution buffer (without CPI). Samples were collected for immunoblot analysis. Bead/protein complexes were processed for MS analysis.

BioID

BioID was carried out using 4–6 × 100 mm tissue culture plates per sample, with the following modifications from the method described [20]. HeLa cells (~ 1.0 × 10^6) were seeded into each plate and after 24 h were transiently transfected with 3–4.5 µg DNA (BirA*-HA or Arl5b-BirA*-HA) per plate. The medium was supplemented with 50 µM sterile filtered biotin (Sigma-Aldrich, North Ryde, NSW, Australia) in c-DMEM (Life Technologies, Scoresby, VIC, Australia) for a period of 16–20 h and the cells harvested 24 h after transfection by scraping in 5 mM EDTA/PBS. For analysis, there was at least 10 × 10^7 cells/sample. Cells were harvested, extracted and incubated with MyOne™ Streptavidin C1 DynaBeads™ (Thermo Fisher Scientific/Invitrogen) (10 µL beads/sample) and the beads washed as per the manufacturer's instructions.

APEX2

Proximity biotinylation using APEX2 was performed according to the protocol described by Lam et al. [21] and Hung et al. [22]. Briefly, HeLa cells (5 × 100 mm tissue culture plates per sample) were transfected with APEX2 constructs for 24 h and treated with 500 µM biotin-phenol (B-P) in C-DMEM for 30 min. Freshly prepared H_2O_2 (100 mM) in PBS was added to a final concentration of 1 mM directly into the culture medium, with cells rocking for 1 min, and then the reaction stopped by the addition of freshly prepared Trolox Quenching Solution (10 mM Sodium Ascorbate, 5 mM Trolox, 10 mM sodium azide in PBS). Cells were harvested in Trolox quenching solution into chilled tubes and washed in cold PBS, pelleted and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% v/v SDS, 0.5% sodium deoxycholate, 1.0% TX-100 in PBS) + cOmplete protease inhibitors + 5 mM Trolox for 20 min on ice. The cell extracts were centrifuged at 16 000 ***g*** 4 °C for 10 min and the solution fraction collected.

In solution trypsin digest for MS

To identify the GFP-Trap immunoprecipitated proteins, GFP-Trap agarose or magnetic beads were incubated with cell extracts and washed as described above. To identify the biotinylated proximity proteins, total cell lysate (TCL)

samples of Arl5b in either BioID and APEX2 fusion proteins or control samples (vector alone or unrelated fusion protein, also biotinylated) were incubated with streptavidin agarose (SA-agarose) or SA-magnetic beads. After rigorous wash steps, bound proteins were directly digested from the GFP-Trap, SA-agarose or SA-magnetic beads by trypsin in the presence of reducing agent [5% trifluoroethanol [TFE], 50 mM TEAB, 1 mM *tris* (2-carboxyethyl) phosphine (TCEP)] and 0.25 mg trypsin per sample. Samples were digested overnight at 37 °C, then either magnetically separated or centrifuged to separate beads from the eluted digested peptides. The resulting peptide samples were then analysed on a LTQ Orbitrap Elite (Thermo Scientific, Scoresby, VIc, Australia) coupled to an Ultimate 3000 RSLC nanosystem (Dionex, Scoresby, VIC, Australia). The nanoLC system was equipped with an Acclaim Pepmap nanotrap column and an Acclaim Pepmap analytical column. 2 μL of the peptide mix was loaded onto the trap column of 3% CH_3CN containing 0.1% formic acid for 5 min before the enrichment column is switched in-line with the analytical column. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, whereby spectra were acquired first in positive mode followed by collision-induced activation (CID). Ten of the most intense peptide ions with charge states ≥2 were isolated and fragmented using normalised collision energy of 35 and activation Q of 0.25. Peptides were identified using MASCOT V2.4 against the human SWISSPROT database with an allowance for three missed cleavages and the fixed modification Carbamidomethyl (C) and variable modification oxidation (M). Protein hits were restricted to those proteins that had 2 or more significant peptides.

Brightfield staining of APEX2

HeLa cell monolayers were transfected with either APEX2 alone or Arl5b-GFP-APEX2 for 24 h. Cells were fixed in 4% PFA for 15 min, quenched in 30 mM glycine/PBS for 10 min, washed with PBS and then incubated with freshly prepared 3,3′-Diaminobenzidine (DAB)/H_2O_2 in PBS for 30 min. Cells were imaged using transmission settings on a Leica confocal SP8.

Immunofluorescence of APEX2 transfected cells

For immunofluorescence of APEX2 transfected cells, cells treated with biotin for 30 min and freshly prepared H_2O_2 for 1 min, then quenched in freshly prepared Trolox as above and washed twice in PBS. Cells were fixed with 4% PFA and quenched with 50 mM NH_4Cl, and permeabilised with 0.1% TX100 4 min, then washed 3 × in PBS, blocked in 5% FBS/PBS for 15 min, then washed once in PBS and then incubated with SA-647 1 : 1000 for 45 min and washed in PBS.

Co-immunoprecipitation of Myc-tagged Arf1 and Arl5b-GFP

HeLa cell monolayers were seeded at 1.0×10^6 in 2 × 10 cm dishes per sample and cultured 24 h. Cells were transfected for 24 h using FuGENE6 (Promega # E2691) according to the manufacturer's protocol, with single transfections of either Arl5bT30N-GFP or Arl5bQ70L-GFP, or double transfections with each Arl5b construct plus Arf1-Myc. After 24 h, transfected cells were harvested by trypsinisation, washed twice in chilled PBS and resuspended in chilled cell lysis buffer [0.5% (v/v) IGEPAL CA-630 (I-3021, Sigma-Aldrich, North Ryde, NSW, Australia)] and 1 × cOmplete™, mini EDTA-free protease inhibitor (CPI) (Roche/ Merck, Bayswater, VIC, Australia), in PBS for 30 min on ice. Lysates were cleared *via* centrifugation at 20 000 ***g*** for 15 min at 4 °C. The soluble lysates were transferred to a fresh tube, and 10% of the volume was kept aside as an input reference. Protein G Dynabeads (Thermo Fisher Scientific) were washed in W + B buffer (0.1 M sodium phosphate buffer, 0.01% v/v Tween®20) (Sigma-Aldrich) containing CPI and used following manufacturer's instructions. Briefly, 50 μl washed Protein G Dynabead slurry were bound to 5 μg rabbit anti-Myc antibody (#ab9106; Abcam) (per sample) on a rotating wheel at room temp for 30 min, separated on magnet and washed thrice in chilled wash buffer (0.1% IGEPAL CA-630 in PBS supplemented with 1 × CPI). Cell lysates were pre-cleared on washed Protein G Dynabeads rotating at room temp for 10 min. Myc-tagged proteins and their precipitates were captured from pre-cleared lysates on Protein G Dynabeads loaded with Myc antibodies rotating at room temp for 30 min, separated on magnet and washed thrice in chilled wash buffer (0.1% IGEPAL CA-630 in PBS that was supplemented with 1 × CPI), with a final PBS wash. Samples were then boiled in 50 μL 1 × NuPAGE SDS sample reducing buffer (Thermo Fisher Scientific) at 100 °C for 10 min for SDS/PAGE and immunoblotting analysis.

Statistical analyses

Quantitation was carried out using GRAPHPAD PRISM software for the indicated number of cells at each time point. All analyses included samples from two or more independent experiments. Data were expressed as mean fluorescence intensity (MFI) ± s.e.m, as indicated, and analysed by an unpaired, two-tailed, Student's *t*-test. $P < 0.05$ (*) was considered significant, $P < 0.01$ (**) was highly significant, and $P < 0.001$ (***) was very highly significant. An absence of a *P*-value indicates that the differences were not significant (n.s.).

Results

Generation of constructs for BioID, APEX2 and GFP-Trap

The identification of effectors of small G proteins is challenging due to their dynamic and transient

interactions. To identify the interacting proteins of Arl5b, we used the constitutive active form of Arl5b, namely Arl5b(Q70L), and incorporated three different approaches. Two of the three approaches used *in vivo* proximity labelling methods in live cells, namely BioID [9] and APEX2 [21]. Both these *in vivo* labelling methods have been widely used in the literature; however, there have been limited studies which have incorporated both methods for the same target. We considered that the incorporation of both *in vivo* labelling methods should yield a larger pool of potential binding partners as different chemistries and schedules are used in the labelling procedures and different amino acids are biotinylated [9,21]. In addition, we included a GFP-Trap pull down to identify direct interactors following cell lysis. The later method has the advantage of a more direct analysis of interacting proteins, but the disadvantage of the potential loss of low-affinity interactive proteins following cell lysis. Figure 1 shows the overall strategy of the approach using the three methods. Although all three methods rely on the overexpression of Arl5b, the use of multiple approaches should increase the capacity to distinguish relevant near neighbours and interactors.

For BioID, we generated a Arl5b(Q70L)-BirA*-HA fusion protein (where * represents the mutation R118G) that was localised at the Golgi in transfected HeLa cells, as detected by antibodies to the HA epitope (Fig. 2A) and was detected as a ~50 kDa species by immunoblotting, the predicted size of Arl5b(Q70L)-BirA*-HA (Fig. S1A). There was extensive overlap of Arl5b(Q70L)-BirA*-HA with the TGN marker, golgin97 (Fig. 2A), indicating that the Arl5b fusion was correctly targeted to the TGN. Addition of biotin to Arl5b-BirA*-HA fusion transfected cells resulted in the biotinylation of proteins, as detected by Streptavidin-488 (SA-AF488), and moreover, the biotinylated proteins localised extensively with Arl5b (Q70L)-BirA*-HA (Fig. 2B). This immunofluorescent data indicate that the majority of proteins biotinylated by Arl5b(Q70L)-BirA*-HA are restricted to the Golgi environment. As a control experiment, we have transfected cells with BirA*-HA (Fig. 2A) alone and observed that the proteins are dispersed in the cytosol.

For APEX2, we generated a Arl5b(Q70L)-GFP-APEX2 fusion protein which was localised to the juxtanuclear location, typical of the Golgi, whereas APEX2 alone was detected throughout the cytosol as detected by peroxidase-mediated DAB staining (Fig. 3A) and by GFP fluorescence (Fig. 3B), indicating Golgi location of both the GFP tag and peroxidase activity. Incubation with biotin-phenol for 30 min and 1 min incubation with 1 mM H_2O_2, then immediately quenched in Trolox, and fixed cells were stained for the Golgi marker GM130, followed by SA-AF647 to detected biotinylated species, showed very strong co-location of the GFP-tagged Arl5b with SA-AF647 indicating the majority of labelling in close proximity to the Golgi (Fig. 3B).

For GFP-Trap, we used a stable cell line expressing Arl5b(Q70L)-GFP, which has been previously described [16] and the control was free GFP expression in the cytosol. Based on our earlier report, there is an eightfold increase in Arl5b mRNA in the Arl5b (Q70L)- GFP stable cell line compared with WT HeLa cells, as assessed by qPCR [16].

Proteomic analysis of biotinylated proteins from *in vivo* labelling and from pull downs

Biotinylated proteins from BioID and APEX2 experiments were purified from lysed cells by affinity chromatography using streptavidin. Analysis of the BioID affinity-purified biotinylated proteins by blotting and probing with streptavidin-HRP detected multiple components with sizes from 20 kD → 200 kD (Fig. S1B). As a control in the analyses, we included cells transfected with BirA*-HA alone, and in contrast to the Golgi-localised Arl5b(Q70L)- BirA*-HA, BirA*-HA protein is distributed throughout the cytosol (Fig. 2A). Purified biotinylated proteins were analysed by MS as described in materials and methods. Peptides with ion score greater than the homology score were considered significant. Proteins identified in the BirA*-HA control were subtracted from the Arl5b(Q70L)-BirA*-HA sample and proteins further filtered in the Arl5b (Q70L)-BirA*-HA sample to contain at least two unique peptide matches. In total, 257 unique proteins were identified with 2 significant peptides from two independent BioID experiments and 43 proteins detected in both experiments (including the bait) (Appendix 1). We also identified biotinylated proteins from transfected cells expressing the Arl5b(Q70L)-GFP-APEX2 fusion protein. Proteins identified in the APEX2 control were subtracted from the Arl5b (Q70L)-GFP-APEX2 sample and proteins further filtered to contain at least two unique peptide matches. One hundred and fifty seven proteins were identified from Arl5b(Q70L)-GFP-APEX2 sample from two independent experiments. The data from the BioID and APEX2 experiments were combined. Of the total 395 unique proteins, 54 proteins were detected in at least 2 of the 4 BioID and APEX2 experiments, listed in Appendix 1.

To identify direct interacting partners of Arl5b (Q70L)-GFP, pull downs were performed on cell

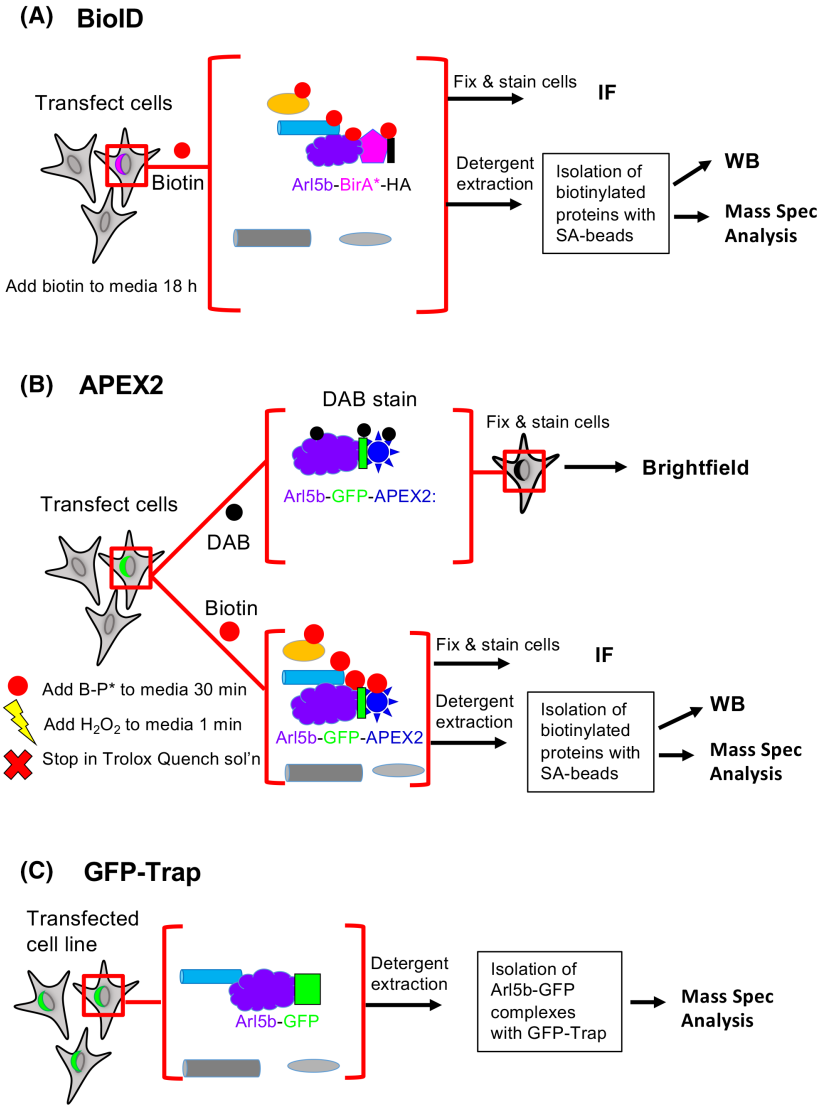


Fig. 1. Outline of experimental protocols to identify interactive partners of Arl5b. (A) BioID, (B) APEX2 and (C) GFP-Trap. In A and B, biotinylated interactive proteins with Arl5b(Q70L) shown as blue rectangles, proximal proteins as orange ovals and non-labelled proteins in grey. In C, proteins bound to Arl5b(Q70L)-GFP depicted as blue rectangles and non-bound proteins in grey.

extracts using GFP-nanotrap and bound material analysed by MS. Peptides with ion score greater than the homology score were considered significant. Proteins identified in the GFP control were subtracted from the Arl5b(Q70L)-GFP, sample and proteins further filtered in the Arl5b(Q70L)-GFP sample to contain at least two unique peptide matches. A protein was designated as a candidate interactor of Arl5b(Q70L)-GFP if it exclusively appeared in at least 2 out of 3 independent experiments; 24 proteins (including Arl5b) were detected in at least 2 out of the 3 independent experiments (Appendix 1 and Fig. 4). Seven of the 24 are known Golgi proteins. Of the 24 proteins, 5 overlapped with both GFP-Trap and BioID/APEX2 (Fig. 4A). The five proteins detected by both *in vivo* proximity and GFP-Trap are Arl5a, Arl5b (bait), Rab10, Arf1/Arf3 and SRPRB. The peptides identified for Arf1 and Arf3 are identical, and the two Arfs could not be distinguished. SRPRB, signal recognition particle receptor subunit beta, is a component of the signal recognition particle (SRP), and it is not clear why this cytosolic and ER subunit would be associated with Arl5b.

Collectively of the 54 proteins detected from 2 independent BioID and APEX2 experiments, and the 24 proteins from the GFP-Trap pull down, 22 (28%) are known to localise to the Golgi. Eleven of the 22 proteins, including the bait, are small G proteins, namely Arl5A, Arl5B, Arf1/3, Arf4, Arf5, Rab1A, Rab6A, Rab6B, Rab8A, Rab10 and Rab14. The remaining proteins include vesicle transport proteins (3) such as SNAREs, membranes proteins (6) including tethering

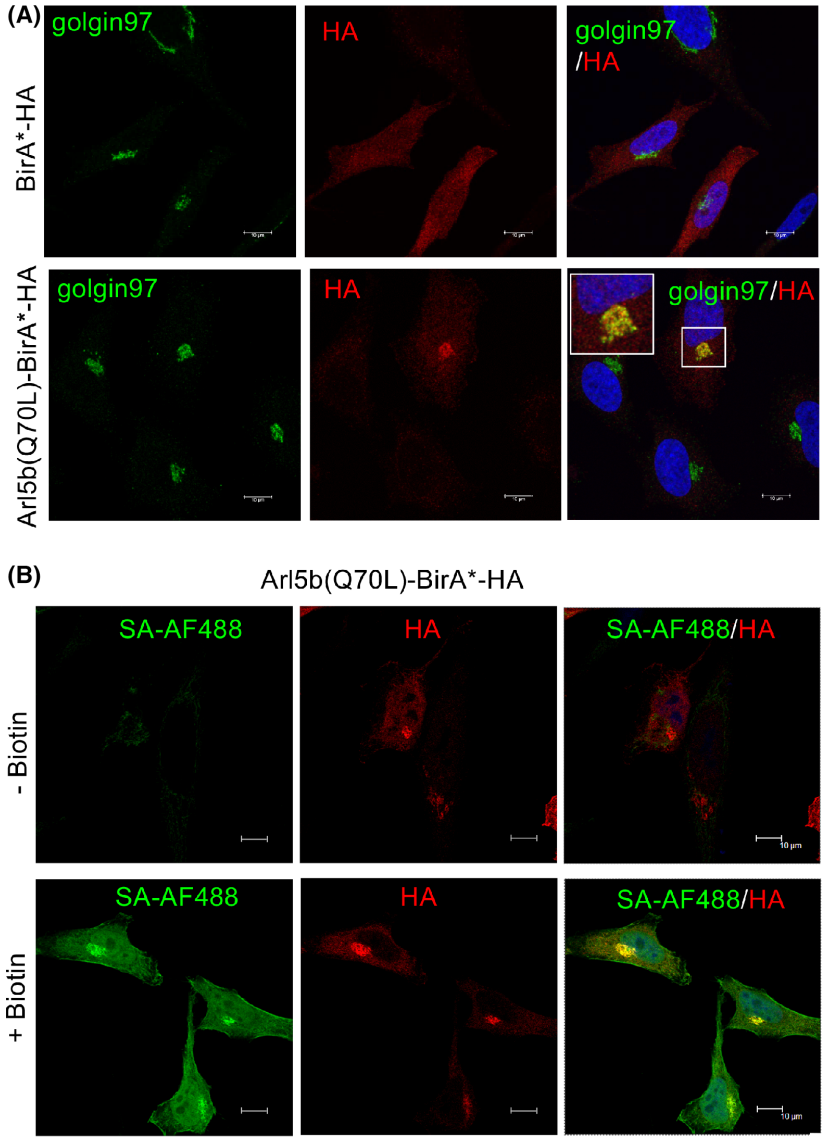


Fig. 2. Localisation of Arl5b (Q70L)-GFP-BirA*, and biotinylated products, in transfected HeLa cells. (A) HeLa cells expressing either HA-tagged vector, BirA*-HA or HA-tagged Arl5b construct, Arl5b (Q70L)- BirA*-HA, were fixed, permeabilised and blocked, followed by staining with antibodies to either golgin97 (green) and HA (red) and DAPI (blue). Higher magnification of the boxed region in the merged image is also shown. Bars represent 10 μm. (B) HeLa cells expressing Arl5b (Q70L)-BirA*-HA were either untreated (−biotin) or treated with 50 μM biotin in c-DMEM for 20 h (+Biotin). Cells were fixed, permeabilised and blocked, followed by staining with strepatavidin-AF488 (SA-AF488) and anti-HA antibodies (red). Bars represent 10 μm.

factors and signal receptors (2) as depicted in the Venn diagram in Fig. 4B. The list of Golgi proteins and the protein scores from the MS are shown in Table 1.

To further investigate the interaction of Arl5b with the candidate small G proteins, we choose Arf1 for further analysis. A reciprocal co-IP was performed by co-transfecting HeLa cells with Arf1-myc and either the Arl5b GDP-restricted mutant (Arl5b(T30N)-GFP) or Arl5b GTP-restricted mutant (Arl5b(Q70L)-GFP) and assessed whether Arf1-myc co-precipitated the Arl5b-GFP variants using anti-myc antibodies and protein G Dynabeads. Immunoblotting of the immunoprecipitates with antibodies to GFP detected a band of ~ 45 kDa, the predicted size of Arl5b-GFP, from the IP with Arl5b(Q70L)-GFP, whereas Arl5b (T30N)-GFP was not detected in the IP, rather was recovered in the Protein G unbound fraction (Fig. S2A). Similar levels of Arf1-myc were precipitated in both IPs (Fig. S2B). Thus, this finding demonstrates that Arf1 can interact with Arl5b and that the interaction is GTP-dependent and/or requires a membrane surface.

Identification of TGN scaffold molecules regulated by Arl5b

Small G proteins are known to recruit scaffolds/tethers to the Golgi membranes, which in turn regulate many of the Golgi functions. From the combined proteomic analysis discussed above, we were interested in

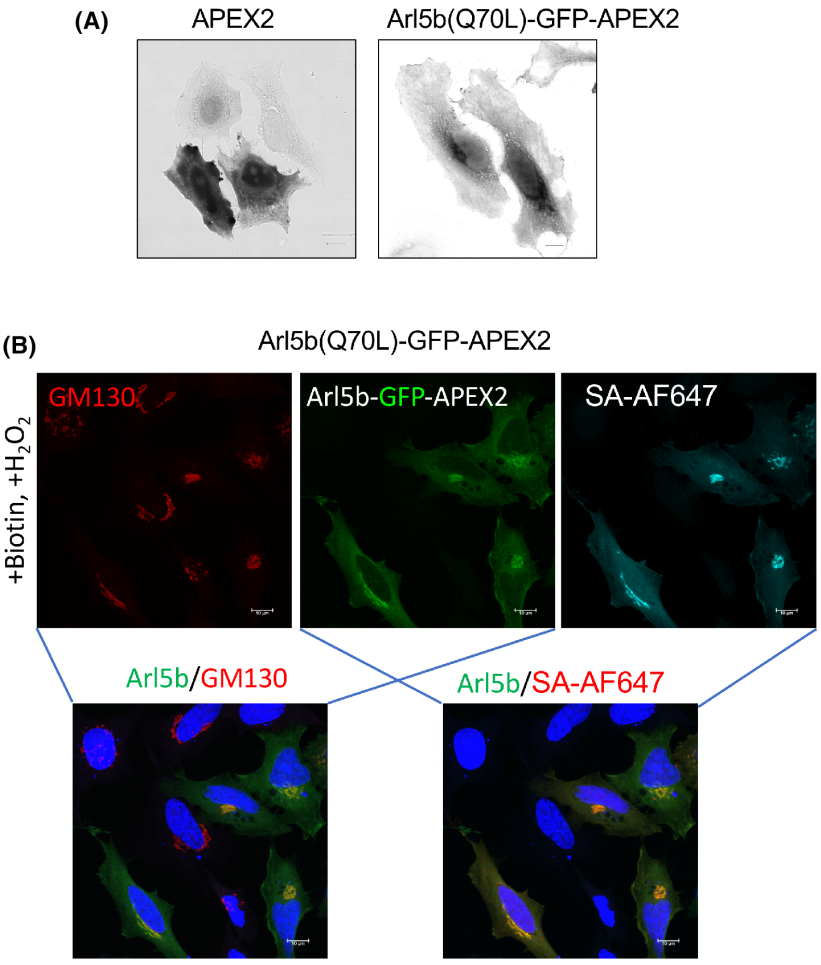


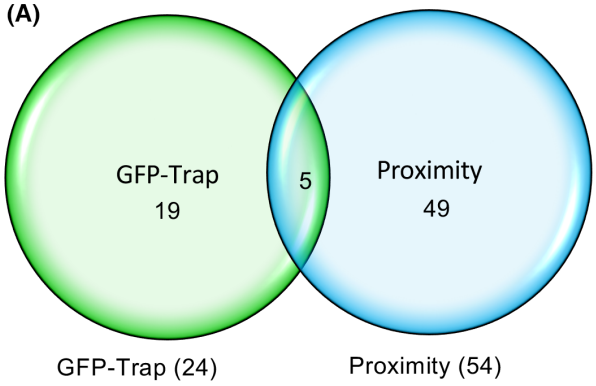
Fig. 3. Localisation of Arl5b(Q70L)-GFP-APEX2, and biotinylated products, in transfected HeLa cells. (A) HeLa cell monolayers were transfected with either APEX2 alone or Arl5b-GFP-APEX2 for 24 h, then fixed in 4% PFA for 15 min, quenched in 30 mM glycine/PBS for 10 min, washed with PBS and then incubated with freshly prepared DAB/H_2O_2 in PBS for 30 min. Cells were imaged by brightfield confocal microscopy. (B) HeLa cell monolayers transfected with Arl5b (Q70L)-GFP-APEX2 (green) were cultured for 24 h. Cells were incubated with biotin-phenol in growth medium for 30 min and then with 1 mM H_2O_2 for only 1 min before quenching in Trolox quenching solution. Cells were fixed in 4% PFA, permeabilised, blocked and then stained with mouse anti-GM130 (red), followed by incubation with streptavidin (SA-AF647) (cyan) to detect biotin and DAPI (blue) to stain the nuclei. Scale bars represent 10 μm. Images are maximum intensity projections of Z-stacks. Note that streptavidin staining is detected at the location of the fusion protein. Bars represent 10 μm.

identifying potential scaffold/tethering molecules, which interact and/or are recruited to the Golgi by Arl5b. It was of particular interest that two scaffold molecules were identified in the set of 22 Golgi proteins from the combined analyses, namely Acyl-CoA-binding domain-containing 3 (ACBD3) (gene *GCP60*) and PIST, a PDZ domain-containing Golgi protein (gene *GOPC*). ACBD3 is a multi-functional protein, which has been reported to be localised to the Golgi, which has been associated with a diverse array of cellular functions including regulation of lipid transport and maintenance of Golgi structure [23,24]. PIST is also a scaffold located on the TGN, which is known to regulate the trafficking of a number of receptors [25,26]. These two proteins were detected in all 4 *in vivo* proximity labelling methods. However, neither were detected by the GFP-Trap pull downs, which may indicate the interactions are weak/transient or that they are in close proximity to Arl5b and do not directly interact.

We firstly analysed the localisation of ACBD3 in HeLa cells to confirm that ACBD3 was localised to the Golgi and to establish whether ACBD3 is located on the TGN. In wild-type (WT) HeLa cells, ACBD3 showed substantial co-localisation with the cis-Golgi marker, GM130, confirming a Golgi location. Line scan analysis of GM130 and ACDB3 showed a partial overlap indicating the location of ACBD3 was not restricted to the *cis*-Golgi. To analyse for TGN location, HeLa cells stably expressing Arl5b(Q70L)-GFP cells were stained with both GM130 and ACBD3. Arl5b and ACBD3 show strong co-localisation. The localisation of Arl5b(Q70L)-GFP and GM130 can be discriminated by line scan analysis. ACBD3 partially colocalises with both markers indicating that ACBD3 is located on the cis-Golgi and also the TGN (Fig. 5A, B). As expected from previous reports [26], PIST also shows strong co-localisation with the TGN marker Arl5b(Q70L)-GFP (Fig. 5C).

Both ACBD3 and PIST are peripheral membrane proteins, and it remains unclear how these proteins are recruited from the cytosol to the TGN membranes. Therefore, we investigated whether the levels of Arl5b

(A)



Techniques	# Proteins in common	Proteins
GFP-Trap, Proximity	5	ARL5B ARL5A RAB10 ARF1/ ARF3 SRPRB
GFP-Trap	19	SMYD3 PLAK SSRD PFKAL S61A1 PTH2 RP9 RPB4 RAB8A HDAC2 ATX10 RAB8B NPRL3 PHF8 DPM1 LMNA RIR1 SRPR TADBP
Proximity	49	ABCD3 AL3A2 ARF4 ARF5 ATPB CD44 DNJA1 DNJA2 DNJB1 GCP60 GOPC GORS2 HLAA HLAC IF5A1 ITA3 ITA5 ITB1 KAISO LAP2A LAT1 LDHB LMAN1 MUC18 NUDC PDLI5 PGRC2 PP1A PRDX2 RAB14 RAB1A RAB6A RAB6B RL40 RPN1 RS27A RTN4 RUVB1 STAT1 TM165 TM87A TPD54 UBB UBC VAMP2 VAMP3 VPS54 XCT ZCCHV

Bait Proteins at Golgi

(B)

Gene Name
ARL5B
ARL5A
ARF1ARF3
ARF4
ARF5
CD44
GCP60
GOPC
GORS2
ITA5
LMAN1
RAB10
RAB14
RAB1A
RAB6A
RAB6B
RAB8A
TM165
TM87A
VAMP2
VAMP3
VPS54

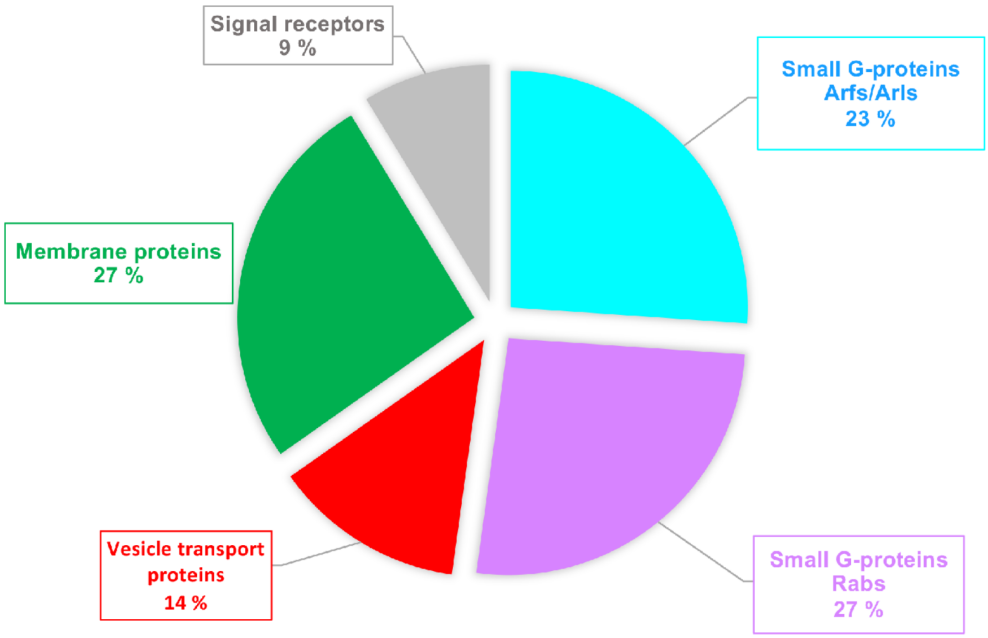


Fig. 4. Analysis of proteins identified from proximity labelling and GFP-Trap. (A) Venn diagram showing the overlap in hits from the proximity labelling techniques with the hits from GFP-Trap affinity isolation and the identity of the proteins. (B) The list of 22 unique proteins from (A) which localise to the Golgi, including the bait, Arl5b, and a pie chart of the classification of these Golgi proteins.

impact on Golgi levels of ACBD3 and PIST. Comparison of staining of WT HeLa cells and the overexpressing Arl5b(Q70L)-GFP HeLa cell line, under identical conditions, showed increased ACDB3 staining in the Golgi of the Arl5b(Q70L)-GFP HeLa cell line (Fig. S3A). Quantitation of ACDB3 levels on the Golgi demonstrates a significant increase in ACBD3 in HeLaArl5b(Q70L)-GFP cells compared with WT HeLa (Fig. 6A). Moreover, knock-down of Arl5b resulted in reduction of intensity of ACBD3 staining

Table 1. Golgi localizing proteins identified from BioID, APEX2 and GFP-Trap. Bait indicated in green. Significant gene/protein lists for each technique were pooled (total 76 genes) and analysed in Gene Ontology (GO) enrichment analysis http://geneontology.org/ filtering on Homo Sapiens and cell components. Of the 76 proteins, three proteins could not be mapped by this analysis (LAP2B, SRPR, SUGT1). Of the 73 remaining proteins, 22 proteins (including bait) were localized to the Golgi apparatus. Dark grey indicates the peptide score was below 30 or not detected, and light grey indicates the peptide score was <40.

		Peptide score per expt						
Gene name	Protein name	BioID-1	BioID-2	APEX2-1	APEX2-2	GFP-Trap-1	GFP-Trap-2	GFP-Trap-3
ARL5B	ADP-ribosylation factor-like protein 5B;ARL5B	152.95	214.73	127.07	40.9	495	616.69	237.47
ARL5A	ADP-ribosylation factor-like protein 5A;ARL5A	101.46	48.9	98.86	41.46	207.7	245.63	73.63
ARF1/ ARF3	ADP-ribosylation factor 1;ARF1/ADP-ribosylation factor 3;ARF3	78.71	186.94	125.07	76.46	43.1		86.96
ARF4	ADP-ribosylation factor 4;ARF4	84.03	117.47	105.37	60.71	30.31	37.45	
ARF5	ADP-ribosylation factor 5;ARF5	56.91	120.91	49.15				
CD44	CD44 antigen;CD44	115.12	137.03	82.94	145.13	231.84	54.72	55.77
GCP60	Golgi resident protein GCP60;ACBD3	61.44	113.54	132.78	112.41			
GOPC	Golgi-associated PDZ and coiled-coil motif-containing protein;GOPC	20.27	67.44	126.42	70.94			
GORS2	Golgi reassembly-stacking protein 2;GORASP2	47.99	51.43					
ITA5	Integrin alpha-5;ITGA5	64.2	225.08			141.89		
LMAN1	Protein ERGIC-53;LMAN1	96.03	150.73			34.75		65
RAB10	Ras-related protein Rab-10;RAB10		58.22	64.05	30.18	128.96	77.61	82.66
RAB14	Ras-related protein Rab-14;RAB14		42.7	46.76	30.18	148.41	49	61.66
RAB1A	Ras-related protein Rab-1A;RAB1A	110.58	248.77	105.73	45.76	115.09	49	89.81
RAB6A	Ras-related protein Rab-6A;RAB6A	87.32	174.32	68.25	30.18	49	61.66	78.92
RAB6B	Ras-related protein Rab-6B;RAB6B	55.73	124.8	46.76	30.18	49	61.66	66.31
RAB8A	Ras-related protein Rab-8A;RAB8A		58.22	58.67	30.18	93.83	55.05	89.81
TM165	Transmembrane protein 165;TMEM165	55.4	133.29			33.03		
TM87A	Transmembrane protein 87A;TMEM87A	42.11	193.46					
VAMP2	Vesicle-associated membrane protein 2;VAMP2	172.52	139.72			48.69		
VAMP3	Vesicle-associated membrane protein 3;VAMP3	195.82	301.82			78.18		
VPS54	Vacuolar protein sorting-associated protein 54;VPS54	78.94	260.14					

in both HeLaArl5b(Q70L)-GFP cells (Fig. 7) and WT HeLa cells (not shown). Quantitation of the fluorescence levels following Arl5b knock-down showed a significant reduction (~50%) of endogenous ACDB3 on the Golgi of either WT HeLa or HeLaAr5b(Q70L)-GFP cells (Fig. 8A). The specificity of the ACBD3 antibodies was confirmed by the reduced staining (> 50%) in cells treated with ACBD3 siRNA (Fig. 8A). Collectively, these results demonstrate that Arl5b is required for recruitment of ~ 50% of ACBD3 to the Golgi.

Phosphatidylinositol 4-kinase beta (PI4KB) has been reported to be recruited to the TGN by ACBD3 and plays an important role for transport carrier formation [23]. Therefore, we examined the potential role of Arl5b in recruitment of PI4KB at the TGN by the analysis of Golgi staining. An increased level of PI4KB was observed at the Golgi in Arl5b-GFP stable cell line, compared with WT cells (Fig. S3B), indicating a role for TGN-localised Arl5b in the recruitment of PI4KB at the TGN.

Quantitation of PIST levels on the Golgi demonstrated no significant change in the level of PIST in HeLaArl5b-GFP cells compared with WT HeLa cells (Fig. 6B). Moreover, and in contrast to ACBD3, knock-down of Arl5b in either WT HeLa or HeLaAr5b(Q70L)-GFP cells did not reduce the level of PIST at the Golgi (Fig. 8B). Hence Arl5b is not required for PIST recruitment. Collectively, these findings show that Arl5b is directly associated with ACBD3 recruitment to the Golgi, but not PIST recruitment. In addition, these findings show the advantage of combining multiple methods in detection of interaction partners.

Discussion

Rabs, Arls and Arfs are major regulators of TGN sorting and trafficking pathways exiting and returning to the TGN [1,12,14]. The interactive partners of these GTPases play an essential role in mediating their activities; however, the identity of the Arl and Arf effectors

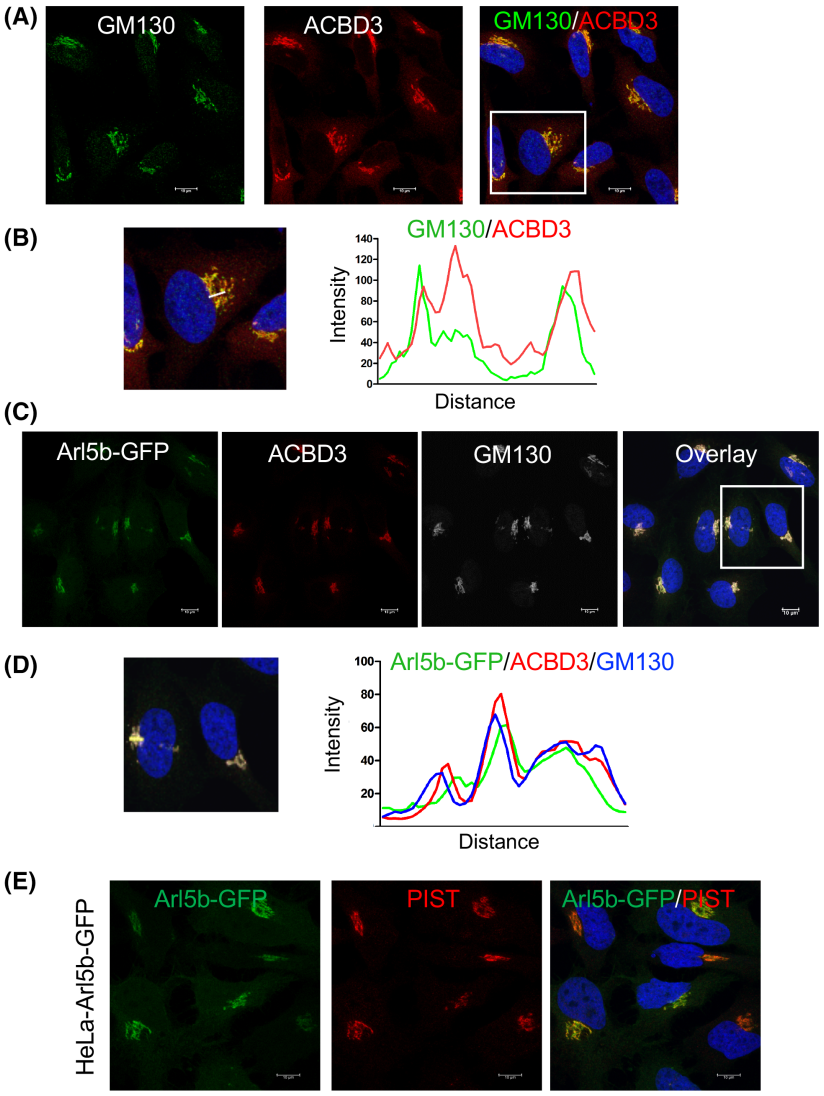


Fig. 5. ACBD3 and PIST co-localise with the marker, Arb5b-GFP. HeLa WT (A) and HeLaArl5b(Q70L)-GFP cells (C–E) were fixed and permeabilised and stained for ACBD3 (red) and GM130 (A green, C greyscale) and PIST (red) (E). For (A) and (C), a line scan profile was generated, B and D, respectively, for the region marked with a white line in the images magnified from the boxed regions. Bars represent 10 μm.

remains poorly characterised. Here, we have sought to identify the interacting partners of Arl5b, which regulates endosomal-TGN transport [16,27]. We incorporated three proteomic approaches in the study to maximise the capture of both membrane-bound and peripheral membrane proteins incorporating both direct pull downs and proximity-based labelling methods. A notable finding from these analyses was the detection of a number of small G proteins, with Rab10 and Arf1/Arf3 detected by all three methods. We also confirmed an interaction between Arl5b and Arf1 by a reciprocal IP experiment where Arl1-myc co-precipitated Arl5b-GFP. The proximity assays also identified Arf4, Arf5 and Rab14, Rab6A and Rab6B and the GFP pull-down assays also identified Rab8A. All these small G proteins have been reported to be associated with the Golgi, and most with the TGN [7,12], whereas the *medial*-Golgi Rab, Rab33B [28] was not detected at significant levels, which provides confidence in the strategy to identify spatially relevant proteins. The identification of a cluster of small G proteins is relevant as it indicates a co-ordinated suite of small G proteins regulating functions at the TGN, rather than each acting independently.

A challenge in the field is to identify the interactome of membrane proteins. Approaches involving relocation of the bait to different organelles have been used [29] to enhance the identification of binding partners using proximity methods; however, these approaches will only detect peripheral membrane proteins which interact with the bait but not membrane proteins, such as small G proteins, which are specifically targeted to

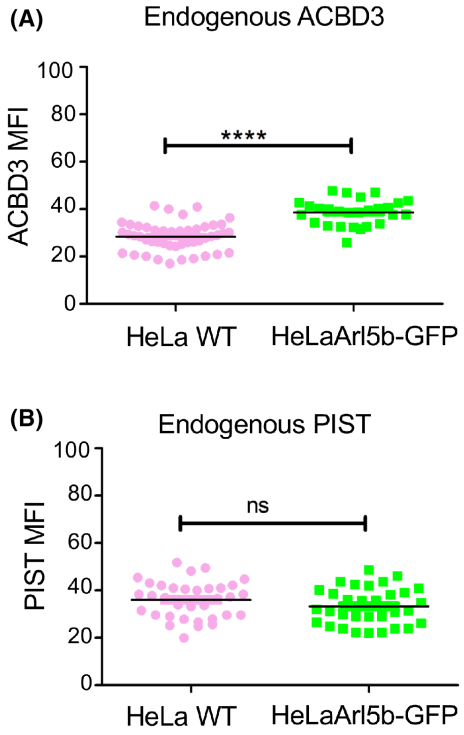


Fig. 6. Expression of HeLaArl5b-GFP results in an increase in endogenous ACBD3 at the Golgi, but not PIST. HeLa WT and HeLaArl5b-GFP cells were fixed and permeabilised and stained for either (A) ACBD3 or (B) PIST and the Golgi staining quantified as described in methods and expressed as mean fluorescence intensity (MFI) ± SEM. Each symbol represents an individual cell ($n \geq 26$). Student's *t*-test, ****$P < 0.0001$, n.s. not significant.

membranes of selected organelles. Hence, approaches to identify effectors *in situ* on their normal membrane sites are required. A strength of the current approach was the application of 3 different strategies to identify the Arb5b neighbourhood within TGN membranes. The findings from this work highlight the complexity of small G protein regulated pathways at the Golgi/TGN and suggest caution should be applied when manipulating the levels of individual Rabs or Arls as they could influence the activities of other G proteins in the same pathway. Of note is that a recent study has also incorporated pull downs and BioID to analyse complexes on different organelles using a uniquely designed single construct [30]. One caveat of our strategy is that it involved overexpression of the Arl5b bait, which may result in non-physiological interactions. Further analysis is required to confirm the interaction of endogenous Arl5b with all of the targets and whether these interactions are dependent or independent on GTP-bound Arl5b.

Why are so many G proteins detected in the current analyses? The findings here suggest that the G proteins either exist as an interactive complex or that they are spatially organised within the same domain. Small G protein cascades have been reported on the early and late endosomes [31,32] and proposed for recycling endosomes [33,34], and our findings suggest that Rab/Arf/Arl cascades may occur on the TGN. A more detailed study is required to determine how these small proteins may regulate each other. One possibility is that GEFs and GAPs are effectors of the TGN small G proteins that regulate the conversion process of small G protein cascades [35], for example, the late Golgi Arf1 has been shown to control the conversion of a Rab in the yeast late Golgi [36]. GEF and GAP proteins were not detected in the current study; however, these may be difficult to label and or pull down.

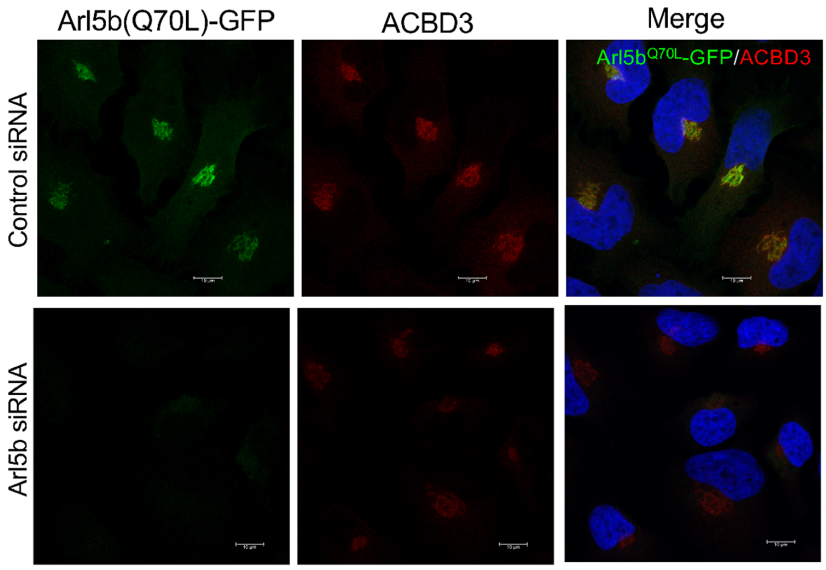


Fig. 7. Impact of silencing of Arf5b on endogenous ACBD3 at the Golgi. Monolayers of HeLaArl5b(Q70L)-GFP cells were transfected with either control siRNA or Arl5b siRNA, as indicated, for 72 h, fixed and permeabilised and stained for ACBD3 (red) and DAPI (blue). Bars represent 10 μm.

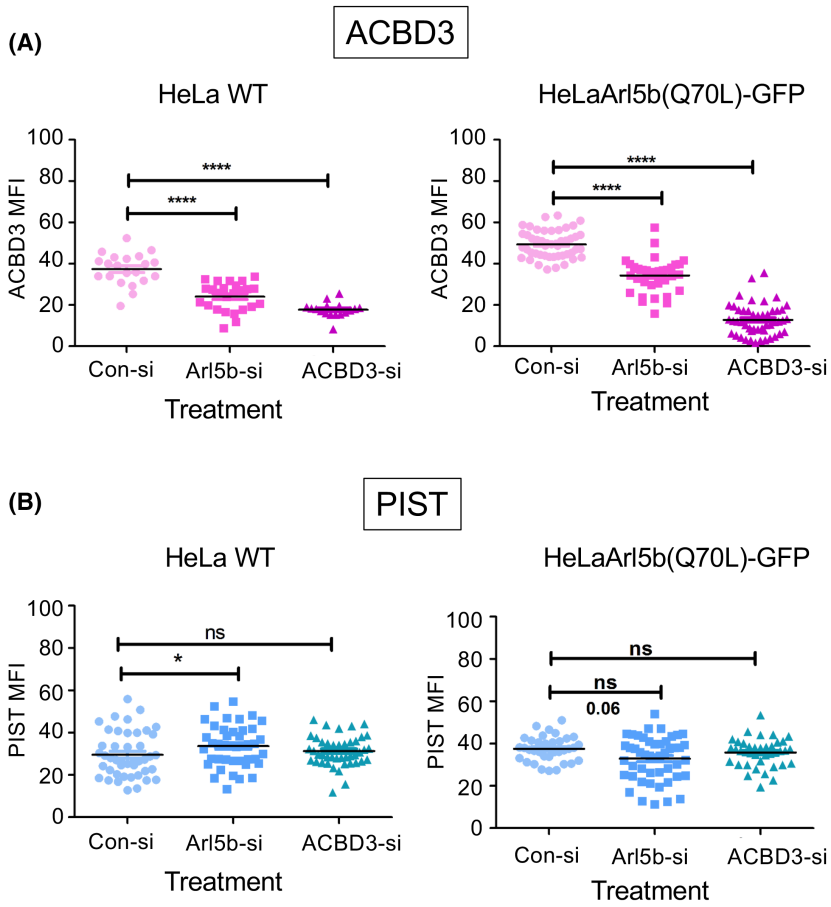


Fig. 8. Quantitation of Golgi-localised ACDB3 and PIST in HeLa WT and HeLaArl5b(Q70L)-GFP cells following Arl5b knock-down. (A,B) Monolayers of HeLa WT cells and HeLaArl5b(Q70L)-GFP cells, as indicated, were transfected with either control siRNA, Arl5b siRNA or ACBD3 siRNA, as indicated, for 72 h, fixed and permeabilised and stained for (A) ACBD3 or (B) PIST. Golgi staining quantified as described in methods and expressed as mean fluorescence intensity (MFI) ± SEM. Each symbol represents an individual cell (n = 17–48). Student's t-test, ****$P < 0.0001$, *$P < 0.05$, ns, not significant.

A recent study has identified the heat shock protein, HSC70, as an interactive partner of Arl5b [37]. HSC70 was not detected in our study; however, as these authors showed that HSC70 bound preferentially to the GDP-bound form of Arl5b [37], the absence of HSC70 from the interactive partners of GTP-bound Arl5b(Q70L) in our study is probably not surprising.

One key function of small G proteins at the Golgi is the recruitment of scaffolds/tethers to the Golgi membranes, which in turn regulate many of the Golgi functions. It was of particular interest that two Golgi-resident proteins PIST (GOPC) and ACBD3 (GCP60) were identified from the combined proximity labelling techniques, but were not detected with the GFP-Trap pull down. ACBD3 endogenous levels were elevated in HeLaArl5b(Q70L)-GFP cells compared with wild-type HeLa cells. ACDB3 has a number of functions associated with the Golgi stack and the TGN. Giantin, which is localised to the *cis*- and *medial*- cisternae, has been reported to bind to ACBD3 [38]. At the TGN, ACBD3 been reported to recruit PI4KB to the Golgi [39], a kinase located to the TGN [40]; however, the mechanisms for the location of ACBD3 to the TGN had not been defined. Here, we showed that an increase in expression of active Arl5b resulted in an increase in ACBD3 at the Golgi with substantial overlap with Arl5b-GFP, indicating that Arl5b was enhancing ACDB3 localisation at the TGN Knock-down experiments demonstrated that Arl5b was required for the recruitment of the tethering factor ACBD3 to the TGN, indicating an association of ACBD3 with Arl5b. There was a partial reduction of ACBD3 at the Golgi following Arl5b knock-down, indicating additional mechanisms for ACBD3 recruitment, such as giantin-mediated recruitment to the cis/medial Golgi. Thus, the approach used was able to identify a tethering factor which interacts with Arl5b either directly or indirectly. The lack of detection of ACBD3 by the GFP-Trap pull-down assay may reflect a low-affinity interaction. These findings, together with previous work, suggest that the tethering factor ACBD3 has multiple roles in the early and late Golgi compartments.

In summary, this study shows the benefit of employing multiple approaches to study *in situ* interactions with small G proteins and highlights a potential

interactive network of small G proteins at the TGN. Further investigation is now required to determine whether these G proteins act in a co-ordinated G protein cascade.

Acknowledgements

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Author contributions

FJH and PAG conceived the project, designed the experiments, interpreted the data and wrote the manuscript. FJH performed the majority of the experiments. EHJC provided tools and expertise for image quantitation analysis. NAW performed the MS analysis on trypsin digests and CM assisted with the analysis of MS data and fluorescence image analysis..

Data accessibility

The data supporting the findings of this study are available on request from the authors.

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Appendix 1. Gene ontology list

Genes and proteins—3 techniques combined

Genes	BioID 2–2	Proximity at least 2–4	GFP-Trap at least 2–3	Accession #	Protein name
ABCD3	ABCD3	ABCD3		P28288	ATP-binding cassette subfamily D member 3
AL3A2	AL3A2	AL3A2		P51648	Aldehyde dehydrogenase family 3 member A2;ALDH3A2
ARF1_ARF3		ARF1_ARF3	ARF1_ARF3	P84077, P61204	ADP-ribosylation factor 1; ARF1 and/or ARF3
ARF4		ARF4		P18085	ADP-ribosylation factor 3; ARF3
ARF5		ARF5		P84085	ADP-ribosylation factor 4; ARF4
ARL5A	ARL5A	ARL5A	ARL5A	Q9Y689	ADP-ribosylation factor-like protein 5A; ARL5A
ARL5B	ARL5B	ARL5B	ARL5B	Q96KC2	ADP-ribosylation factor-like protein 5B; ARL5B
ATG2B	ATG2B			Q96BY7	Autophagy-related protein 2 homolog B; ATG2B
ATPB	ATPB	ATPB		P06576	ATP synthase subunit beta, mitochondrial; ATP5F1B
ATX10			ATX10	Q9UBB4	Ataxin-10; ATXN10; ortholog
CD44	CD44	CD44		P16070	CD44 antigen;CD44
DNJA1	DNJA1	DNJA1		P31689	DnaJ homolog subfamily A member 1; DNAJA1
DNJA2	DNJA2	DNJA2		O60884	DnaJ homolog subfamily A member 2; DNAJA2
DNJB1	DNJB1	DNJB1		P25685	DnaJ homolog subfamily B member 1; DNAJB1
DPM1			DPM1	O60762	Dolichol-phosphate mannosyltransferase subunit 1; DPM
GCP60		GCP60		Q9H3P7	Golgi resident protein GCP60; ACBD3
GOPC		GOPC		Q9HD26	Golgi-associated PDZ and coiled-coil motif-containing protein; GOPC
GORS2	GORS2	GORS2		Q9H8Y8	Golgi reassembly-stacking protein 2; GORASP2
HDAC2			HDAC2	Q92769	Histone deacetylase 2; HDAC2
HLAA	HLAA	HLAA		P04439	HLA class I histocompatibility antigen, A alpha chain; HLA-A
HLAC	HLAC	HLAC		P10321	HLA class I histocompatibility antigen, C alpha chain; HLA-C
IF5A1	IF5A1	IF5A1		P63241	Eukaryotic translation initiation factor 5A-1; EIF5A
ITA3	ITA3	ITA3		P26006	Integrin alpha-3; ITGA3
ITA5	ITA5	ITA5		P08648	Integrin alpha-5; ITGA5
ITB1	ITB1	ITB1		P05556	Integrin beta-1; ITGB1
KAISO	KAISO	KAISO		Q86T24	Transcriptional regulator Kaiso; ZBTB33
LAP2A	LAP2A	LAP2A		P42166	Lamina-associated polypeptide 2, isoform alpha; TMPO
LAT1	LAT1	LAT1		Q01650	Large neutral amino acid transporter small subunit 1; SLC7A5
LDHB	LDHB	LDHB		P07195	L-lactate dehydrogenase B chain; LDHB
LMAN1	LMAN1	LMAN1		P49257	Protein ERGIC-53; LMAN1
LMNA			LMNA	P02545	Prelamin-A/C; LMNA
MUC18	MUC18	MUC18		P43121	Cell surface glycoprotein MUC18; MCAM
NPRL3			NPRL3	Q12980	GATOR complex protein NPRL3; NPRL3
NUDC	NUDC	NUDC		Q9Y266	Nuclear migration protein nudC; NUDC
PDLI5	PDLI5	PDLI5		Q96HC4	PDZ and LIM domain protein 5; PDLIM5
PFKAL			PFKAL	Q9UPP1	Histone lysine demethylase PHF8; PHF8
PGRC2	PGRC2	PGRC2		O15173	Membrane-associated progesterone receptor component 2; PGRMC2
PFKAL			PFKAL	P17858	ATP-dependent 6-phosphofructokinase, liver type; PFKL
PLAK			PLAK	P14923	Junction plakoglobin; JUP
PP1A		PP1A		P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit; PPP1CA
PRDX2		PRDX2		P32119	Peroxiredoxin-2; PRDX2
PTH2			PTH2	Q9Y3E5	Peptidyl-tRNA hydrolase 2, mitochondrial; PTRH2
RAB1A		RAB1A		P62820	Ras-related protein Rab-1A; RAB1A
RAB6A	RAB6A	RAB6A		P20340	Ras-related protein Rab-6A; RAB6A
RAB6B	RAB6B	RAB6B		Q9NRW1	Ras-related protein Rab-6B; RAB6B
RAB8A			RAB8A	P61006	Ras-related protein Rab-8A; RAB8A

Appendix 1. (Continued).

Genes	BioID 2–2	Proximity at least 2–4	GFP-Trap at least 2–3	Accession #	Protein name
RAB8B			RAB8B	Q92930	Ras-related protein Rab-8B; RAB8B
RAB10		RAB10	RAB10	P61026	Ras-related protein Rab-10; RAB10
RAB14		RAB14		P61106	Ras-related protein Rab-14; RAB14
RIR1			RIR1	P23921	Ribonucleoside-diphosphate reductase large subunit; RRM1
RL40	RL40	RL40		P62987	Ubiquitin-60S ribosomal protein L40; UBA52
RP9			RP9	Q8TA86	Retinitis pigmentosa 9 protein; RP9
RPB4			RPB4	O15514	DNA-directed RNA polymerase II subunit RPB4; POLR2D
RPN1	RPN1	RPN1		P04843	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1; RPN1
RS27A	RS27A	RS27A		P62979	Ubiquitin-40S ribosomal protein S27a; RPS27A
RTN4	RTN4	RTN4		Q9NQC3	Reticulon-4; RTN4
RUVB1	RUVB1	RUVB1		Q9Y265	RuvB-like 1; RUVBL1
S61A1			S61A1	P61619	Protein transport protein Sec61 subunit alpha isoform 1; SEC61A1
SMYD3			SMYD3	Q9H7B4	Histone-lysine *N*-methyltransferase SMYD3; SMYD3
SRPR			SRPR	Q6IAX9	Signal recognition particle receptor
SRPRB	SRPRB	SRPRB	SRPRB	Q9Y5M8	Signal recognition particle receptor subunit beta; SRPRB
SSRD			SSRD	P51571	Translocon-associated protein subunit delta; SSR4
STAT1		STAT1		P42224	Signal transducer and activator of transcription 1-alpha/beta; STAT1
TADBP			TADBP	Q13148	TAR DNA-binding protein 43; TARDBP
TM165	TM165	TM165		Q9HC07	Transmembrane protein 165; TMEM165
TM87A	TM87A	TM87A		Q8NBN3	Transmembrane protein 87A; TMEM87A
TPD54	TPD54	TPD54		O43399	Tumor protein D54; TPD52L2
UBB	UBB	UBB		P0CG47	Polyubiquitin-B; UBB
UBC	UBC	UBC		P0CG48	Polyubiquitin-C; UBC
VAMP2	VAMP2	VAMP2		P63027	Vesicle-associated membrane protein 2; VAMP2
VAMP3	VAMP3	VAMP3		Q15836	Vesicle-associated membrane protein 3; VAMP3
VPS54	VPS54	VPS54		Q9P1Q0	Vacuolar protein sorting-associated protein 54; VPS54
XCT	XCT	XCT		Q9UPY5	Cystine/glutamate transporter; SLC7A11
ZCCHV		ZCCHV		Q7Z2W4	Zinc finger CCCH-type antiviral protein 1; ZC3HAV1
74	43	54	24	Total	

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Immunoblot analysis of Arl5b (Q70L)-BirA* fusion protein and biotinylated products of *in vivo* labelling.

Fig. S2. Arf1-myc co-immunoprecipitates Arl5b-Q71L-GFP.

Fig. S3. Comparison of ACBD3 and PI4PB staining in HeLa WT and HeLaArl5b(Q70L)-GFP cells.