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# The membrane-tethering protein p115 interacts with GBF1, an ARF guanine-nucleotide-exchange factor

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**The membrane-transport factor p115 interacts with diverse components of the membrane-transport machinery. It binds two Golgi matrix proteins, a Rab GTPase, and various members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family. Here, we describe a novel interaction between p115 and Golgi-specific brefeldin-A-resistant factor 1 (GBF1), a guanine-nucleotide exchange factor for ADP ribosylation factor (ARF). GBF1 was identified in a yeast two-hybrid screen, using full-length p115 as bait. The interaction was confirmed biochemically, using *in vitro* and *in vivo* assays. The interacting domains were mapped to the proline-rich region of GBF1 and the head region of p115. These proteins colocalize extensively in the Golgi and in peripheral vesicular tubular clusters. Mutagenesis analysis indicates that the interaction is not required for targeting GBF1 or p115 to membranes. Expression of the p115-binding (pro-rich) region of GBF1 leads to Golgi disruption, indicating that the interaction between p115 and GBF1 is functionally relevant.**

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## INTRODUCTION

The transport of proteins and lipids between distinct compartments of the secretory pathway is mediated by the formation, movement and fusion of transport intermediates. Each stage of this process is regulated by proteins that act in a sequential and coordinated manner. One of these proteins, p115, has been shown to be required for membrane traffic from the endoplasmic reticulum (ER) to and through the Golgi, and for Golgi reassembly after mitosis (Alvarez *et al.*, 1999; Shorter & Warren, 1999; Waters *et al.*, 1992). Although intensely studied in the past few years, the exact molecular mechanism of p115 function is not completely understood. p115 is a parallel homodimer, with two globular heads and an extended rod-like coiled-coil tail (Sapperstein *et al.*, 1995). Distinct domains of p115 interact with different proteins, including components of the Golgi matrix (GM130 and giantin), a small GTPase of the Rab family (Rab1a), and *N*-ethylmaleimide-sensitive-factor attachment-protein-receptor (SNARE) family members (syntaxin 5, Sly1p, GOS-28, membrin, Ykt6p, rSec22p, Bet1p, and GS15). Both GM130 and giantin interact with the same highly acidic carboxy-terminal 25-amino-acid domain in p115 (Linstedt *et al.*, 2000; Nelson *et al.*, 1998). Interactions between p115, GM130 and giantin appear to be important for ER–Golgi traffic, and for Golgi reassembly after cytokinesis (Nakamura *et al.*, 1997; Shorter & Warren, 1999; Lesa *et al.*, 2000; Alvarez *et al.*, 2001). The interaction between p115 and Rab1 is thought to mediate the recruitment of p115 to membranes (Allan *et al.*, 2000). Because this recruitment precedes the interaction of p115 with SNAREs, it has been suggested that p115 recruitment 'primes' transport intermediates for subsequent targeting and fusion (Allan *et al.*, 2000). All interactions between p115 and the various SNAREs involve the coiled-coil 2 region of p115, and a subset of SNAREs also interact with the p115 coiled-coil 4 region (Shorter *et al.*, 2002). The interaction between p115 and specific SNARE pairs has been shown to facilitate the formation of the 'SNAREpin' (also known as the trans-SNARE complex) that precedes membrane fusion (Shorter *et al.*, 2002).

In this study, we characterize a newly discovered interaction between p115 and the ADP ribosylation factor (ARF) guanine-nucleotide exchange factor, GBF1 (Golgi-specific brefeldin-A-resistant factor 1). GBF1 catalyses the exchange of GDP for GTP required for the activation of ARFs. *In vitro*, GBF1 shows preferential activity towards class II ARFs (ARF5), whereas *in vivo* GBF1 activates both class II (ARF5) and class I (ARF1) ARFs. GBF1-mediated activation of ARFs promotes the recruitment of COPI to membranes both *in vitro* and *in vivo* (Claude *et al.*, 1999; Kawamoto *et al.*, 2002). GBF1 localizes to the Golgi and to peripheral ER–Golgi intermediate structures, and cycles between these compartments (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Zhao *et al.*, 2002). Our results suggest that the interaction between p115 and GBF1 does not have a role in membrane recruitment of either protein, indicating that the interaction may function in GBF1-mediated ARF activation.

## RESULTS

To identify proteins that interact with p115, we used a yeast two-hybrid system. Full-length rat p115 was cloned into the 'bait' vector pLexA (p115-LexA) and used to screen a mouse kidney pB42AD cDNA library. Figure 1A summarizes the results of this screen: 88 positive clones were obtained and were re-tested for the interaction. Thirteen false positives were discarded after this step. PCR and

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restriction digests were performed on the 75 remaining clones. Sixty PCR products were obtained, and were grouped according to their restriction pattern. Forty clones were positively identified as corresponding to known genes, and sequencing showed that 20 of these contained an open reading frame encoding a guanine-nucleotide exchange factor, GBF1 (Claude *et al.*, 1999). GM130, which is known to bind p115, was encoded in three positive clones, confirming the validity of the assay. GBF1 was identified in two independent groups, represented by clones 4 and 12, which contained inserts of different sizes (~1 kb and 2 kb, respectively). As shown in Fig. 2A, clone 12 encodes amino acids 1,430–1,859 of GBF1 and overlaps clone 4 (amino acids 1,752–1,859) in a C-terminal stretch of 108 amino acids that are highly enriched in proline. Transformation of yeast cells with purified plasmids from the two groups and the full-length p115-LexA construct confirmed that both GBF1 fragments interact with p115.

To confirm the p115 interaction with GBF1 biochemically, we expressed and purified from bacteria the p115-binding domain of GBF1 (GBFpro; encoded by clone 4) fused to glutathione *S*-transferase (GST). The fusion protein (GST–GBFpro) was incubated with rat liver cytosol, and recovered by adsorption on glutathione-sepharose 4B (GSH) beads. The samples were washed, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and analysed by western blotting using anti-p115 antibodies. As shown in Fig. 1B, p115 was recovered from cytosol incubated with GST-GBFpro, but not with GST alone. To confirm that full-length p115 and GBF1 interact, we immunoprecipitated rat liver cytosol with beads bound to covalently coupled anti-p115 or non-immune IgGs. After washing, proteins were eluted from the beads, separated by SDS–PAGE, and analysed by western blotting using anti-p115 or anti-GBF1 antibodies. As shown in Fig. 1C, GBF1 co-immunoprecipitated with p115 when cytosol was incubated with beads bound to anti-p115 antibodies. Tubulin and β-COP were not detected in the precipitates. In the control immunoprecipitation, using non-immune IgGs, neither p115 nor GBF1 were detected.

The GBF1 clones isolated allowed us to map the p115-binding region of GBF1 to a C-terminal, 108-amino-acid, proline-rich domain, which is encoded by clone 4 (Fig. 2A). Removal of this region (construct GBF1-12Δ) prevents interactions with p115, showing that amino acids 1,762–1,859 are sufficient and required for binding between p115 and GBF1 (Fig. 2B). To map the region of p115 involved in GBF1 binding, we made several deletion constructs of p115, and tested them for interactions with clones 4 and 12. As shown in Fig. 2B, the regions of GBF1 encoded by clones 4 and 12 bind to a p115 region containing amino acids 1–766. This region of p115 encompasses the globular head, coiled-coil 1 and part of coiled-coil 2. The coiled-coil domains do not seem to be important for binding, as a construct containing all four coiled-coil regions and the acidic tail (amino acids 710–959) does not bind GBF1. Within the globular-head region, a construct containing only the H1 domain (amino acids 1–150; see Fig. 2B) and a construct with a deletion in the H2 region (deletion of amino acids 62–371) did not bind either of the proteins encoded by clones 4 and 12. None of these constructs activated either the empty vector or the GBF1-12Δ construct in which the p115-binding residues were deleted. These results show that amino acids 1–766 of p115 are sufficient to bind GBF1, and that amino acids 150–371 are required

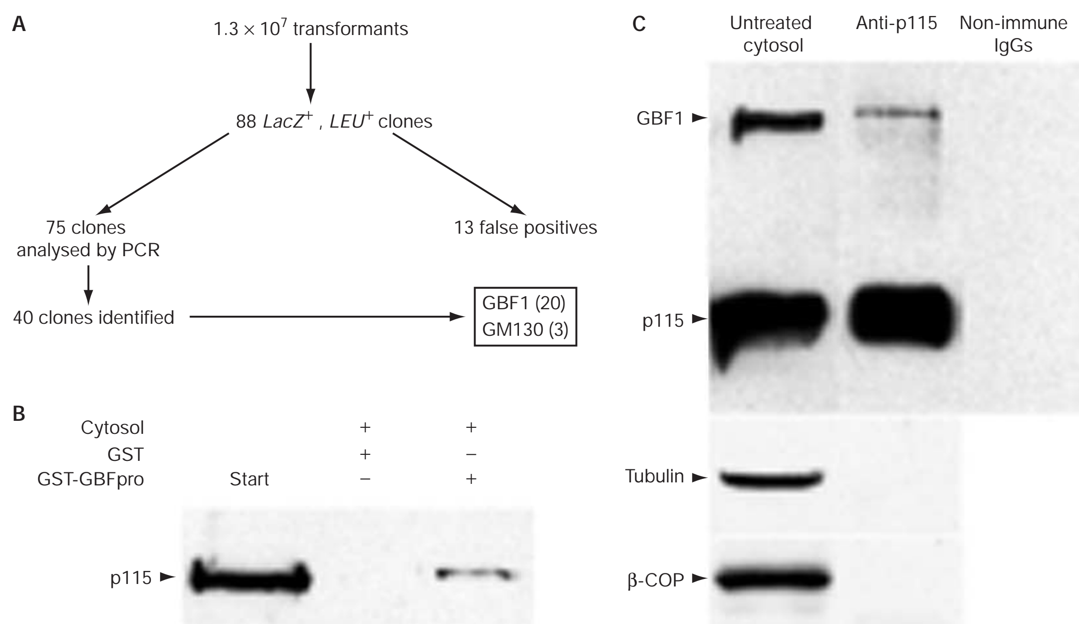


**Fig. 1** | p115 interacts with GBF1. (**A**) Full-length p115 was used to screen a mouse kidney cDNA library in a yeast two-hybrid system, yielding 88 positive clones. After re-testing, 13 false positives were discarded. PCR and restriction digest analysis was performed on the 75 remaining clones. Sequencing identified 40 clones that matched known sequences, 20 of which encoded Golgi-specific brefeldin-A-resistant factor 1 (GBF1). Three clones encoding GM130 were also recovered. (**B**) The interaction between p115 and GBF1 was tested in a glutathione *S*-transferase (GST) pull-down assay, as described in the Methods section. p115 was recovered from cytosol incubated with GST–GBFpro, but not with control GST beads. (**C**) The interaction between p115 and GBF1 was tested in a co-immunoprecipitation assay, as described in the Methods section. GBF1 was co-immunoprecipitated with p115 from cytosol with protein A beads cross-linked to anti-p115 antibodies, but not with control beads cross-linked to non-immune IgGs.

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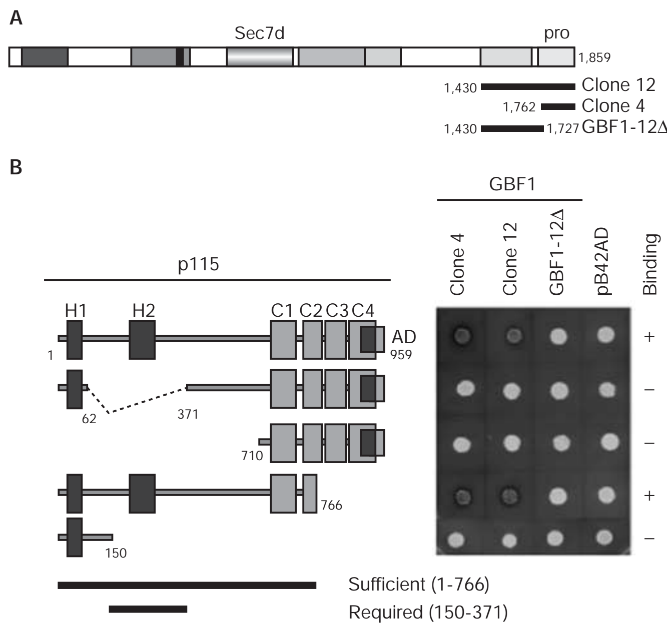


**Fig. 2** | Mapping of interacting domains. (**A**) Full-length GBF1, clones 4 and 12, and construct GBF1-12 Δ. (**B**) Left-hand panel, full-length p115 and p115 deletion constructs. H1 and H2 correspond to domains with high homology to yeast Uso1. A third homology domain, H3, is illustrated as a dark grey square within C4. These constructs were tested for interaction with GBF1 clones 4 and 12 and the deletion construct GBF1-12 Δ, based on clone 12, in which the binding site for p115 is deleted. The p115 constructs were also tested for interaction with the pB42AD vector only as a control. The constructs were tested in the yeast two-hybrid assay for their ability to induce *lacZ* and *LEU* reporter-gene expression. The results obtained with each of the p115 constructs are shown in the corresponding row of the photograph in the right-hand panel. Blue colonies (grey) indicate interaction. C1–C4, coiled-coil domains; Pro, proline-rich domain.

for binding. The region between amino acids 150 and 371 includes the H2 domain (amino acids 200–252), which shows the most homology (61% identity; 77% similarity) to Uso1, the yeast homologue of p115.

GBF1 has been shown to localize to the Golgi region and to peripheral elements identified as vesicular tubular clusters (VTCs) (Claude *et al.*, 1999; Kawamoto *et al.*, 2002). A similar pattern of localization has been observed for p115, which localizes to the Golgi and to peripheral VTCs (Nelson *et al.*, 1998). Both p115 and GBF cycle between the Golgi and pre-Golgi elements, as shown by their redistribution to enlarged peripheral VTCs on incubation at 15 °C (Nelson *et al.*, 1998; Zhao *et al.*, 2002).

We used affinity-purified anti-GBF1 antibodies to confirm the colocalization of GBF1 and p115. As shown in Fig. 3A, p115 and GBF1 colocalize significantly in the Golgi, as well as in the peripheral structures. Quantitation analysis showed that 63% of the peripheral structures containing GBF1 also contained p115, and 69% of p115-containing structures contained GBF1.

Both GBF1 and p115 are peripherally associated with membranes, and are assumed to be recruited from cytosol to membranes by interacting with other proteins. We therefore explored the possibility that the p115–GBF1 interaction might target either protein to the membrane. To determine whether interaction with p115 is required for targeting of GBF1 we made a Myc-epitope-tagged construct, GBF1Δpro, which lacks the proline-rich p115-binding domain, and compared its localization to that of Myc-tagged full- length GBF1. As shown in Fig. 3B, GBF1Δpro localization is indistinguishable from that of full-length GBF1, indicating that targeting does not depend on p115 interaction. The reverse also seems to be true, as in cells treated with brefeldin A (BFA), p115 and GBF1 associate with different membrane compartments. BFA causes the disassembly of the Golgi and the relocation of Golgi proteins into either the ER or ER exit sites (Jackson, 2000). As shown in Fig. 3C, BFA causes GBF1 to localize to the ER, whereas p115 is found at peripheral punctate structures, corresponding to arrested ER exit sites and immature VTCs (Nelson *et al.*, 1998; Ward *et al.*, 2001; Zhao *et al.*, 2002). These results show that the interaction between p115 and GBF1 is likely to have a role other than in targeting to membranes.

As shown in Figs 1 and 2, the proline-rich domain at the C terminus of GBF1 is sufficient to bind p115. To determine whether the interaction between endogenous p115 and GBF1 is functionally relevant, we overexpressed a green fluorescent protein (GFP)-tagged construct encoding the proline-rich domain of GBF1 (GFP-GBFpro) to compete with wild-type GBF1 for p115 binding. As shown in Fig. 3D and F, GFP-GBFpro localized to the Golgi (Fig. 3D and F, arrows) when expressed at low levels, and did not significantly affect Golgi structure. However, high-level expression of GFP-GBFpro caused Golgi disassembly, as shown by the redistribution of p115 (Fig. 3E, arrowhead) and giantin (Fig. 3F) from the Golgi area to the periphery of the cell. Golgi disruption was not a result of protein overexpression, as expression of GFP (at both low and high levels) had no effect on p115 or giantin localization and Golgi structure (Fig. 3G, arrowhead). Our results suggest that the interaction between p115 and GBF1 is important for the maintenance of Golgi integrity.

## DISCUSSION

Here, we report that the membrane-tethering protein p115 binds specifically to GBF1, a guanine-nucleotide-exchange factor for ARF. We identified GBF1 as p115-binding protein in a yeast two-hybrid screen, and confirmed this interaction *in vitro* and *in vivo*. The interacting domains were mapped to a poorly conserved proline-rich domain in GBF1, and to the head region of p115. GBF1 and p115 colocalize extensively in the Golgi and in peripheral VTCs. Overexpression of the p115-binding proline-rich domain of GBF1 fused to GFP causes Golgi disruption, indicating that the interaction between p115 and GBF1 might be functionally important.

## SPECULATION

We propose that p115 might function to facilitate interactions between various components required for ER–Golgi transport. These might include the active form of Rab1, specific SNAREs and GBF1. Especially interesting is the possibility that p115 may promote interactions between active Rab1 and GBF1, and that this event is required for ARF activation and COPI recruitment. Our model is consistent with the findings that Rab1, p115 and GBF1 seem to function at the same stage of ER–Golgi transport (Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Pind *et al.*, 1994; Alvarez *et al.*, 1999; Allan *et al.*, 2000; Puthenveedu & Linstedt, 2001; García-Mata *et al.* 2003). Other lines of evidence also support a functional relationship between Rab1, p115, GBF1 and COPI. First, GBF1 has been shown to be involved in


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COPI recruitment (Claude *et al.*, 1999; Kawamoto *et al.*, 2002). Second, GBF1 binds to p115 (this study). Third, p115 interacts directly with Rab1, and their yeast homologues (Uso1 and Ypt1, respectively) interact genetically (Sapperstein *et al.*, 1995; Allan *et al.*, 2000). Last, Ypt1 interacts genetically with Gea1 and Gea2—the yeast homologues of GBF1—and with ARF1, indicating that Rabs and ARFs participate in the same pathway leading to COPI recruitment (Jones *et al.*, 1999). In agreement with this, overexpression of a constitutively inactive Rab1 protein results in COPI dissociation and Golgi disassembly, and this can be rescued by overexpression of GBF1 (Alvarez *et al.*, 2003). More experiments are required to characterize the temporal and spatial relationship between Rab1, p115 and GBF1.

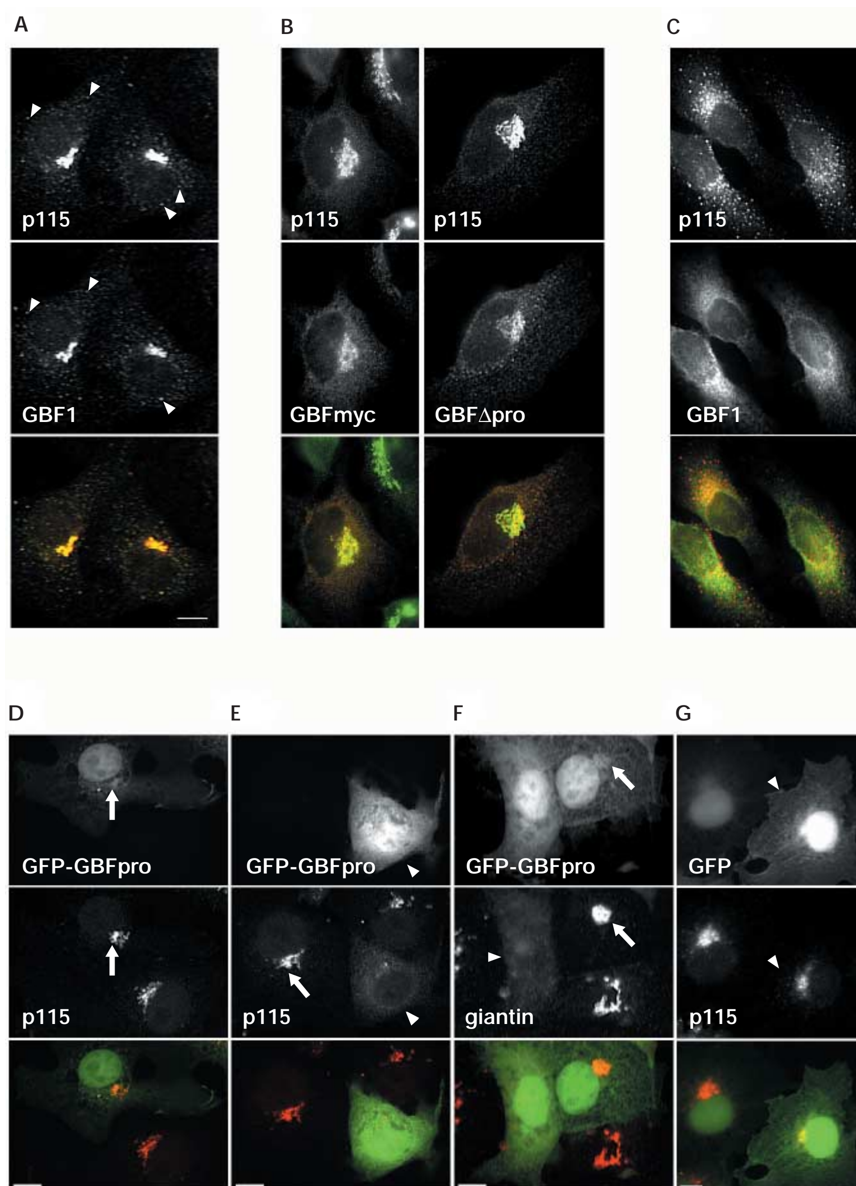


**Fig. 3** | Characterization of the localization of p115 and GBF1, and of the effects of disrupting their interaction. (**A–C**) Localization of GBF1 and p115 is independent of their interaction. (**A**) COS-7 cells were fixed and analysed by immunofluorescence using anti-p115 and anti-GBF1 antibodies. GBF1 and p115 colocalize at the Golgi and at peripheral vesicular tubular clusters (VTCs) (arrowheads). (**B**) HeLa cells expressing a Myc-tagged full-length GBF1 or GBF1Δpro construct (lacking the p115-binding domain) analysed by immunofluorescence using anti-Myc and anti-p115 antibodies. GBF1Δpro targets to membranes in a pattern identical to that of full-length GBF1. (**C**) Cells were treated with 5 μg $ml^{-1}$ brefeldin A for 30 min and analysed by immunofluorescence using anti-p115 and anti-GBF1 antibodies. Whereas GBF1 localizes to the endoplasmic reticulum, p115 localizes to peripheral VTCs. (**D–G**) Overexpression of the p115-binding domain of GBF1 causes Golgi disassembly. COS-7 cells expressing either green fluorescent protein (GFP) only, or a GFP-tagged construct encoding the carboxy-terminal proline-rich domain of GBF1 (GFP-GBF1pro), were processed for immunofluorescence and analysed using anti-p115 or anti-giantin antibodies. (**D**) At low levels of expression, GFP-GBF1pro has no effect on Golgi structure. (**E, F**) At high levels of expression, the Golgi is completely disassembled, as shown by the localization pattern of the anti-p115 (**E**) and anti-giantin (**F**) antibodies. Golgi disassembly is not observed in control cells expressing GFP only (**G**). Scale bar, 10 μm.

## METHODS

**Antibodies.** COS-7 cells and HeLa cells were grown on coverslips at 37 °C in the following media: COS-7 cells in DMEM, 10% FBS; HeLa cells in EMEM, 1 mM sodium pyruvate, 10% FBS. BFA was from Sigma, and was used at 5 μg ml$^{-1}$. GSH (reduced glutathione)–agarose and GSH were from Sigma. The production of rabbit polyclonal antibodies against p115 was described previously (Nelson *et al.*, 1998). Rabbit polyclonal anti-GBF1 antibodies were made by immunization with a purified $His_6$-tagged construct encoding the last 107 amino acids of mouse GBF1, and were affinity-purified as described previously (Nelson *et al.*, 1998). Anti-GBF1 antibodies were crosslinked to protein A–benzamidine Sepharose 4 Fast Flow (Amersham Pharmacia) using dimethyl pimelimidate (Pierce) in accordance with the manufacturer's instructions. Anti-giantin antibodies were provided by H.-P. Hauri. Anti-Myc monoclonal antibodies were from Invitrogen. Monoclonal anti-β-tubulin and anti-β-COP antibodies were from Sigma. Goat anti-rat and anti-mouse antibodies conjugated with Texas Red-X or Oregon Green were from Molecular Probes.

**Transfection of tissue culture cells.** Cells were transfected with TransIT transfection reagent (Mirus) according to the manufacturer's instructions.

**DNA constructs.** A partial human GBF1 complementary DNA (KIAA0248) was obtained from the Kazusa DNA Research Institute, Japan. KIAA0248 is a 5,634-bp cDNA, and lacks 0.5 kb of the GBF1 open reading frame. The missing fragment was amplified by PCR from a human lung cDNA library (provided by C. Wu). The PCR product was then subcloned into the KIAA0248 cDNA using the internal *Eco*RI site at nucleotide position 1,124 and an engineered external *Xho*I site.

To generate Myc-tagged full-length GBF1, GBF1 was amplified by PCR and subcloned into pcDNA4.0/TO/myc-His (Invitrogen). GFP-GBFpro was made by subcloning the insert in clone 4 from the two-hybrid screen into pEGFP-C2 (Clontech) using *Eco*RI and *Xho*I. All constructs were verified by sequencing at the University of Alabama sequencing facility.

**Two-hybrid screening.** A LexA-based yeast two-hybrid system (Clontech) was used to screen for p115-interacting proteins. Full-length p115 was cloned into the 'bait' vector pLexA (p115-LexA) and used to screen a mouse kidney pB42AD cDNA library (>1.8 × $10^6$ independent clones) (provided by B. Yoder). Individual colonies containing potentially positive clones were isolated, re-tested for interaction with p115, and false positives were discarded. The inserts from the positive clones were amplified by PCR and grouped according to their restriction pattern. Representative clones from each group were sequenced.

**Co-immunoprecipitation.** Rat liver cytosol (1 ml) was incubated at 4 °C with anti-p115 or non-immune antibodies cross-linked to protein A beads (200 μg IgG per 100 μl beads) for 1 h and then washed four times with 20 mM HEPES, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (HKMED) and 1% Triton X-100. Precipitates were eluted with 0.1 M glycine, pH 2.8, separated by SDS–PAGE, transferred to nitrocellulose and detected with anti-GBF1 and anti-p115 antibodies. Precipitates were also tested for the presence of non-related proteins with anti-tubulin and anti-β-COP antibodies. Between 2 and 4% of total GBF1 was recovered in the precipitates.

**GST pull-down assays.** Rat liver cytosol (6 mg ml$^{-1}$) was prepared as described previously (Alvarez *et al.*, 1999) and precleared using 2 ml of GSH–agarose beads. GST-GBFpro and GST (10 μg each) were bound to GSH–agarose beads, washed with HKMED and incubated with 500 μl precleared cytosol for 1.5 h at 4 °C. Samples were washed ten times with HKMED, 0.2% Tween 20. GSH beads were then resuspended in SDS sample-buffer, boiled for 3 min and separated on a 10% acrylamide gel. Proteins were transferred to nitrocellulose membranes and detected with anti-p115 antibodies. Between 1 and 3% of total p115 was recovered in the precipitates in different experiments.

**Immunofluorescence microscopy**. Cells grown on coverslips were washed in PBS, fixed in 3% paraformaldehyde for 10 min, quenched with 10 mM $NH_4Cl$ and permeabilized with 0.1% Triton X-100 in PBS. Coverslips were then washed with PBS and blocked in PBS, 2.5% goat serum, 0.2% Tween 20 for 5 min, followed by further blocking in PBS, 0.4% fish skin gelatin, 0.2% Tween 20. Cells were incubated with primary antibody for 1 h at 25 °C. Coverslips were washed with PBS, 0.2% Tween 20 and incubated with secondary antibodies for 45 min. Coverslips were washed three times with PBS, 0.2% Tween 20, and mounted in a 9:1 glycerol/PBS solution containing 0.1% p-phenylenediamine.

**Quantification of protein colocalization.** Images of cells double-labelled with two different fluorophore-conjugated antibodies were acquired and analysed for signal overlap as follows: using Adobe Photoshop 6.0 (Adobe Systems), structures were individualized by drawing a rectangle around them, and the percentage of colocalization of the fluorophores was calculated. A structure is defined by an intensity signal greater than 50 arbitrary units (0–255 range) and an area of ≥ 4 $pixels^2$ (0.16 $μm^2$ using a 50 × objective lens).

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