DjA1 maintains Golgi integrity via interaction with GRASP65

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ABSTRACT **In mammalian cells, the Golgi reassembly stacking protein of 65 kDa (GRASP65) has been implicated in both Golgi stacking and ribbon linking by forming *trans*-oligomers. To better understand its function and regulation, we used biochemical methods to identify the DnaJ homolog subfamily A member 1 (DjA1) as a novel GRASP65-binding protein. In cells, depletion of DjA1 resulted in Golgi fragmentation, short and improperly aligned cisternae, and delayed Golgi reassembly after nocodazole washout. In vitro, immunodepletion of DjA1 from interphase cytosol reduced its activity to enhance GRASP65 oligomerization and Golgi membrane fusion, while adding purified DjA1 enhanced GRASP65 oligomerization. DjA1 is a cochaperone of Heat shock cognate 71-kDa protein (Hsc70), but the activity of DjA1 in Golgi structure formation is independent of its cochaperone activity or Hsc70, rather, through DjA1-GRASP65 interaction to promote GRASP65 oligomerization. Thus, DjA1 interacts with GRASP65 to enhance Golgi structure formation through the promotion of GRASP65 *trans*-oligomerization.**

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INTRODUCTION

The Golgi apparatus is a central intracellular membrane organelle responsible for trafficking and modification of proteins and lipids. In mammalian cells, the Golgi is characterized by stacks of five to seven flattened cisternae, which are laterally linked into a ribbon-like structure (Lowe, 2011) adjacent to the centrosome and the nucleus. The Golgi structure is highly dynamic and undergoes rapid disassembly and reassembly during mitosis, or under stress and pathological conditions (Wang and Seemann, 2011; Huang and Wang, 2017). Numerous Golgi matrix proteins, including the Golgi Re-Assembly Stacking Proteins GRASP65 and GRASP55 (GRASPs, also called GORASP1 and GORASP2, respectively) and golgins, have been demonstrated to work together to maintain normal Golgi structure and function (Xiang and Wang, 2010). However, the exact mechanisms of Golgi structure formation remain largely unknown.

GRASP55/65 are peripheral proteins targeted to the cytoplasmic face of Golgi membranes by myristoylation and concentrated at the interface between the cisternae where stacking occurs (Shorter *et al.*, 1999; Yoshimura *et al.*, 2001). Both GRASPs contain a conserved N-terminal GRASP domain and a C-terminal serine/proline-rich (SPR) domain (Zhang and Wang, 2015). Several groups have reported their roles in Golgi stacking and ribbon-linking (Barr *et al.*, 1997; Wang *et al.*, 2003; Puthenveedu *et al.*, 2006; Veenendaal *et al.*, 2014), as well as cargo transportation, unconventional secretion, cell cycle regulation, apoptosis and cell migration (Lane *et al.*, 2002; Wang *et al.*, 2008; Ramirez and Lowe, 2009; Cheng *et al.*, 2010; Wei and Seemann, 2010; Vinke *et al.*, 2011; Ji *et al.*, 2013; Tang and Wang, 2013; Kim *et al.*, 2016). GRASPs from adjacent Golgi cisternae form *trans*-oligomers through the N-terminal GRASP domain and tether the cisternae into a stack (Wang *et al.*, 2003, 2005). Depletion of either GRASP65 or GRASP55 reduces the number of cisternae in the Golgi stack, while depletion of both

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Abbreviation used: ARS, ATP-regenerating system; BSA, bovine serum albumin; CHC, clathrin heavy chain; CNBr, cyanogen bromide; DjA1, DnaJ homologue subfamily A member 1; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EM, electron microscopy; EndoH, endoglycosidase H; ER, endoplasmic reticulum; GAK, cyclin-G–associated kinase; GRASP55, Golgi reassembly-stacking protein of 55 kDa; GRASP65, Golgi reassembly stacking protein of 65 kDa; Hsc70, heat shock cognate 71-kDa protein; IC, interphase cytosol; IgG, immunoglobulin G; MC, mitotic cytosol; MGF, mitotic Golgi fragments; PDZ, PSD95/Dlg1/ZO-1 domain; RLG, rat liver Golgi; RUSH, retention using selective hooks; SPR domain, serine/proline-rich domain; TGN, *trans*-Golgi network; VSV-G, vesicular stomatitis virus glycoprotein; WCL, whole cell lysate.

GRASPs leads to disassembly of the entire Golgi stack (Sutterlin *et al.*, 2005; Xiang and Wang, 2010; Bekier *et al.*, 2017). During mitosis, phosphorylation by Cdk1 and Plk1 inhibits GRASP65 oligomerization and leads to cisternal separation. At the end of mitosis, dephosphorylation by PP2A allows the reformation of GRASP *trans*-oligomers and stacking of newly formed cisternae (Wang *et al.*, 2003; Tang *et al.*, 2010b; Xiang and Wang, 2010). GRASPs are also involved in Golgi ribbon linking, as depletion of either GRASP65 or GRASP55 results in Golgi ribbon unlinking (Puthenveedu *et al.*, 2006; Feinstein and Linstedt, 2008).

One way to better understand the role of GRASP65 in Golgi structure formation is to identify its interacting proteins. Indeed, our group has recently identified the mammalian actin elongation factor Mena as a binding partner of GRASP65. We found that Mena is targeted to the Golgi through the interaction with GRASP65 to enhance Golgi ribbon linking via GRASP65 oligomerization and local actin polymerization (Tang *et al.*, 2016). However, Mena is unlikely the sole modulator of GRASP65, as Mena mainly modulates GRASP65 in Golgi ribbon linking but has only mild effects on Golgi stack formation (Tang *et al.*, 2016), which indicates that additional, unidentified cytosolic proteins may also interact with GRASP65 and regulate its function in Golgi stacking. Therefore, we examined the mass spectrometry results previously used to discover Mena as a GRASP65-binding protein (Tang *et al.*, 2016) and identified a set of protein chaperones as novel GRASP65 interacting proteins, including DjA1 and Hsc70. Further investigation revealed that DjA1, but not Hsc70, directly interacts with GRASP65 and enhances GRASP65 oligomerization. This is the first report that a J-domain protein cochaperone modulates Golgi structure formation through GRASP65.

RESULTS

Identification of DjA1 as a novel GRASP65 binding protein

In our previous studies, we devised a bead aggregation assay to demonstrate that GRASP65 could link adjacent cisternal surfaces (Wang *et al.*, 2003, 2005). Beads coated with purified GRASP65 aggregate extensively when treated with interphase cytosol (IC) compared with buffer containing the same concentration of bovine serum albumin (BSA) (Wang *et al.*, 2003), suggesting the presence of cytosolic factors that enhance GRASP65 oligomerization. The activity was sensitive to heat, salt, and proteinase K, suggesting that the active components are proteins (Tang *et al.*, 2016). We then utilized the bead aggregation assay to monitor the activity during the procedures to enrich and purify the cytosolic factors (Tang *et al.*, 2016), and identified DjA1 and its interacting protein Hsc70 in this process (Figure 1A and Supplemental Table 1).

When purified rat liver Golgi (RLG) membranes were incubated with interphase or mitotic HeLa cell cytosol and reisolated, cytosolic DjA1 and Hsc70 were copurified with the Golgi membranes under both interphase and mitotic conditions (Figure 1B). Noticeably, the interaction of DjA1 and Hsc70 with interphase Golgi membranes was stronger than with mitotic Golgi membranes, which was previously shown by quantitative proteomic studies (Chen *et al.*, 2010, 2012). These results suggest a role of these proteins in maintaining the Golgi structure under interphase conditions. Using immunofluorescence microscopy, we found that endogenous DjA1 was mostly cytosolically localized in cells as previously reported (Terada and Mori, 2000), but with a higher concentration in the perinuclear region, which partially colocalized with GRASP65 (Figure 1C). However, Hsc70 localized in the cytoplasm without detectable colocalization with GRASP65 (Figure 1D). The Golgi localization of DjA1 and Hsc70 was more obvious when cells were permeabilized with saponin before fixation (Supplemental Figure 1).

To confirm the interaction among GRASP65, DjA1, and Hsc70, we incubated BSA- or GRASP65-coated cyanogen bromide–(CNBr) activated beads with HeLa whole cell lysate (WCL) and analyzed bound proteins by Western blotting. As shown in Figure 1E, DjA1 and Hsc70 were copurified with GRASP65 from the WCL but not with BSA. Further experiments showed that endogenous DjA1 and Hsc70 coimmunoprecipitated with GRASP65 from the WCL when a GRASP65 antibody was used (Figure 1F). Conversely, immunoprecipitation of DjA1 also brought down GRASP65-GFP (Figure 1G). Nickel (Ni)-beads pull-down assay using purified His-tagged DjA1 or Hsc70 further confirmed the binding (Figure 1H).

Considering that DjA1 has been reported to bind Hsc70 and functions as its cochaperone, we conducted an in vitro binding assay by incubating BSA- or GRASP65-coupled-CNBr beads with purified His-tagged DjA1, Hsc70, or both to determine whether the interactions between the three proteins were direct. The result showed that DjA1 directly bound to GRASP65 regardless of the presence of Hsc70, while Hsc70 weakly bound to GRASP65 only when DjA1 was present in the system (Figure 1I). This result suggests direct interaction between DjA1 and GRASP65 and therefore links Hsc70 to it. However, Hsc70 lacks direct interaction with GRASP65 in vitro. To clarify the specificity of the DjA1-GRASP65 interaction, we compared the affinities of DjA1 and DjA4, two Type 1 J-domain protein family members with similar structures (Supplemental Figure 2), with GRASP65 in parallel. As shown in Figure 1J, only DjA1-, but not DjA4-, coupled beads pulled down GRASP65-GFP from HeLa cells lysate. Moreover, unlike GRASP65, GRASP55 was not pulled down by DjA1 (Figure 1K). Combined, these results indicate a specific interaction between GRASP65 and DjA1.

Mapping the DjA1 and GRASP65 interaction regions

To further understand the DjA1-GRASP65 interaction, we mapped the interaction regions on both proteins using the pull-down assay. Like other members in the Type 1 J-domain protein family, DjA1 contains an N-terminal J domain bearing the highly conserved histidine-proline-aspartic acid (HPD) tripeptide critical for the interaction with Hsc70 (Tsai and Douglas, 1996). Adjacent to the J-domain, there is a glycine/phenylalanine (G/F)-rich region followed by a central cysteine-rich region (CRR) consisting of four repeats of the CXXCXGXG Zinc finger motif, and then a C-terminal region involved in binding of client proteins (Figure 2A) (Lu and Cyr, 1998; Li *et al.*, 2003; Borges *et al.*, 2005). We prepared recombinant DjA1 fragments based on its structural domains and used them as the baits for pull down. The results showed that the C-terminus of DjA1, amino acids 206–397, was required for its binding to GRASP65 (Figure 2B).

On the other hand, GRASP65 contains an N-terminal GRASP domain consisted of two PDZ domains, and a C-terminal serine/proline-rich (SPR) domain (Wang *et al.*, 2005; Tang *et al.*, 2010b). To determine the binding region of DjA1 on GRASP65, we used GRASP65 fragments (Figure 2C) to pull down endogenous DjA1 from cell lysate. As shown in Figure 2D, full-length GRASP65 and its C-terminal SPR domain interacted with DjA1, demonstrating that the interaction is mediated by the SPR domain of GRASP65 instead of the N-terminal GRASP domain. Further truncations of the SPR domain demonstrated that amino acids 202–320 of GRASP65 serves as the minimal fragment for DjA1 binding (Figure 2E). In addition, in vitro binding assay using both recombinant proteins confirmed direct interaction between GRASP65 amino acids 202–320 and DjA1 C-terminal region (Figure 2F).

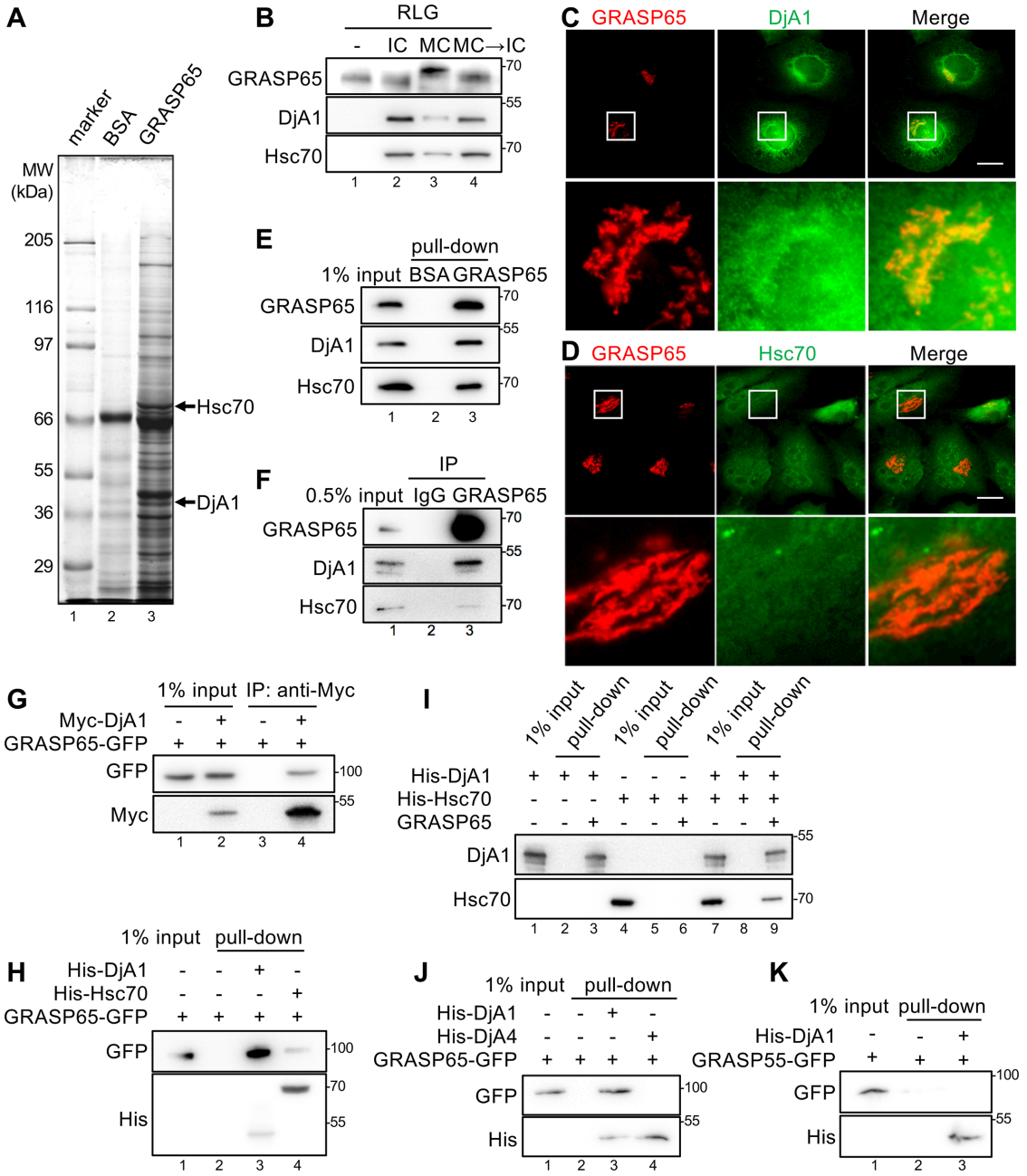

FIGURE 1: Identification of DjA1 as a GRASP65 binding protein. (A) Affinity purification of GRAS65-interacting proteins. Interphase cytosol (IC) fractionated by 15–30% ammonium sulfate precipitation was incubated with either BSA- or His-GRASP65-coupled CNBr activated beads, bound proteins were analyzed by SDS–PAGE and Coomassie blue staining. Arrows indicate the DjA1 and Hsc70 bands that were excised and identified by mass spectrometry. (B) Purified rat liver Golgi membranes (RLG) were incubated with interphase (IC) or mitotic (MC) cytosol, or sequentially incubated with MC and then IC (MC→IC), reisolated, and blotted for indicated proteins. (C, D) HeLa cells were immunostained for indicated proteins (GRASP65, TRITC, red; DjA1 or Hsc70, FITC, green). Bars, 20 μm. Boxed regions in the top panels are enlarged and shown in the bottom panels. (E) BSA- or full-length GRASP65-coupled CNBr beads were incubated with HeLa whole cell lysate (WCL, input), bound proteins were analyzed by Western blotting. (F) HeLa cell lysate was immunoprecipitated (IP) with either nonspecific IgG or an anti-GRASP65 antibody followed by Western blotting. (G) HeLa cells were cotransfected with myc-tagged DjA1 and GFP-tagged GRASP65, immunoprecipitated with an anti-myc antibody followed by Western blotting. (H) Purified His-tagged DjA1 or Hsc70 were incubated with the WCL of HeLa cells expressing GRASP65-GFP followed by Nickel (Ni)-beads pull down. Bound proteins were analyzed by Western blotting for GFP (GRASP65) and His (DjA1 or Hsc70). (I) BSA- or GRASP65-coupled CNBr beads were incubated with purified His-tagged DjA1, Hsc70, or both. Bound proteins were analyzed by Western blotting. (J) Purified His-tagged DjA1 or DjA4 was incubated with the WCL of HeLa cells expressing GRASP65-GFP followed by Ni-beads pull down. Bound proteins were analyzed by Western blotting for GFP (GRASP65) and His (DjA1 or DjA4). (K) His-tagged DjA1 was incubated with the WCL of HeLa cells expressing GRASP55-GFP followed by Ni-beads pull down. Bound proteins were analyzed by Western blotting for GFP (GRASP55) and His (DjA1).

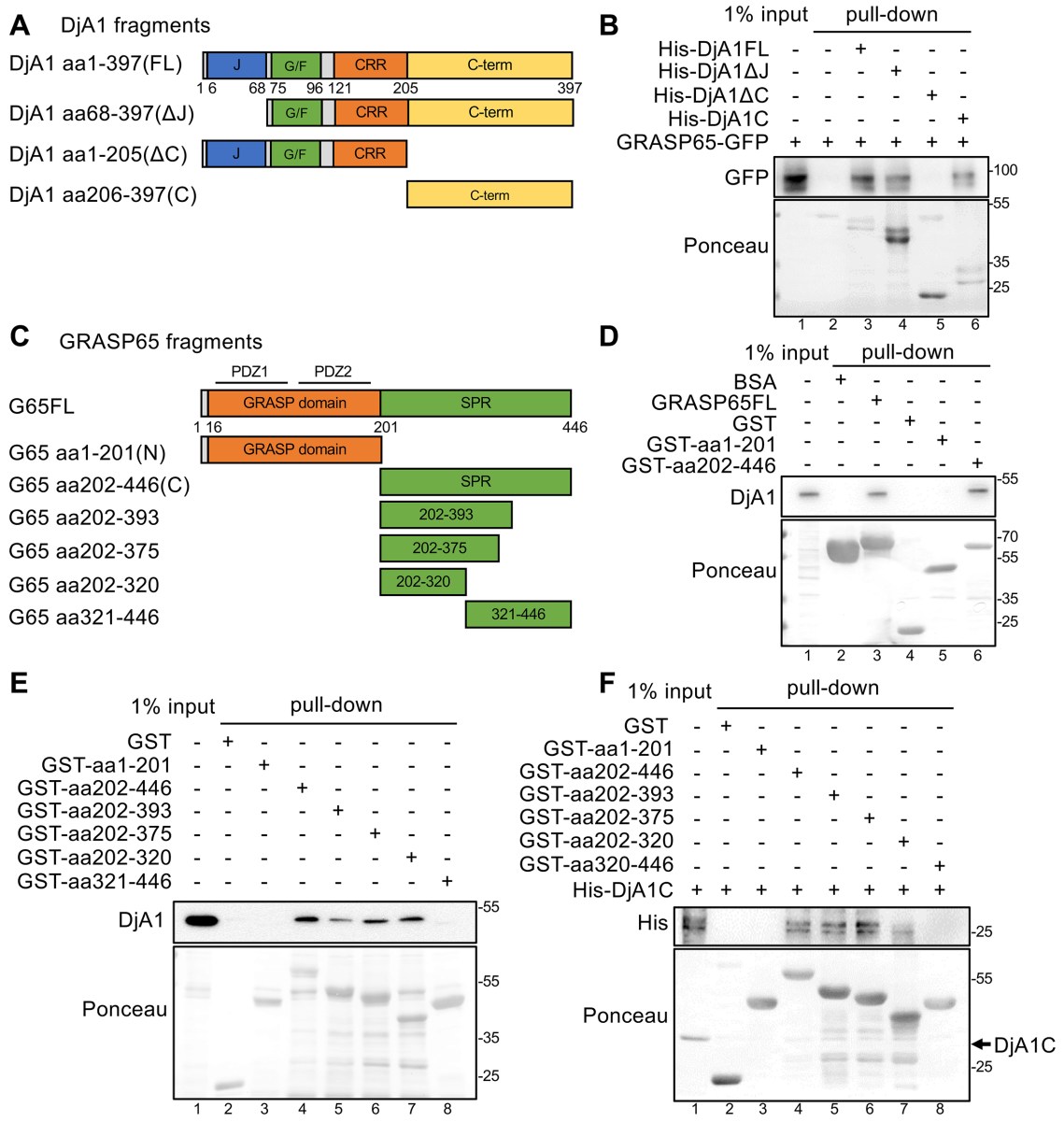

FIGURE 2: Mapping DjA1-GRASP65 interacting regions. (A) Schematic structure of DjA1. J, J-domain; G/F, glycine and phenylalanine-rich domain; CRR, central cysteine-rich region (CR-type zinc finger). (B) Purified His-tagged DjA1 fragments were incubated with WCL of HeLa cells expressing GRASP65-GFP followed by Ni-beads pull down. Bound proteins were analyzed by Western blotting for GFP (GRASP65). (C) Schematic structure of GRASP65. PDZ, PSD95/Dlg1/ZO-1 domain; SPR, serine/proline-rich domain. (D) CNBr beads coated with BSA or full-length GRASP65 (GRASP65FL), or glutathione agarose coupled with GST or GST-tagged GRASP65 fragments were incubated with HeLa WCL. Bound proteins were analyzed by Western blotting for DjA1. (E) Glutathione agarose coupled with GST or purified GST-tagged GRASP65 fragments were incubated with WCL. Bound proteins were analyzed by Western blotting for DjA1. (F) Glutathione agarose coupled with GST or GST-tagged GRASP65 fragments were incubated with purified His-DjA1C. Bound proteins were analyzed by Western blotting for His (DjA1C).

DjA1 is required for Golgi integrity and cisternae stacking

To determine the role of DjA1 in Golgi structure formation, we knocked down DjA1 in HeLa cells and analyzed the Golgi structure by immunofluorescence microscopy using several Golgi markers including GM130, GRASP65, GCC88, and TGN46. The results showed that DjA1-depletion did not affect GRASP65 Golgi localization, but caused Golgi disorganization, with more detectable Golgi elements detached from each other (Figure 3A; Supplemental Figure 3), which was rescued by exogenously expressing small interfering RNA (siRNA)-resistant GFP-DjA1 but not by GFP (Figure 3, A–C). Quantitation of these cells showed that there were 9.6 ± 1.7 Golgi elements/cell in DjA1-depleted cells, significantly higher than that in control cells (3.8 ± 1.0), and exogenously expressing DjA1 rescued this phenotype (4.9 ± 2.1) (Figure 3B). These results suggest that DjA1 is involved in Golgi integrity.

We then examined the Golgi structure more closely by electron microscopy (EM) after DjA1 depletion and GFP or siRNA-resistant GFP-DjA1 expression. When cells were treated with control siRNA, the Golgi stacks were well-organized regardless of whether the cells were transfected with GFP- or GFP-DjA1. When DjA1 was depleted,

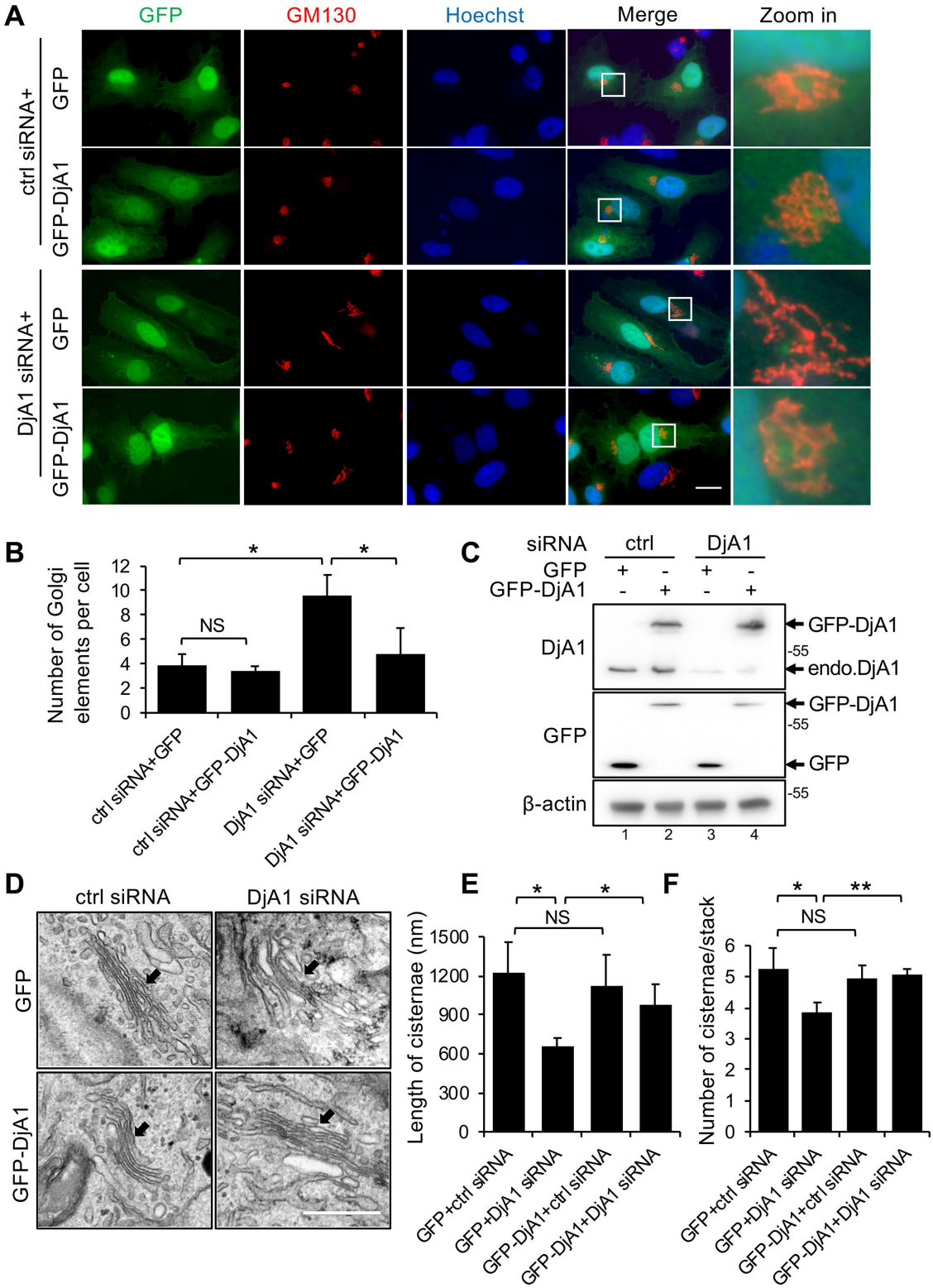

FIGURE 3: DjA1 is required for Golgi integrity. (A) HeLa cells treated with control (ctrl) or DjA1-specific siRNA for 48 h were transfected with a GFP vector or siRNA-resistant GFP-DjA1 for 24 h. Cells were fixed and stained for GM130 (TRITC, red) to show the Golgi structure. Bar, 20 μm. Boxed regions are enlarged and shown on the right. (B) Quantification of detectable Golgi elements per cell. (C) Cells used in A were analyzed by Western blotting for endogenous DjA1 and expressed GFP-DjA1. endo. DjA1, endogenous DjA1. (D) HeLa cells expressing GFP or siRNA-resistant GFP-DjA1 were enriched by flowcytometry, transfected with control or DjA1 siRNA, and analyzed by EM. Representative EM images are shown. Arrows, Golgi stacks. Bar, 500 nm. (E) Quantification of the length of Golgi cisternae. (F) Quantification of the number of cisternae per stack. The results are presented as mean ± SD. Statistics was performed using Student's *t* test. NS, nonspecific; *$p < 0.05$; **$p < 0.01$.

the Golgi stacks became abnormal, with the cisternae irregular in shape, sometimes swollen, improperly aligned, less stacked, and significantly shorter in length than in control cells. This phenotype was rescued by exogenous expression of siRNA-resistant GFP-DjA1 (Figure 3D and Supplemental Figure 4). The average length of Golgi stacks reduced from 1223.2 ± 235.1 nm in control cells to 659.8 ± 61.2 nm in DjA1-depleted cells, which was restored to 977.2 ± 157.2 nm when GFP-DjA1 was expressed (Figure 3E). Meanwhile, the average count of cisternae per stack reduced from 5.2 ± 0.7 to 3.8 ± 0.3, which was restored to 5.1 ± 0.2 by GFP-DjA1 expression (Figure 3F). These results indicate that DjA1 is required for the maintenance of cisternae shape and alignment in the stacks.

DjA1 depletion impedes Golgi ribbon formation after nocodazole washout

To determine whether DjA1 plays a role in maintaining the dynamic structure of the Golgi in cells, we induced Golgi ribbon unlinking by nocodazole treatment, which causes reversible depolymerization of microtubules and results in dispersed Golgi ministacks in the cytosol. Washout of nocodazole allows microtubules to repolymerize and a Golgi ribbon to reform (Minin, 1997; Thyberg and Moskalewski, 1999). As shown in Figure 4, DjA1 depletion had no effect on nocodazole-induced Golgi fragmentation, which, however, significantly delayed the reformation of the Golgi ribbon (Figure 4A). The effect was most dramatic 15 and 30 min after nocodazole removal, seen as more free Golgi elements unfused with the Golgi core in DjA1-depleted cells. After 60 min, all the Golgi elements were concentrated at the cell center in control cells, and the Golgi ribbon became intact in control cells. However, in DjA1-depleted cells, more Golgi elements remained unconnected. Quantitation results showed that DjA1 depletion increased the number of detectable Golgi elements per cell at multiple time points, indicating a defect in accumulation and fusion of the Golgi elements (Figure 4B).

DjA1 is required for Golgi membrane fusion

Given that DjA1 depletion in cells reduces the length of the Golgi cisternae (Figure 3, D and E), we wondered whether DjA1 plays a role in Golgi membrane fusion. We previously devised an in vitro assay to reconstitute the Golgi disassembly and reassembly processes in the cell cycle (Wang *et al.*, 2003; Tang *et al.*, 2010a). Upon treatment with mitotic cytosol (MC), the stacked cisternae in purified Golgi membranes disassemble into mitotic Golgi fragments (MGFs) comprising vesicles, tubules and cisternal remnants. Further incubation of MGFs with IC induces membrane fusion to generate single cisternae, which subsequently form multilayer stacks (Tang *et al.*, 2010a). To determine the role of DjA1 in Golgi membrane fusion, we immunoblocked the IC with a DjA1-specific antibody, while nonspecific immunoglobulin G (IgG) was used as a negative control. When incubated with control IgG-treated cytosol, the MGFs (Figure 5A) reassembled into long Golgi cisternae (Figure 5B). The Golgi membrane fusion activity was measured by quantifying long cisternal membranes generated from vesicles after incubation. Compared to nonspecific IgG control, membrane fusion activity was reduced by over 40% when DjA1 was blocked (Figure 5, C and D). Meanwhile, proportion of long cisternae in stacks reduced from 57.8 ± 3.8% to 40.4 ± 7.4% (Figure 5E). These results demonstrate that DjA1 is involved Golgi membrane fusion and stacking.

DjA1 depletion accelerates protein trafficking

As a central membrane organelle for trafficking and processing of membrane and secretory proteins in all eukaryotic cells, the structure of the Golgi and its function in protein trafficking are tightly linked (Zhang and Wang, 2016). To determine whether DjA1 depletion affects protein trafficking, we performed the vesicular stomatitis virus glycoprotein (VSV-G) trafficking assay using the well-established RUSH (retention using selective hooks) system (Boncompain and Perez, 2012). Control or DjA1 knockdown cells were transfected with a Str-Ii_VSVGwt-SBP-EGFP plasmid and cultured at 37°C for 24 h. The endoplasmic reticulum (ER)–retained VSV-G was then released by treatment with 40 μM biotin at 37°C for indicated times (chase). The cell lysates were then treated with endoglycosidase H (EndoH) and analyzed by Western blotting to differentiate the EndoH-resistant (Golgi and post-Golgi) and -sensitive (ER) forms of VSV-G-GFP (Figure 6A). Quantitation of the results showed that DjA1 knockdown significantly accelerated VSV-G trafficking after 30–90 min (Figure 6B), the time points while VSV-G was traversing the Golgi stack (Presley *et al.*, 1997; Xiang and Wang, 2010). Thus, DjA1 depletion accelerates VSV-G trafficking through the Golgi membranes.

DjA1 enhances GRASP65 oligomerization

The next question concerned the mechanism of DjA1 in Golgi structure formation. On the basis of the literature, we speculated three possible roles for DjA1 (and Hsc70) on the Golgi. First, given that DjA1 and Hsc70 are well-established protein chaperones (Terada and Mori, 2000), it is possible that they are recruited to the Golgi by GRASP65 and involved in Golgi structural protein folding, which could be a part of the Golgi protein quality control system. If this is the case, then depletion of DjA1 of Hsc70 could cause destabilization of some Golgi proteins. Therefore, we tested the protein levels of multiple key Golgi structural proteins by Western blotting. However, no significant change was detected in GRASP65 or other Golgi proteins (Supplemental Figure 5, lane 2 vs. lane 1).

It has been previously reported that clathrin cages are disassembled by Hsc70 and some of its J-domain containing cochaperones, such as cyclin-G–associated kinase (GAK) and its neuronal-specific homolog auxilin (also known as DNAJC6) (Krantz *et al.*, 2013; Park *et al.*, 2015). Therefore, an alternative possibility is that DjA1 could be an unidentified Hsc70 cofactor in clathrin uncoating. However, DjA1 knockdown affected neither clathrin distribution in the cell nor the concentration of clathrin heavy chain (CHC) in the perinuclear region (Supplemental Figure 6A). Additionally, when purified Golgi membranes were incubated with purified DjA1, with or without Hsc70, and reisolated, the amount of Golgi-associated clathrin did not significant change (Supplemental Figure 6, B and C). These results ruled out the role of Hsc70 and DjA1 in the disassembly of the clathrin coat on Golgi membranes.

Exclusion of the first two hypotheses leaves the last possibility that DjA1 may modulate the Golgi structure through GRASP65, possibly via regulating its oligomerization. To test this possibility, we employed the bead aggregation assay that we established earlier (Wang *et al.*, 2003, 2005). In this assay, Dyna magnetic beads were first coated with purified GRASP65 at the surface, and further incubated with a buffer or cytosol. Interactions between GRASP65 molecules on different beads could bring the bead together to form aggregates, indicating the efficiency of GRASP65 oligomerization and its capability to link surfaces together as in Golgi stacking. Thus, bead aggregation can be used as a direct readout for GRASP65 oligomerization (Tang *et al.*, 2016), which was used here to study the role of DjA1 in GRASP65 oligomerization. As shown in Figure 7, A and B, GRASP65-coated beads formed large aggregates when incubated with IC, whereas blocking DjA1 using a DjA1-specific antibody significantly reduced this activity. Consistently, immunodepletion of DjA1 from IC significantly reduced GRASP65

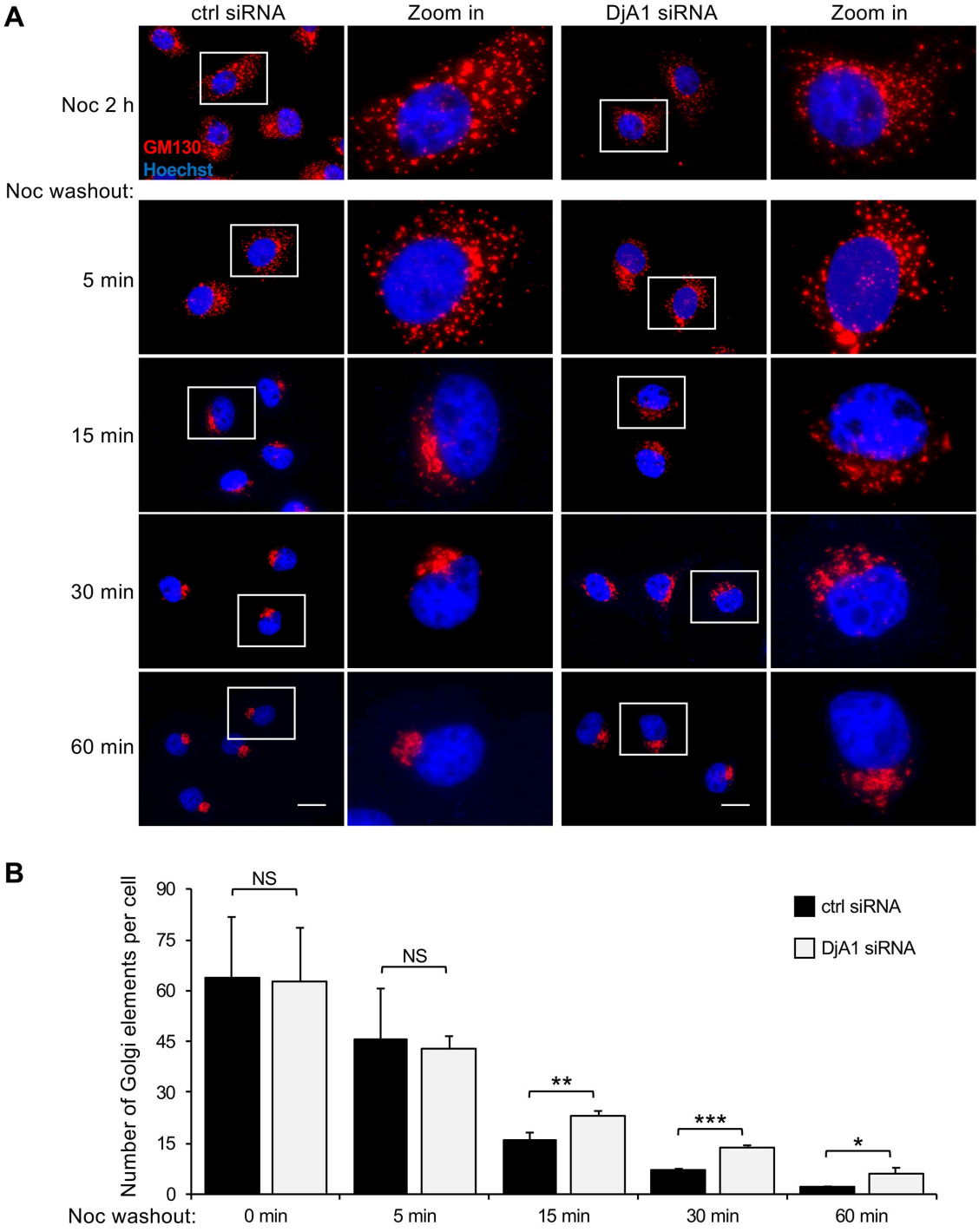

FIGURE 4: DjA1 depletion impairs Golgi ribbon formation after nocodazole washout. (A) HeLa cells transfected with ctrl or DjA1 siRNA for 48 h were treated with nocodazole (Noc) for 2 h. After nocodazole removal for indicated time periods, cells were fixed and stained for GM130 (TRITC, red) to show the Golgi morphology. Bar, 20 μm. Boxed regions are enlarged and shown on the right. (B) Quantification of detectable Golgi elements per cell. The results are presented as mean ± SD. Statistics was performed using Student's *t* test. NS, nonspecific; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

oligomerization (Figure 7, C–E), suggesting that DjA1 is a major component in the IC that enhances GRASP65 oligomerization. Moreover, adding back purified DjA1 full-length (DjA1FL) protein, or the fragments that interacted with GRASP65, including DjA1ΔJ and DjA1C, rescued this effect; whereas adding DjA1ΔC, which does not interact with GRASP65, had no effect (Figure 7, C and D). To confirm a direct role of DjA1 in GRASP65 oligomerization, we incubated GRASP65-coated beads with purified DjA1 fragments without cytosol. As expected, full-length DjA1 and its GRASP65-binding fragments directly enhanced aggregation of GRASP65-coated beads, although at a lower efficiency than complete IC. On the contrary, purified Hsc70 failed to induce GRASP65 oligomerization or to further enhance DjA1-induced GRASP65 oligomerization (Figure 7, F and G).

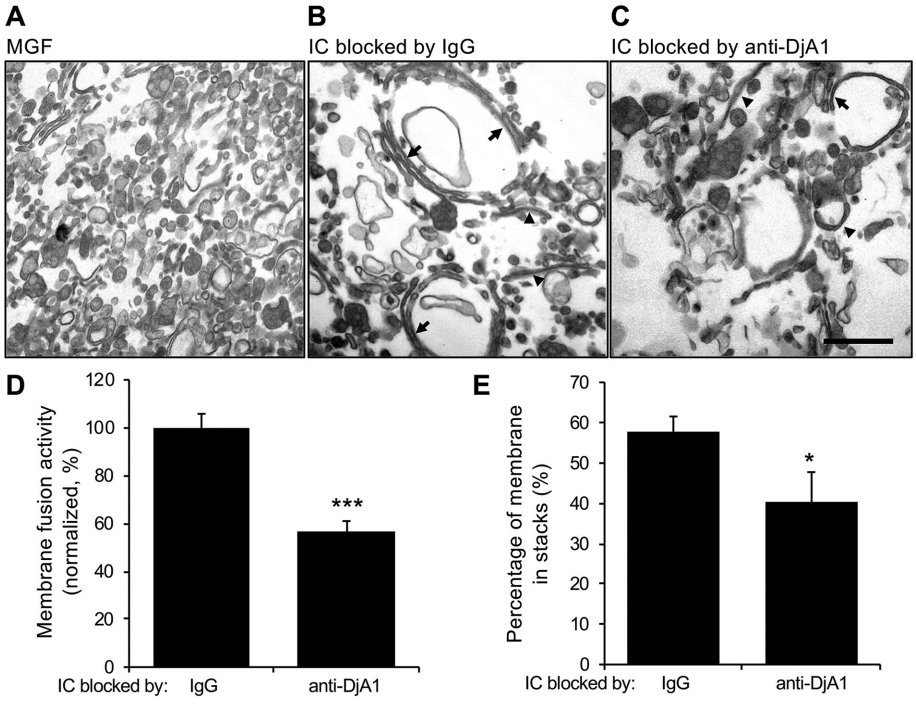

FIGURE 5: DjA1 is required for Golgi membrane fusion. Purified Golgi membranes were disassembled by MC treatment. The resulting mitotic Golgi fragments (MGF, A) were reisolated and further incubated with IC immunoblocked with IgG (B) or anti-DjA1 antibody (C). Membranes were processed for EM and imaged. Arrows, cisternal membranes in stacks. Arrow heads, unstacked cisternal membranes. Bar, 500 nm. (D) The relative Golgi membrane fusion efficiencies were quantified. (E) The percentage of cisternae membranes in stacks was quantified. The results are presented as mean ± SD. Statistical significance was assessed by comparison to MGF using Student's *t* test. *$p < 0.5$. ***$p < 0.001$.

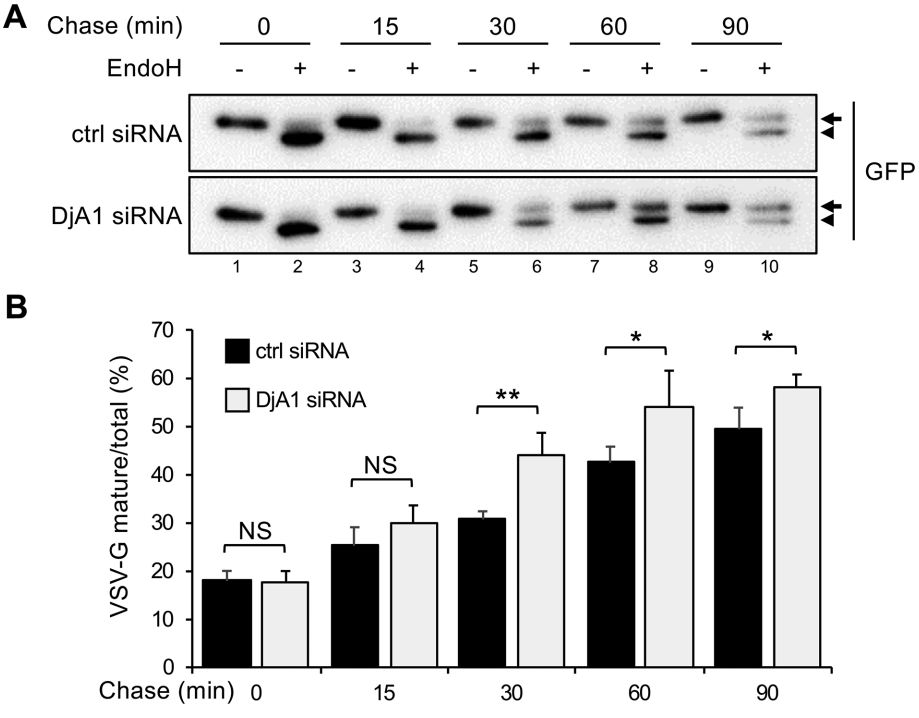

FIGURE 6: DjA1 depletion accelerates protein trafficking. Control (ctrl) or DjA1 siRNA-treated HeLa cells were transfected with a Str-Ii_VSVGwt-SBP-EGFP plasmid and cultured at 37°C for 24 h. Cells were then treated with 40 μM biotin at 37°C for indicated time (chase). (A) Cell lysates were treated with or without EndoH and analyzed by Western blotting for VSV-G-GFP. Arrows indicate the EndoH resistant (Golgi and post-Golgi) form, and arrowheads indicate the EndoH-sensitive (ER) form of VSV-G. (B) Quantitation of A for the percentage of VSV-G in EndoH-resistant form from three independent experiments. The results are presented as mean ± SD. Statistics was performed using Student's *t* test. NS, nonspecific. *$p < 0.05$. **$p < 0.01$.

To further confirm that the effect of DjA1 on GRASP65 oligomerization depends on its interaction with GRASP65, we tested the GRASP domain (GRASP65N) that formed oligomers (Wang *et al.*, 2005) but did not interact with DjA1 (Figure 2, D–F) in the bead aggregation assay. Similarly to GRASP65FL-coated beads, GRASP65N-beads formed aggregates when incubated with IC. However, incubation with DjA1 or its fragments exhibited no effects on the aggregation (Figure 7, H and I). These results demonstrate that the activity of DjA1 in promoting GRASP65 oligomerization requires the interaction between these two proteins.

To demonstrate that DjA1 enhances GRASP65 oligomerization using an alternative approach, we tested the binding affinity between His-GRASP65 and MBP-GRASP65 with or without purified DjA1 fragments. In the absence of DjA1, His-GRASP65 was co-purified when MBP-GRASP65 was pulled down using amylose beads, as previously described (Wang *et al.*, 2003). Adding full-length DjA1 enhanced the efficiency of the copurification. Furthermore, adding DjA1ΔJ or DjA1C, which interacts with GRASP65, had a similar effect as full-length DjA1; whereas adding DjA1ΔC, which does not interact with GRASP65, had no effect (Figure 7J). Taken together, our results demonstrate that DjA1 interacts with GRASP65 to enhance its oligomerization.

DISCUSSION

In this study, we identified a type I J domain protein, DjA1, as a novel interacting partner of the Golgi stacking protein GRASP65. DjA1 is involved in maintaining Golgi integrity via promoting GRASP65 oligomerization and membrane tethering. In vivo and in vitro binding assays revealed a direct interaction between DjA1 C-terminus and GRASP65 amino acids 202–320 region in the SPR domain. Immunofluorescence and EM microscopy showed disrupted Golgi on DjA1 depletion, as Golgi stacks had shorter, irregularly shaped, less-well-stacked, and improperly aligned cisternae. In vitro bead aggregation and copurification assay supported that DjA1 promotes GRASP65

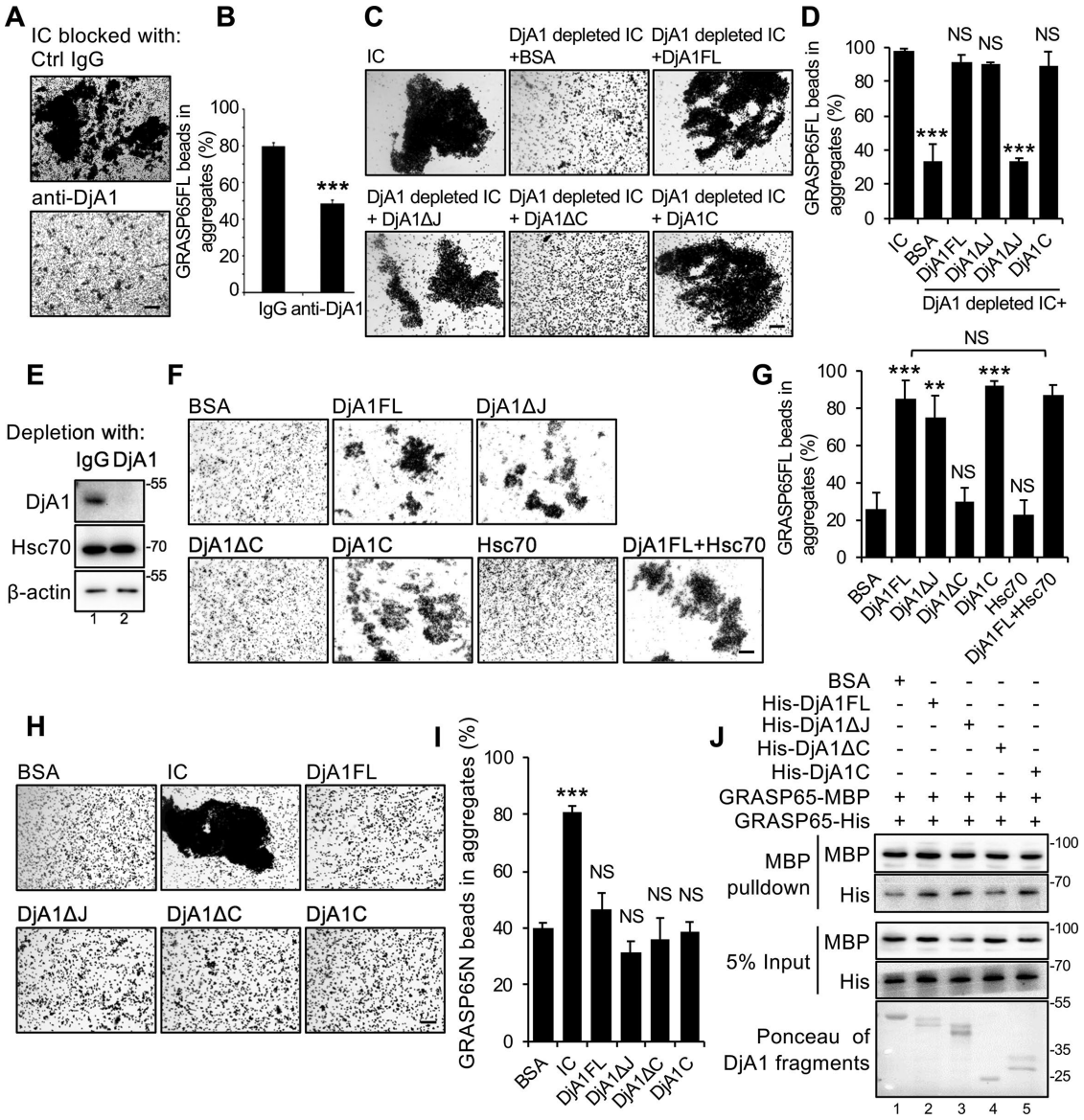

FIGURE 7: DjA1 enhances GRASP65 oligomerization via direct interaction. (A, B) GRASP65-coated beads were incubated with IC immunoblocked by nonspecific IgG or DjA1 antibody. Beads were imaged by microscopy (A), and the percentage of beads in aggregates was quantified (B). Statistical significance was assessed by comparison to IgG-treated IC. (C, D) GRASP65-coated beads were incubated with IC immunodepleted by control IgG (IC) or DjA1 antibodies (DjA1-depleted IC), which was supplemented with BSA or indicated DjA1 fragments. Beads were imaged by microscopy (C), and the percentage of beads in aggregates was quantified (D). (E) Immunodepletion efficiency was analyzed by Western blotting. (F, G) GRASP65-coated beads were incubated with BSA or indicated purified proteins. Beads were imaged by microscopy (F), and the percentage of beads in aggregates was quantified (G). Statistical significance was assessed by comparison to BSA-treated beads. (H, I) GRASP65 N-terminal GRASP domain- (GRASP65N) coated beads were incubated with BSA or indicated DjA1 fragments. The results are presented as mean ± SD. Statistical significance was assessed by comparison to BSA-treated beads using Student's *t* test. NS, nonspecific; $^{**}p < 0.01$; $^{***}p < 0.001$. Bars, 100 μm. (J) Purified MBP- and His-tagged GRASP65 were incubated in the presence of BSA or DjA1 fragments followed by Amylose resin pull down. Bound proteins were analyzed by Western blotting.

oligomerization. However, depletion of DjA1 impeded Golgi ribbon formation after nocodazole washout, which indicated that DjA1 may partially function in ribbon linking.

DjA1 was first identified as a novel GRASP65 interacting protein by affinity purification and mass spectrometry, and was subsequently shown to be associated with interphase Golgi membranes. Since its binding partner Hsc70 was identified at the same time, we originally raised three hypotheses in their roles in Golgi structure formation and function. First, considering that they are protein chaperones involved in protein folding (Terada and Oike, 2010), we initially speculated that they may be recruited to the Golgi membranes by GRASP65 to function as part of the Golgi protein quality control system. However, subsequent experiments failed to detect an impact of DjA1 depletion on the level of several Golgi structural proteins (Supplemental Figure 5). Second, clathrin functions in vesicles budding from the *trans*-Golgi network (TGN) in the sorting of newly synthesized lysosomal enzymes for delivery to the endosomal system (Robinson and Neuhaus, 2016; Staudt *et al.*, 2017), where

Hsc70 and its J domain protein cofactors are required for clathrin uncoating (Ungewickell *et al.*, 1995; Greener *et al.*, 2000). We therefore determined whether DjA1 and Hsc70 could function together in clathrin uncoating at the TGN. However, DjA1 depletion showed no significant effect on the Golgi localization of clathrin in cells (Supplemental Figure 6A). In addition, treatment of Golgi membranes with purified DjA1, with or without Hsc70, did not affect the association of clathrin with Golgi membranes (Supplemental Figure 6, B and C). Therefore, in the following studies we focused on their roles in GRASP65-related Golgi morphology regulation and later excluded the role of Hsc70 in this process.

A notable phenomenon seen in DjA1 knockdown cells was the misshaped and improperly aligned cisternae under the EM. Although it has been confirmed that DjA1 enhances GRASP65 oligomerization, the exact mechanism remains unknown. It is possible that DjA1 may affect the size of GRASP65 oligomers and the arrangement of GRASP65 molecules in them. Considering that DjA1 forms dimer while binding to its client proteins, DjA1 dimerization may provide a "tethering" effect between different GRASP65 molecules and therefore facilitates their assembly. Alternatively, DjA1 interaction with the SPR domain of GRASP65 may free the N-terminal GRASP domain for oligomerization. Consequently, DjA1 depletion may affect GRASP65 distribution and organization in the gap between the cisternae. It has been suggested that membrane adhesion dictates Golgi stacking and cisternal morphology (Lee *et al.*, 2014), it is reasonable to speculate that improper or uneven localization of GRASP65, or disorganization of GRASP65 molecules in the oligomers, may affect the morphology and organization of Golgi cisternal membranes in the stacks. Future structure studies of DjA1 and GRASP65 may shed light on the exact mechanism.

DjA1 depletion accelerates proteins trafficking, similarly to GRASP depletion (Xiang *et al.*, 2013). As a central hub of protein processing and trafficking, the function of the Golgi is tightly linked to its structure. Previously, we have shown that Golgi structure disruption by GRASP65 and GRASP55 double-depletion enhances the trafficking of VSV-G, integrin, and cathepsin D (Xiang *et al.*, 2013). Microinjection of GRASP65 antibodies inhibits stacking and results in accelerated CD8 trafficking (Wang *et al.*, 2008). One explanation is that Golgi unstacking increases the membrane surface and thus the efficiency of coat protein complex I (COPI) vesicle formation (Wang *et al.*, 2008; Xiang *et al.*, 2013). As DjA1 depletion impairs Golgi stack formation, it is reasonable to speculate that it also accelerates protein trafficking, as seen in GRASP-depleted cells.

Although GRASP65 was originally identified as a Golgi stacking factor (Barr *et al.*, 1997; Shorter *et al.*, 1999), it was later reported to be also important in Golgi ribbon linking (Puthenveedu *et al.*, 2006; Duran *et al.*, 2008; Feinstein and Linstedt, 2008). Both stacking and ribbon linking are mediated by GRASP65 *trans*-oligomers (Tang and Wang, 2013). Interestingly, the size of GRASP65 *trans*-oligomers (Feng *et al.*, 2013) corresponds well with the gap between the cisternae of the Golgi stack, but is too small for the much larger and heterogeneous gap between stacks (Cluett and Brown, 1992), suggesting the involvement of other bridging proteins in GRASP65-mediated ribbon formation. Indeed, our group has previously identified Mena as a GRASP65 interacting protein, which promotes local actin polymerization and facilitates Golgi ribbon linking. In the current study, DjA1 depletion results in shorter and fewer cisternae in the stacks, which is similar to the effect of single GRASP65 interference (Xiang and Wang, 2010; Bekier *et al.*, 2017) and indicates its function in cisternae stacking. Interestingly, knockdown of DjA1 has a relative more severe phenotype on Golgi morphology than knockdown or knockout of GRASP65 itself. Although the exact mechanism is unknown, one possibility is that it could be due to the compensation effect of GRASP55 in GRASP65- but not DjA1-depleted cells. As shown in Supplemental Figure 5, the level of GRASP55 increased in GRASP65 knockout but not DjA1-depleted cells. Because GRASP55 and GRASP65 play complementary roles in Golgi stacking (Xiang and Wang, 2010; Bekier *et al.*, 2017), increased GRASP55 level may reduce or diminish the effect caused by GRASP65 depletion.

However, restrained ribbon formation after nocodazole washout was also observed after DjA1 depletion, which indicated its potential role in ribbon linking. However, given that stacking defects may subsequently affect ribbon linking, it is unknown at this time whether DjA1 directly affects ribbon linking via GRASP65 function in stacking. The possibility that DjA1 (and Hsc70) affects Golgi ribbon linking via collaboration with other (GRASP-related) factors can also not be excluded.

Although DjA1 has been established as a Hsc70 cochaperone, the results showing DjA1 functions independently of Hsc70 in Golgi structure maintenance are not surprising since DjA1 mainly cooperate with Hsc70 via its J-domain and accumulating reports have emerged to support the idea that J proteins do not "completely" rely on their J domains to carry out their cellular functions. In some cases, the J domain becomes dispensable (Ajit Tamadaddi and Sahi, 2016). As for DjA1, Cao *et al.* discovered that DjA1 associates with the bPB2 and PA subunits of the influenza A virus complex and enhances viral RNA synthesis; this function depends on its C-terminal substrate-binding domain but not Hsp70 (Cao *et al.*, 2014). In this study, we demonstrate that DjA1 interacts with GRASP65 to promote its oligomerization and Golgi structure formation, a novel function of DjA1 that is independent of Hsc70.

In summary, we have identified that DjA1 directly interacts with GRASP65 to enhance GRASP65 oligomerization and facilitates Golgi stack and ribbon formation. Together with our previous research, this study further confirms GRASP65 as a multifaceted protein in Golgi structure formation through oligomerization and interaction with an array of binding proteins.

MATERIALS AND METHODS

Reagents, plasmids, antibodies, and siRNA

All reagents were purchased from Calbiochem (Gibbstown, NJ), EMD Millipore (Billerica, MA), Roche (Mannheim, Germany), Sigma-Aldrich (St. Louis, MO), Santa Cruz Biotechnology (Paso Robles, CA), and Thermo Fisher Scientific (Waltham, MA), unless otherwise stated. The following antibodies were used: monoclonal antibodies against β-actin (AC-15, SigmaAldrich), clathrin (X22, Affinity Bioreagent), DjA1 (sc-59554, Santa Cruz), GM130 (610823, BD Biosciences), and histidine (His) tag (66005, Proteintech); and polyclonal antibodies against DjA1 (11713, Proteintech), GCC88 (16271, Proteintech), human GRASP65 (Joachim Seemann, UT Southwestern, Dallas, TX), human GRASP55 (Proteintech), Man1A1(M3694, Sigma-Aldrich), syntaxin 5 (Vladimir Lupashin, University of Arkansas for Medical Sciences), and TGN46 (AHP500G, Serotec).

Control siRNA (Silencer Select Negative Control #1 siRNA) was purchased from Applied Biosystems (Thermo Fisher Scientific). DjA1 specific siRNA was synthesized by Sigma-Aldrich targeting to human DjA1 (5′-GGACATACAGCTCGTTGAA-3′). Constructs of GST- or His-tagged GRASP65 fragments or full-length proteins were prepared as described previously (Wang *et al.*, 2005). DjA1 cDNA was constructed into the pEGFP-C1 vector using pPROEX-DjA1 (gifted by Jason C. Young, Department of Biochemistry, McGill University, Canada) as template. siRNA-resistant mutant of DjA1 was generated by mutating the siRNA targeting sequence to 5′-GGATATTCAACTGGTAGAG-3′. His-tagged DjA1 fragments were constructed into pET30a(+) vector

based on pPROEX-DjA1. The Str-Ii_VSVGwt-SBP-EGFP plasmid was a gift from Franck Perez (Addgene, plasmid #65300). All constructs were confirmed by DNA sequencing.

Cell culture

HeLa cells were routinely cultured in DMEM supplemented with 10% GemCell SuperCalf serum (Gemini Bio-products, West Sacramento, CA). For knockdown experiments, HeLa cells were transfected using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions; cells were analyzed 48 h after transfection. For expression of exogenous proteins, HeLa cells were transfected with indicated constructs using Polyethylenimine (PEI) and analyzed 24 h afterward. For DjA1 knockdown and rescue, cells were transfected with DjA1-specific siRNA for 48 h and then with either an EGFP-C1 vector or the siRNA-resistant mutant of GFP-DjA1 for another 24 h.

Identification of GRASP65-interacting proteins

This was done as previously (Tang *et al.*, 2016). Briefly, interphase HeLa cell cytosol was diluted to 1 mg/ml and precipitated with 15% ammonium sulfate. After centrifugation in a bench-top minicentrifuge at full speed for 30 min at 4°C, the supernatant was increased to 30% ammonium sulfate followed by centrifugation as above. Proteins in the pellet were dissolved and dialyzed into KHM buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]–KOH, pH 7.0, 0.2 M sucrose, 60 mM KCl, 5 mM Mg(OAc)2, 2 mM ATP, 1 mM GTP, 1 mM glutathione, and protease inhibitors). Dissolved proteins were incubated with BSA or His-GRASP65-coupled, CNBr-activated Sepharose 4B beads (Sigma-Aldrich) in the presence of an ATP-regenerating system (ARS) (10 mM creatine phosphate, 1 mM ATP, 20 μg/ml creatine kinase, and 20 ng/ml cytochalasin B) in KHM buffer at 4°C overnight following manufacture's instruction. The beads were washed extensively and boiled in SDS buffer. Bound proteins were analyzed by SDS–PAGE. Specific bands that appeared in the lane of GRASP65 beads but not in the BSA beads were analyzed by mass spectrometry (Proteomics and Peptide Synthesis Core, University of Michigan) and searched against International Protein Index human databases as previously described (Chen *et al.*, 2010, 2012).

Preparation of recombinant proteins

All the recombinant proteins were expressed in BL21(DE3) Gold bacteria with induction performed on cultures (OD_{600} 0.6) at 25°C overnight with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). His-tagged GRASP65 full-length, DjA1 full-length and fragments were purified using HisPurTM Ni-NTA Resin (Thermo Fisher Scientific, Waltham, MA). GST-tagged GRASP65 fragments were purified on glutathione–SepharoseTM 4B Resin (GE Healthcare Life Sciences, Pittsburgh, PA). MBP-tagged GRASP65 full length was purified on Amylose Resin (New England BioLabs, Ipswich, MA).

In vitro binding assay

To confirm that DjA1 interacts with GRASP65 in vitro, 50 μg of purified DjA1 full-length protein was incubated with BSA- or GRASP65-coated CNBr beads in the presence of an ARS in KHM buffer at 4°C overnight. The beads were washed extensively, and the proteins enriched on the beads were boiled in SDS loading buffer and analyzed by SDS–PAGE and Western blotting. To map the interaction domains of GRASP65 and DjA1 in vitro, 20 μg purified GST- or GST-tagged GRASP65 fragments were coupled to glutathione beads and incubated with 50 μg His-tagged DjA1C in the presence of an ARS in KHM buffer at 4°C overnight. The beads were washed and analyzed by SDS–PAGE and Western blotting for GST or His tag.

Coimmunoprecipitation

For coimmunoprecipitation of endogenous DjA1 and GRASP65, HeLa cells were collected and lysed in immunoprecipitation buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, with protease inhibitors) at 4°C for 40 min and cleared by centrifugation in a bench-top centrifuge at 12,000 rpm at 4°C for 10 min. GRASP65 antibody and protein A beads or myc antibody and protein G beads were added to the lysate and incubated overnight at 4°C with gentle rotation. The beads were washed with immunoprecipitation buffer three times and then analyzed by SDS–PAGE and Western blotting.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (Wang *et al.*, 2005; Tang *et al.*, 2010a, 2016). Briefly, cells were fixed with 4% paraformaldehyde (PFA) for 15 min and quenched with 50 mM NH_4Cl for 10 min, followed by permeabilization with 0.2% Triton X-100 for 10 min. Cells were blocked with 1% BSA for 30 min and then stained with indicated antibodies. To reveal DjA1 and Hsc70 associated with intracellular membranes, HeLa cells were permeabilized before fixation as previously described (Villeneuve *et al.*, 2018). Briefly, HeLa cells were washed twice with wash buffer (125 mM potassium acetate, 25 mM HEPES, pH 7.2, and 2.5 mM magnesium acetate) at room temperature, permeabilized by incubation in wash buffer containing 0.1% saponin for 5 min on ice, washed again with wash buffer for 5 min at room temperature, fixed with 4% PFA, and stained with indicated antibodies. Images were taken using Zeiss Observer Z1 epifluorescence microscope with 63× oil lens. For protein colocalization, single layer scans are used. To quantify the Golgi elements, z-stack images were captured using fixed parameters with 0.27 μm intervals at *z*-axis. Images were then processed using ImageJ 3D Objects Counter tool with threshold set as MaxEntropy. More than 300 cells from three independent experiments were imaged and quantified. For the nocodazole washout experiment, cells were treated with 500 ng/ml nocodazole for 2 h with or without washout for indicated times. Cells were then fixed and stained for GM130.

Bead aggregation assay

Bead aggregation assays were performed as previously described (Wang *et al.*, 2003, 2005; Tang *et al.*, 2016). In brief, purified GRASP65 full-length or N-terminal fragment was coupled onto the surface of Dynabeads M-500 Subcellular or M-450 Tosylactivated (Invitrogen). The coupled beads were then incubated with IC or purified proteins as indicated in the presence of an ARS at 37°C with rotation for 1 h. The beads were then submitted for imaging and quantitation. Random phase contrast digital images (10–20) of each sample were captured using Zeiss Observer Z1 epifluorescence microscope with a 10× lens. Images from three independent experiments were analyzed using MATLAB7.4 software to determine the surface area of objects, which was used to calculate the number of beads in the clusters. Aggregates were defined as those with six or more beads. For immunodepletion of DjA1, IC prepared from HeLa S3 cells (Rabouille *et al.*, 1995) was incubated with nonspecific control IgG or DjA1 antibody (ProteinTech) and protein A-agarose beads at 4°C overnight. The beads were pelleted, and supernatant was analyzed by Western blotting of DjA1.

Quantification of Golgi cisternal length and stacking

HeLa cells expressing GFP or siRNA-resistant GFP-DjA1 were first enriched by fluorescence activated cell sorting (FACS; Synergy Head cell sorter) and then transfected with ctrl or DjA1 siRNA for

48 h to knock down endogenous DjA1. Cells were processed for EM as previously described (Tan *et al.*, 2017). Sections of 60 nm were mounted on Formvar-coated copper grids and contrasted with 3% lead citrate f or 5 min. Grids were imaged using a JOEL transmission electron microscope. Images of >20 cells were captured at 5000–20,000× magnification. Golgi stacks were identified using morphological criteria and quantified using standard stereological techniques (Wang *et al.*, 2005; Tang *et al.*, 2010a; Tan *et al.*, 2017). Cisternae were defined as long membrane profiles with a length greater than four times their width, and the latter did not exceed 60 nm. The longest cisternae in a Golgi stack were measured as the length of cisternae using the ImageJ software Measure tool, and the number of cisternae in the Golgi stack with the most cisternae layers was counted as the number of cisternae per stack.

Golgi disassembly and reassembly assay and quantitation

The Golgi disassembly assay was performed as described previously (Satoh *et al.*, 2003; Tang *et al.*, 2010a). Briefly, purified Golgi membranes (20 µg) were mixed with 2 mg of mitotic cytosol, 1 mM GTP, and ARS in MEB buffer (Tris–HCl 50 mM, pH 7.4, 0.2 M sucrose, 50 mM KCl, 20 mM β-glycerophosphate, 15 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 10 mM $MgCl_2$, 2 mM ATP, 1 mM GTP, 1 mM glutathione, and protease inhibitors) in a final volume of 200 µl. After incubation for 1 h at 37°C, MGFs were isolated by centrifugation (135,000 × *g* for 30 min in a TLA55 rotor) through a 0.4 M sucrose cushion in KHM buffer onto 6 µl of 2 M sucrose cushion. The membranes were resuspended in KHM buffer and either fixed and processed for EM (Tang *et al.*, 2010a) or used in reassembly reactions. For Golgi reassembly, 20 µg of mitotic Golgi fragments was mixed with 400 µg of IC (blocked with either control IgG or DjA1 antibody) in the presence of an ARS in a final volume of 100 µl in KHM buffer and incubated at 37°C for 1 h. The membranes were fixed and pelleted by centrifugation and processed for EM. To quantify the reassembly of the Golgi, EM images of Golgi membranes were captured at 11,000× magnification. The percentage of membranes in cisternae or vesicles was determined by the intersection method (Misteli and Warren, 1994; Tang *et al.*, 2010a). Cisternae were defined as long membrane profiles with a length greater than four times their width, and the latter did not exceed 60 nm. Quantification of the relative Golgi membrane fusion efficiency was performed as described previously (Tang *et al.*, 2010a, 2016).

VSV-G trafficking using RUSH system

VSV-G trafficking assay was performed using a protocol based on the previous report (Boncompain and Perez, 2012). In brief, HeLa cells transfected with indicated siRNA were transfected with the Str-Ii_VSVGwt-SBP-EGFP plasmid and cultured at 37°C for 24 h. Cells were then treated with 40 µM biotin at 37°C for indicated times (chase). Cell lysates were treated with or without EndoH and analyzed by Western blotting for VSV-G-GFP. The percentage of VSV-G in EndoH resistant form was quantified using ImageJ software.

Quantitation and statistics

Quantitation was based on three independent experiments. The results are presented as mean ± SD. Statistics was performed using Student's *t* test. The significance is shown as nonspecific (NS); $*p < 0.05$; $**p < 0.01$ or $***p < 0.001$.

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