Supplementary Information

This file contains Supplementary Methods, Supplementary Results, Supplementary References, Figures S1 and S2, and Tables S1 and S2.

Supplementary Methods

Strains, plasmids and antibodies

Stains and plasmids used in this study are listed in Tables S1 and S2, respectively. All constructs were confirmed by DNA sequencing. The CJY110 *GEA1::13xMyc* strain was constructed from BY4742 *erg6Δ* by tagging the chromosomal GEA1 gene at its C-terminus with 13 consecutive Myc epitopes as described (Longtine *et al.*, 1998). Briefly, PCR was performed using pFa6a-13Myc-His3MX6 as template and an appropriate *GEA1* primer pair designed for homologous recombination at the chromosomal *GEA1* locus. The products of 4 PCR reactions (20 μL/each) were pooled, column-purified (Qiagen), and concentrated in 20 μL of elution buffer (Qiagen). This concentrated DNA was transformed into BY4742 *erg6Δ* using the lithium acetate method. For the LexA two-hybrid constructs, PCR products were ligated into pEG202 (bait vector) or pJG-4-5 (prey vector) to generate in-frame fusions with the LexA DNA binding domain and activation domain, respectively. Plasmids expressing 5HA-Gea1p(5-1408), 5HA-Gea1p truncations, 2HA-GBF1(1-663), or full-length GBF1 were constructed in vector YEp352 (2μ, URA). YFP-GBF(1-894) was constructed in vector pEYFP-C1 (Clontech). pVenus-C1 was constructed by subcloning from pVenus-N1. Venus-GBF(1-1440) and Venus-GBF(1-1273) were constructed in pVenus-N1. GST–Gea1p(434-521) and GST-Gea2p(759-1459) were cloned into pGEX4T-1 (Amersham Biosciences) using EcoR1-XhoI sites and EcoR1-SalI sites, respectively. Sec21pC(666-935)-His6 was obtained by in-frame cloning into SacI-SalI sites of pET22b

(Novagen). The GST fusion proteins and the HIS-tagged protein were produced by transformation of the *Escherichia coli* strain BL21 (DE3) pLysS (Novagen) and purified according to the manufacturer's instructions.

The anti-β'-COP antibodies were a generous gift from Felix Wieland. The following mouse monoclonal antibodies were used: HA.11 (Covance) or clone HA-7 ascites fluid (Sigma) against the HA tag, 9E10 against the Myc tag, sc138 (Santa Cruz) against GST, anti-GBF1 and anti-GM130 (BD Biosciences), anti-GFP (Roche diagnostics). The following polyclonal antibodies were also used: rabbit anti-LexA antibody (Invitrogen), rabbit anti-HIS antibody (Covance), rabbit anti-β-COP (Affinity Bioreagents), sheep anti-human TGN46 (Serotec) and HRP-conjugated anti–mouse or anti–rabbit IgG (Amersham Biosciences).

Two- hybrid assays

Reporter strain EGY48 was co-transformed with plasmid pSH18-34 (*URA3*), a bait (*HIS3*) and a prey (*TRP1*) fusion construct, using the lithium acetate method. Transformants were selected on –HIS–TRP–URA plates. To assess growth on media lacking leucine, three independent transformants were streaked onto –HIS–TRP–URA–LEU plates containing 2% galactose/1% raffinose, and incubated at 30 °C for 3-7 days. Each experiments was repeated at least three times.

GST pull-downs

Purified GST, GST-Gea1p(434-521), GST-Gea2p (5-535) (GST-Gea2pN), or GST-Gea2p (759-1459) (GST-Gea2pC) proteins were immobilized on glutathione-sepharose 4B beads (Amersham Biosciences). The bound beads were blocked with 500 μL 1% BSA in binding buffer (20 mM

HEPES pH 7.2, 100 mM KCl, 5 mM $MgCl_2$) at 4 °C for 1 hr, and then were incubated with purified His6-tagged protein in binding buffer at 4 °C for 1 hr. The beads were washed six times with washing buffer (20 mM HEPES pH 7.2, 200 mM KCl, 5 mM $MgCl_2$, 1% T Triton X-100). Bound protein was resolved by SDS-PAGE (NUPAGE Norvex 4-12 % Bis-Tris gradient gel, Invitrogen) and revealed by immunoblotting using antibodies to His-tag followed by chemiluminescent detection (Amersham Biosciences).

Co-immunoprecipitation assays in yeast

Yeast were grown in 100 mL synthetic medium at 30°C to an optical density of 0.6-0.8. Yeast cells were harvested and resuspended in 500 μL of ice-cold lysis buffer (20 mM HEPES pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1% T Triton X-100) containing complete protease inhibitor cocktail (Roche) and 1 mM PMSF, and then 2/3 volumes of acid-washed glass beads (Sigma) were added. The cells were disrupted by vigorous vortexing 10 times, 1 min each, and 1 min chilling on ice between each vortexing. The cell lysate was centrifuged at 500 X *g* and supernatant then subjected to a 2500 X *g* centrifugation at 4 °C for 10 min. The cleared cell lysate was diluted 2.5-fold with lysis buffer lacking Triton X-100 and incubated with antibody or control IgG at 4°C for 1 hr, followed by incubation with 50 μL pre-equilibrated Dynabeads Protein G (DYNAL) at 4°C for 1 hr. The resin was then washed six times with washing buffer (20 mM HEPES pH 7.2, 500 mM KCl, 5 mM $MgCl_2$, 1% T Triton X-100) and resuspended in 40 μL NUPAGE SDS-sample buffer (Invitrogen). Bound proteins were resolved by SDS-PAGE (NUPAGE Norvex 4-12 % Bis-Tris gredient gel, Invitrogen) and revealed by immunoblotting followed by chemiluminescent detection (Amersham Biosciences). For experiments carried out in the presence of BFA, a 10 mg/mL stock solution in ethanol was added to 100 mL yeast culture

growing at 30 °C with optical density of 0.6 to a final concentration of 100 μg/mL and the yeast culture was grown for another 10 min at 30 °C before harvesting. The BFA concentration, 100 μg/mL, was maintained through the remainder of the co-immunoprecipitation procedure.

Co-immunoprecipitation assays in mammalian cells

For co-immunoprecipitation experiments in mammalian cells (Fig 3D), a Venus (YFP derivative)-tagged version of human GBF1 (Niu *et al.*, 2005) and *B. taurus* γ1-COP with an HA epitope tag (Wu *et al.*, 2000) were co-expressed in COS7 cells, and GBF1 was immunoprecipitated. COS7 cells in 6 cm culture dishes were co-transfected with the plasmids expressing *Bos taurus* HA-γ1-COP (pCDNA3-Btγ1-COP) and either Venus-GBF1, Venus-GBF(1-1440), Venus-GBF(1-1273) or YFP-GBF(1-894) expressing, respectively, human wild type GBF1 and GBF1 deleted of the C-terminal 416, 583 and 962 amino acids. After 20 hours of expression, cells were washed two times with 5 mL of cold potassium buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 19 mM Na_2HPO_4, 1,8 mM KH_2PO_4), then disrupted in 200 μL of cold Lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP40). After centrifugation at 4°C, supernatants were incubated with 0.8 μg of anti-GFP antibodies (Roche) for 1.5 hours at 4°C. Then, 20 μL of protein G Sepharose 4 Fast Flow (GE Healthcare) was added and the mixtures were incubated at 4°C for 1.5 hours. The resin was washed four times with 1 mL of buffer W100 (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) then two times with 1 mL PBS. Proteins were then eluted by incubation with 50 μL of SDS-PAGE sample buffer for 5 minutes at 95°C. Eluted proteins were separated on a 10% SDS-PAGE gel and analyzed by Western blotting using anti-HA antibodies (Sigma). In supplementary Fig 2C, the experiment shown in the upper panel was carried out as indicated above. The experiment shown

in the bottom panel was the same except that the resin was washed two times with 1 mL of buffer W100, two times with 1 mL buffer W250-0.5NP (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40) then two times with 1 mL PBS. In Fig 3E, F and G (upper panel), the resin was washed only twice with W100, then once with PBS. In Fig 3G (lower panel), Fig 3H and Fig 4C, COS7 cells were transfected with the indicted GBF1 constructs and *Homo sapiens* HA-γ1-COP (pCDNA3-Hsγ1-COP). The resin was washed four times with W100, then twice with PBS.

Quantification of the ratio of endogenous COPI signal obtained in co-IPs from cells expressing YFP alone or YFP-GBF1 was carried out as follows. In three separate experiments, the amounts of COPI detected by anti-β'-COP antibodies as well as the amounts of YFP or YFP-GBF1 in the corresponding immunoprecipitates were determined. In each experiment, the ratio of the β'-COP signal for YFP and for YFP-GBF1 were calculated, and mean and standard error determined (0.83 ± 0.24). A similar calculation was performed for the ratio of YFP signals (47 ± 8). Hence the normalized ratio of the β'-COP signal for YFP-GBF1 to YFP is 57 ± 8. Note that the β'-COP signal in the co-IPs from cells expressing YFP alone is due to the presence of YFP, since controls in which lysates from cells not expressing YFP or YFP-GBF1 were mock-immunoprecipitated with anti-GFP antibodies showed no detectable β'-COP signal (Fig 3G upper panel, first lane).

RNA interference

For GBF1, BIG1 and BIG2 expression silencing, siGENOME: GBF1, siGENOME: BIG1 and siGENOME: ARFGEF2 SMARTpool reagents from Dharmacon (Lafayette, CO) were used. As a control, a SMARTpool reagent against an unrelated mRNA was used. Two consecutive

transfections of HeLa cells were performed using 50 nM siRNA and Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) at 24-h intervals, and cells were harvested 48 hours after first transfection.

Supplementary Results

Specificity of COPI recruitment is mediated by Arf GEF GBF1 in mammalian cells

Overexpression of a given large Arf GEF (either wild type or mutant) has effects on specific coats (Kawamoto *et al*., 2002; Shinotsuka *et al*., 2002a; Shinotsuka *et al*., 2002b), and microinjection of antibodies against GBF1 or depletion of GBF1 in cells with inhibitory RNAs causes release of COPI from Golgi membranes into the cytosol, but has no effect on other coats such as the AP1/clathrin coat (Casanova, 2007; Ishizaki *et al*., 2008; Lefrancois & McCormick, 2007; Manolea *et al*., 2007). We carried out depletion experiments using small interfering RNAs (siRNAs) directed against GBF1, BIG1 and BIG2. Depletion of either BIG1 or BIG2 or both together had no effect on localization of COPI to the Golgi (Fig. S1 A-D). However, depletion of GBF1 had a dramatic effect, causing complete relocation of COPI from the Golgi to the cytosol (Fig. S1E). This redistribution was not due to disassembly of the Golgi apparatus, as the Golgi was still intact in cells treated with GBF1 siRNAs (Fig. S1 F,G). Although both the *cis*-Golgi (marked by GM130) (Fig. S1 F,G upper panels) and the *trans*-Golgi network (TGN) marked by TGN46 (Fig. S1 F,G lower panels) were present, their structure was affected to some extent in GBF1-depleted cells (Fig S1 F,G). When a plasmid expressing YFP-GBF1 was transfected at the same time as GBF1 siRNA, no expression of the YFP-tagged protein was detectable at either 24 or 48 hours after transfection (Fig S1H), indicating a very efficient knock-down of GBF1

expression in these experiments. The level of endogenous GBF1 protein was reduced to 10% or less its normal level (Fig S1 H). We obtained similar levels of depletion of BIG1 and BIG2 in siRNA experiments (E. Smirnova & C.L. Jackson, unpublished results).

Supplementary References

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Supplementary Figure Legends

Figure S1. Depletion of GBF1, but not BIG1 or BIG2, causes release of COPI into the cytosol. HeLa cells were transfected with siRNAs targeting (A) lamin (negative control), (B) BIG1, (C) BIG2, (D) BIG1+BIG2, or (E) GBF1 and prepared for immunofluoresence analysis using antibodies against GBF1 (first row) and β-COP (middle row); the merge image is shown in the third row. HeLa cells transfected with (F) control siRNAs and (G) siRNAs targeting GBF1 were prepared for immunofluoresence using antibodies against GM130 (top panels) or TGN46 (lower panels). (H) HeLa cells were transfected with YFP-GBF1 and siRNAs against GBF1 (lanes 1 and 2) or control siRNAs (lane 3), and cell lysates prepared either 24 or 48 hours after transfection. Samples were analyzed by Western blot using antibodies against GFP (left panel) or against GBF1 (right panel). The positions of exogenously expressed YFP-GBF1 and endogenous GBF1 are indicated.

Figure S2. The N-terminus of yeast Gea1p interacts with Sec21p (yeast γ-COP). (A) Bait plasmid pYD150 (pEG202 carrying full-length γ-COP) was co-transformed into yeast strain EGY48 along with prey plasmid pJG4-5 alone or with pJG4-5 plasmids carrying the indicated portions of Gea1p (numbers indicate amino acid positions). Cells were grown on –HIS–TRP medium to select for the plasmids, or on –HIS–TRP–LEU medium to monitor expression of the reporter. -, no interaction, +, strong interaction, +/-, weak interaction. (B) Lysates from yeast strain CJY104 *SEC21::3xGFP* carrying plasmid pRC4 (full-length wild type 5xHA-Gea1p), pYD110 5xHA-Gea1(5–754), pYD111 5xHA-Gea1(5–611), pYD112 5xHA-Gea1(5-539), pYD113 5xHA-Gea1(5–521) or pYD114 (YEp352-5xHA-GEA1(5-423)) were prepared and incubated with anti-GFP antibodies (+) or control IgGs (-). Bound material was analyzed by

Western using anti-HA antibodies. (C) Lysates from COS7 cells expressing YFP alone or human Venus-GBF1, and bovine HA-γ1-COP, were incubated with anti-GFP antibodies. Immunoprecipitates were subjected to Western blot analysis using anti-HA antibodies. (D) BFA sensitivity of strain CJY104 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 erg6Δ::KANMX4 SEC21::3xGFP*. The *erg6Δ* deletion was introduced into the parent strain to render it sensitive to BFA, since wild type strains of *Saccharomyces cerevisiae* are resistant to the drug. Cells were imaged either before BFA treatment (0 min) or 5 minutes after treatment with 100 μg/mL BFA (5 min). Sec21p-3xGFP was released from membranes into the cytosol upon BFA treatment.

Table S1. Strains used in this study

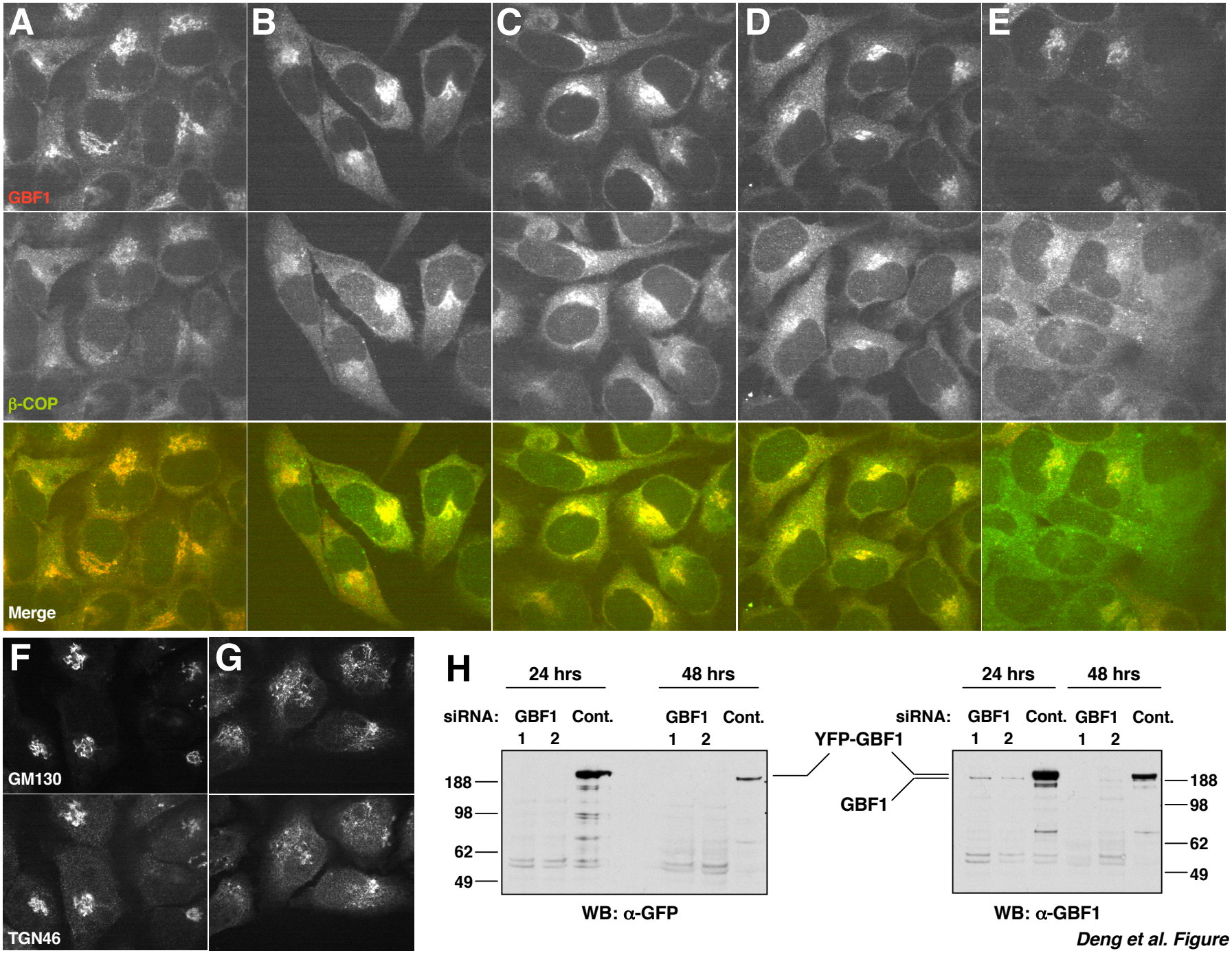
Strain	Genotype	Reference
CJY104	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 erg6Δ::KANMX4 SEC21::3xGFP	Park et al. 2005
CJY110	MATα ura3-52 leu2-Δ1 his3Δ200 lys2-801 ade2-101 erg6Δ GEA1::13xMyc-HIS3MX6	This study
EGY48	MATa trpl his3 ura3 6lexAop-LEU2	E. Golemis

Table S2. Plasmids used in this study

Plasmid	Vector and insert information	Reference
pYD150	pEG202-SEC21	This study
PYD173	pJG4-5-GEA1(1-521)	This study
PYD174	pJG4-5-GEA1(1-433)	This study
pYD175	pJG4-5-GEA1(1-310)	This study
pYD176	pJG4-5-GEA1(1-166)	This study
pYD177	pJG4-5-GEA1(167-521)	This study
pYD178	pJG4-5-GEA1(167-433)	This study
pYD179	pJG4-5-GEA1(167-310)	This study

pYD180	pJG4-5-GEA1(311-521)	This study
pYD181	pJG4-5-GEA1(311-433)	This study
pYD182	pJG4-5-GEA1(434-521)	This study
pRC4	YEp352-5HA-GEA1	This study
pYD110	YEp352-5xHA-GEA1(5-754)	This study
pYD111	YEp352-5xHA-GEA1(5-611)	This study
pYD112	YEp352-5xHA-GEA1(5-539)	This study
pYD113	YEp352-5xHA-GEA1(5-521)	This study
pYD114	YEp352-5xHA-GEA1(5-423)	This study
pYD115	YEp352-5xHA-GEA1(5-310)	This study
pYD116	YEp352-5xHA-GEA1(5-223)	This study
pYD124	YEp352-SEC21-3xHA	This study
pYD126	YEp352-SEC21(1-665)-3xHA	This study
pYD127	YEp352-SEC21(666-935)-3xHA	This study
pYD191	pGEX4T-1-GEA1(434-521)	This study
pCLJ662	pGEX4T-1-GEA2(5-535) (N-term)	This study
pYD194	pGEX4T-1-GEA2(759-1459) (C-term)	This study
pYD195	pGEX4T-1-SEC7(466-825) (N-term)	This study
pYD137	pET22b-SEC21(666-935) (C-term)	This study
pYD212	Yep352-GBF1	This study
pYD213	Yep352-GBF1(1-663)-2xHA	This study

pYD154	pEG202-LexA-γ1-COP	This study
pcDNA3-Btγ1-COP	pcDNA3-HA-γ1-COP (*Bos taurus*)	Wu et al. 2000
pcDNA3-Hsγ1-COP	pcDNA3-HA-γ1-COP (*Homo sapiens*)	This study
pTKN101	Venus-GBF1	This study
pTKN110	Venus-GBF1(1-1440)	This study
pTKN109	Venus-GBF1(1-1273)	This study
pTKN107	YFP-GBF1(1-894)	This study
pEYFP-C1	EYFP	Clontech
pVenus-C1	Venus (YFP - F46L, F64L, M153T, V163A, S175G)	George Patterson

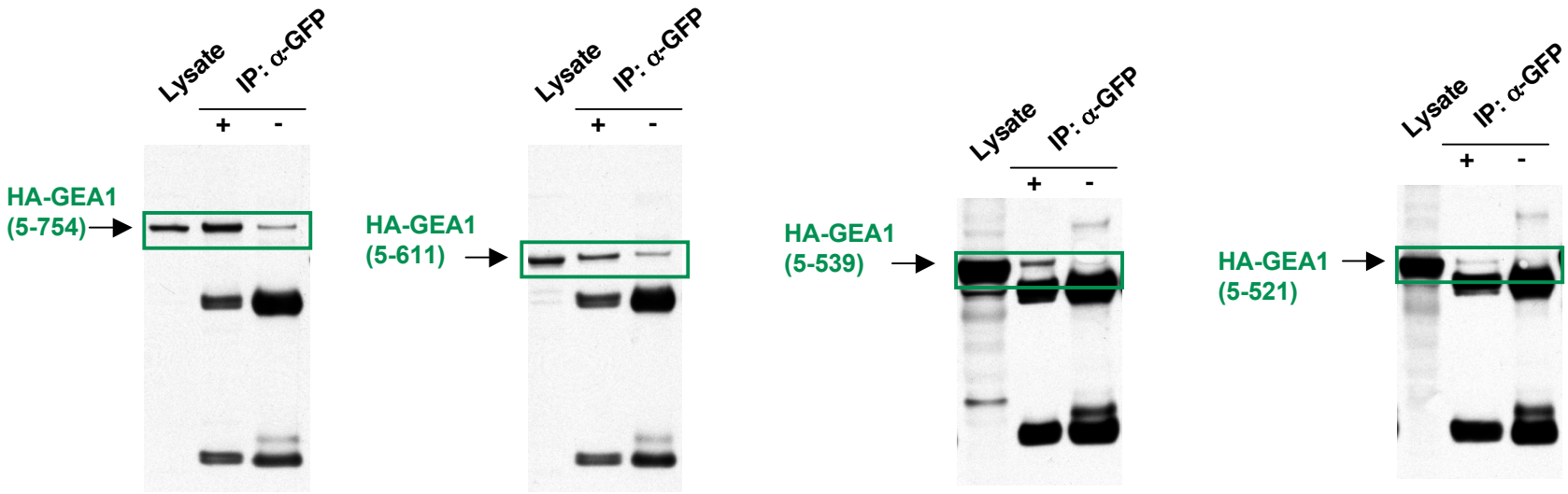


Deng et al. Figure S1

A

pJG4-5:	Gea1 1-521	Gea1 1-433	Gea1 1-310	Gea1 1-166	Gea1 167-521	Gea1 167-433	Gea1 167-310	Gea1 311-521	Gea1 311-433	Gea1 434-521
pEG202-Sec21	+	-	-	-	-	-	-	-	-	+/-

B



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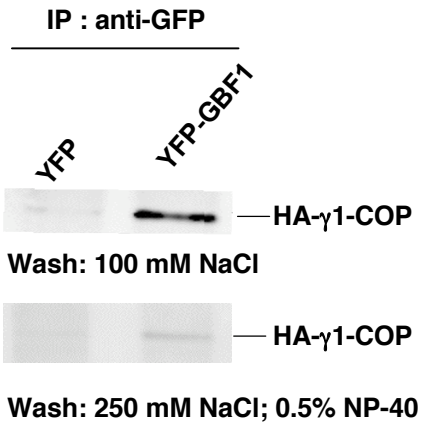

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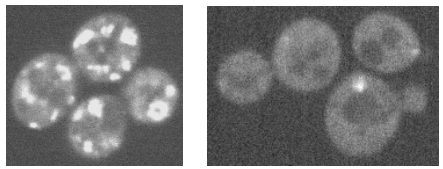

Deng et al. Figure S2