GRASP55 and GRASP65 play complementary and essential roles in Golgi cisternal stacking

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In vitro studies have suggested that Golgi stack formation involves two homologous peripheral Golgi proteins, GRASP65 and GRASP55, which localize to the cis and medial-trans cisternae, respectively. However, no mechanism has been provided on how these two GRASP proteins work together to stack Golgi cisternae. Here, we show that depletion of either GRASP55 or GRASP65 by siRNA reduces the number of cisternae per Golgi stack, whereas simultaneous knockdown of both GRASP proteins leads to disassembly of the entire stack. GRASP55 stacks Golgi membranes by forming oligomers through its N-terminal GRASP domain. This process is regulated by phosphorylation within the C-terminal serine/proline-rich domain. Expression of nonphosphorylatable GRASP55 mutants enhances Golgi stacking in interphase cells and inhibits Golgi disassembly during mitosis. These results demonstrate that GRASP55 and GRASP65 stack mammalian Golgi cisternae via a common mechanism.

Introduction

The Golgi complex is a membrane-bound organelle that serves as a central conduit for the processing of membrane and secretory proteins in all eukaryotic cells. It comprises stacks of flattened cisternae that are laterally linked to form a ribbon in mammalian cells. Formation of stacks is thought to be significant in that it facilitates the accurate localization and function of enzymes that modify *N*- and *O*-linked oligosaccharides (Kornfeld and Kornfeld, 1985). However, the mechanism that mediates stacking is poorly understood. GRASP65 (Golgi reassembly stacking protein of 65 kD), a peripheral Golgi protein that is associated with membranes via N-terminal myristoylation, was identified as the first Golgi-stacking factor through the use of an in vitro assay that reconstitutes the cell cycle–regulated Golgi disassembly and reassembly process. Adding either GRASP65 antibodies or a soluble GRASP65 mutant inhibited restacking of newly formed Golgi cisternae in this assay (Barr et al., 1997). Consistent with this finding, microinjection of anti-GRASP65 antibodies into mitotic cells inhibited subsequent Golgi stack formation in the daughter cells (Wang et al., 2003). Knockdown of the sole GRASP protein in *Drosophila*, dGRASP, and its interacting protein dGM130 led to the disassembly of the Golgi stacks into single cisternae and vesicles (Kondylis et al., 2005). In one study, depletion of GRASP65 in mammalian cells reduced the number of cisternae per stack from 6 to 3 (Sütterlin et al., 2005), although a separate study reported that knockdown of GRASP65 resulted in unlinking of the Golgi ribbon (Puthenveedu et al., 2006). Further biochemical studies revealed that GRASP65 forms stable homodimers, and that homodimers residing on adjacent Golgi membranes form oligomers. These trans-oligomers are capable of holding the cisternal membranes together into stacks (Wang et al., 2003, 2005). Oligomerization is regulated by cdc2- and polo-like kinase (plk)–mediated phosphorylation (Tang et al., 2008; Wang, 2008). Collectively, these results provided strong evidence that GRASP65 is essential for Golgi structure formation. However, as GRASP65 is localized only at the cis Golgi (Shorter et al., 1999), the molecular machinery that links medial-trans cisternae into stacks remains unknown.

GRASP55, a homologue of GRASP65 primarily localized to the medial-trans cisternae, was identified by database searching (Shorter et al., 1999). As was the case for GRASP65, recombinant GRASP55 and anti-GRASP55 antibodies blocked the stacking of Golgi cisternae in vitro. GRASP55 exhibits a high level of sequence identity with GRASP65. Its N-terminal GRASP domain is 80% similar and 66% identical to that of GRASP65 in rat. Its C-terminal serine/proline-rich (SPR) domain, like that of GRASP65, also contains a number of potential

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Abbreviations used in this paper: CIP, calf intestine alkaline phosphatase; GRASP, Golgi reassembly stacking protein; IC, interphase cytosol; ManII, mannosidase II; MC, mitotic cytosol; MEK1, mitogen-activated protein kinase kinase 1; plk, polo-like kinase; SPR, serine/proline rich.

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phosphorylation sites. The sequence similarity and distinct localizations suggest that two GRASP proteins may contribute to the stacking of different regions of the Golgi (Pfeffer, 2001). However, whether GRASP55 plays a direct role in stacking in vivo and what the underlying mechanism is remain unclear. In this study, we provide evidence that GRASP55 is directly involved in Golgi stacking in a manner similar to GRASP65, in particular that mitotically regulated trans-oligomerization of GRASP55 plays a role in medial-trans Golgi stack formation. Our results define the mechanism by which GRASP55 functions as a Golgi stacking factor and provide evidence that GRASP65 and GRASP55 function collaboratively to stack Golgi cisternal membranes.

Results

Knockdown of GRASP55 reduces the number of cisternae per stack

To determine the function of GRASP55 in vivo, we depleted GRASP55 in HeLa cells using an siRNA strategy published previously (Feinstein and Linstedt, 2008). Both Western blotting and immunofluorescence microscopy showed that the knockdown efficiency was time dependent. It was most efficient (>98% reduction based on Western blotting) 96 h after transfection (Fig. S1, A–E). GRASP55 depletion had no effect on the expression level of other Golgi proteins, GRASP65, GM130, and Gos28. Using GM130 (Fig. 1, A and B) and mannosidase II (ManII, not depicted) as Golgi markers, no obvious change in Golgi morphology or localization was detected in a majority of the cells. As light microscopy lacks the necessary resolution to determine Golgi stacking, we analyzed the cells by electron microscopy (EM). Cells transfected with GRASP55 siRNA exhibited a reduced number of cisternae in individual stacks (Fig. 1, F vs. E). The average number of cisternae per stack dropped from 5.7 ± 0.2 in cells transfected with control siRNA to 3.7 ± 0.1 in GRASP55-depleted cells (Fig. 1 J). Most of the stacks in GRASP55-depleted cells contained 3–4 cisternae, a significant reduction from 5–6 in cells treated with control siRNA (Fig. 1 K). Collectively, these results suggest that GRASP55 is involved in Golgi stacking in vivo.

The effect of GRASP55 knockdown was rescued by expression of exogenous GRASP55

To ensure that the observed effects of GRASP55 siRNA on Golgi stacking were directly caused by GRASP55 depletion, we expressed rat GRASP55 in HeLa cells in which endogenous GRASP55 was depleted. We generated HeLa cell lines that stably expressed GFP-tagged rat GRASP55 using an inducible retroviral expression system (Heusser et al., 2006). This allowed us to control the expression time and the level of the exogenous protein and to avoid possible effects of continuous overexpression of this protein (or its mutants) on cell growth (Duran et al., 2008). In addition, this approach ensured that all cells expressed rat GRASP55, thus permitting EM analysis. These cells were transfected with control or GRASP55 siRNA, and 48 h later, doxycycline was added to induce expression of rat GRASP55. In the presence of control siRNA, cells expressing rat GRASP55 showed little change in the expression of other Golgi markers, such as GM130 and Gos28, although the levels of endogenous GRASP55 and GRASP65 were reduced. The total GRASP55 in the cell line was about twofold that of endogenous GRASP55 in the GFP cell line (Fig. 1 I). After transfection with siRNA targeted to human but not rat GRASP55, the endogenous GRASP55 was efficiently knocked down (>98% depletion), whereas both rat GRASP55-GFP and GFP were well expressed (Fig. 1 I). When examined under a confocal microscope, GRASP55-GFP colocalized with GM130, whereas GFP was cytosolic. Overall Golgi morphology was not significantly affected in cell lines expressing either GFP or rat GRASP55, with or without endogenous GRASP55 (Fig. 1, C and D). When examined by EM, depletion of GRASP55 in the GFP cell line significantly reduced stacking (Fig. 1 G). Expression of GRASP55-GFP restored the average number of cisternae per stack to 5.6 ± 0.2 (Fig. 1, H and L; Fig. S2, A–D). These results confirmed that the reduced Golgi stacking was caused by GRASP55 depletion.

Effects of GRASP55 depletion on Golgi ribbon formation

Recently it was shown that GRASP55 depletion led to fragmentation of the Golgi ribbon (Feinstein and Linstedt, 2008), although this effect was not observed in a similar study by a different group (Duran et al., 2008). To address this discrepancy, we carefully analyzed Golgi morphology in GRASP55-depleted cells by fluorescence microscopy. We divided the cells into three groups according to Golgi morphology (Fig. 2, A–C): (1) intact, with all the membranes linked in a compact structure; (2) mildly fragmented, with mini Golgi elements less connected, but still clustered and adjacent to the nucleus; and (3) scattered, with Golgi elements dispersed in the cytoplasm, as previously observed (Feinstein and Linstedt, 2008). Using GM130, GRASP65, and Gos28 as Golgi markers, most cells treated with control siRNA (96–97%) had intact Golgi, with a small number of cells containing either mildly fragmented (∼1%) or scattered (∼1%) Golgi (Fig. 2 D). Depletion of GRASP55 led to mild fragmentation of the Golgi in 22–24% of the cells (Fig. 2 D). However, a majority (∼75%) of the cells had intact Golgi, and the number of cells with scattered Golgi (1–2%) did not increase, indicating a mild effect of GRASP55 depletion on Golgi ribbon formation. To determine more precisely whether GRASP55 depletion affects the continuity of Golgi membranes, we performed FRAP analysis (Duran et al., 2008; Feinstein and Linstedt, 2008). HeLa cells were transfected with ManII-GFP 72 h after the initial siRNA transfection and analyzed 24 h later. A region of the Golgi was bleached with an argon laser at maximal intensity, and the recovery of fluorescence in this area was recorded for 480 s by time-lapse imaging. As shown in Fig. 2, E–G, cells treated with GRASP55 siRNA exhibited a reduced recovery rate compared with cells treated with control siRNA. It is necessary to note that significant variation existed among cells with different Golgi morphologies (Fig. 2, A–C). Nevertheless, this experiment demonstrated that GRASP55 depletion affected the continuity of the

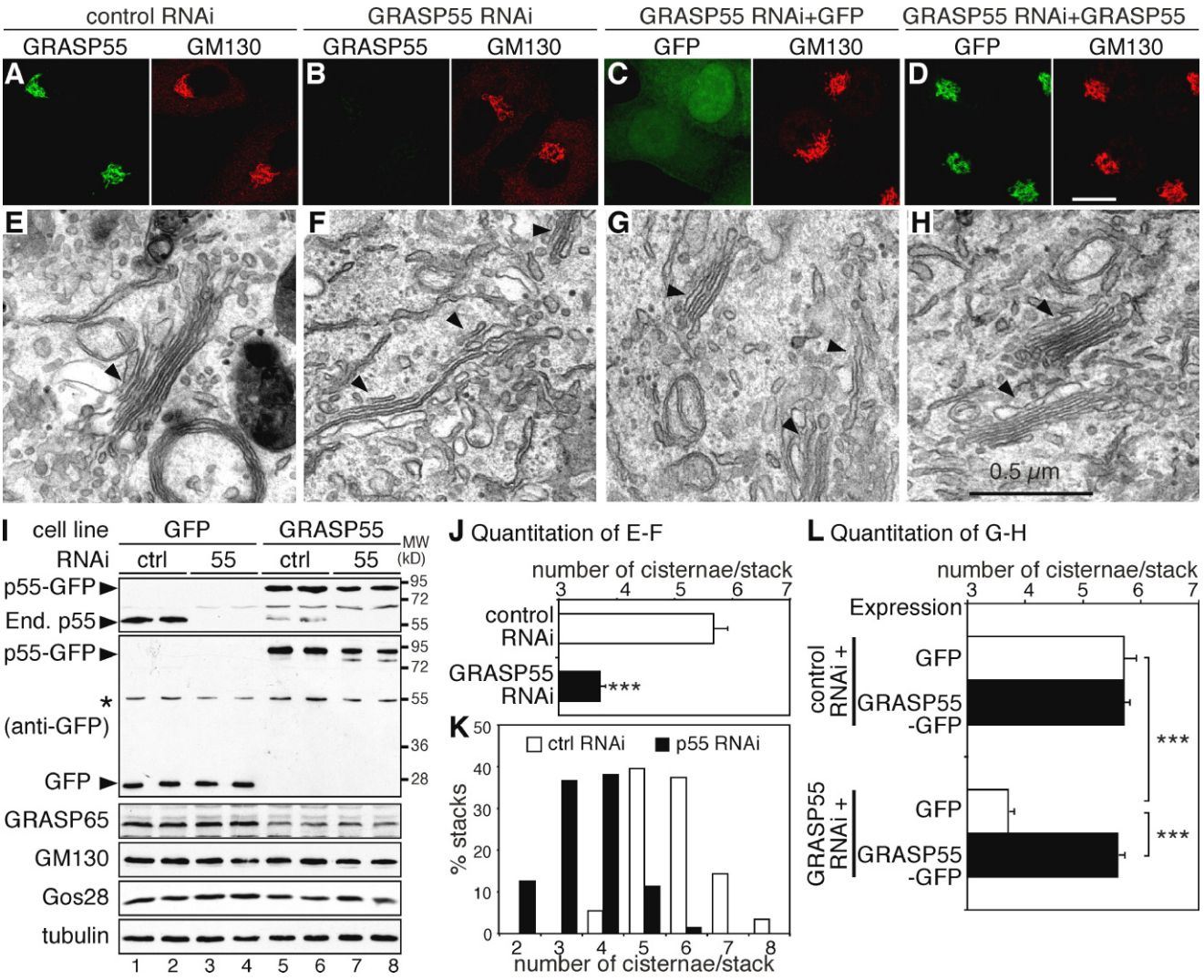


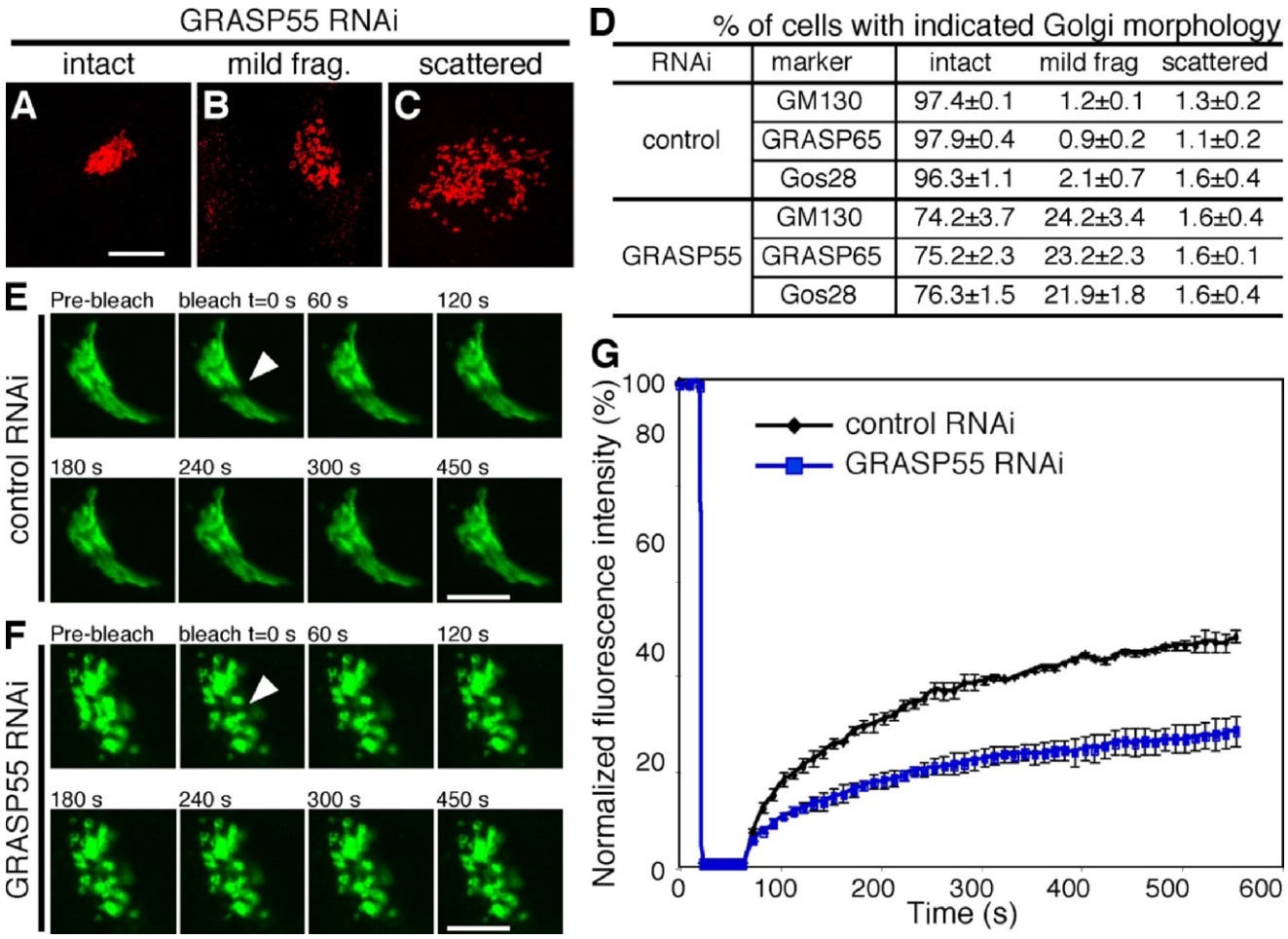
Figure 1. **Knockdown of GRASP55 reduces the number of cisternae per stack.** (A and B) Confocal fluorescence images of GRASP55 knockdown cells. HeLa cells transfected with indicated siRNA were fixed after 96 h and immunostained for GRASP55 and GM130. (C and D) Fluorescence images of HeLa cells in which endogenous GRASP55 was replaced by exogenous GRASP55. HeLa cells expressing GFP or rat GRASP55-GFP using an inducible retroviral expression system were transfected with control siRNA or siRNA specific for human GRASP55. Doxycycline was added 48 h after transfection. Cells were fixed and stained for GM130. (E–H) Representative EM micrographs of cells described in A–D. Arrowheads indicate Golgi stacks. Note that the number of cisternae in the stacks was reduced in GRASP55 knockdown cells (F), which was restored by the expression of rat GRASP55 (H), but not by GFP (G). (I) Immunoblots of cells described in C and D. Duplicate samples were loaded; asterisk indicates a nonspecific band. "End. p55", endogenous GRASP55. (J) Quantitation of the EM images in E and F from three sets of independent experiments. Results are expressed as the mean ± SEM. (K) The numbers of cisternae per stack from one representative experiment were presented in a histogram format. Note that most stacks contained 5–6 cisternae in control RNAi cells, which was reduced to 3–4 in GRASP55 RNAi cells. (L) Quantitation of G–H. Note that GRASP55 knockdown significantly reduced the number of cisternae per stack, whereas expression of rat GRASP55 restored it. ***, $P < 0.001$.

Golgi membranes, indicating a role for GRASP55 in Golgi ribbon linking, which may be directly or indirectly caused by Golgi unstacking.

Knockdown of both GRASP55 and GRASP65 leads to disassembly of the entire Golgi stack

Because GRASP55 and GRASP65 localize to different compartments of the Golgi, we suspected that the two homologues might stack different parts of the Golgi, and therefore, knockdown of both proteins might lead to complete unstacking of the Golgi. To test this, we transfected HeLa cells with siRNAs for both GRASP55 and GRASP65. Both fluorescence microscopy (Fig. 3, A and B) and Western blotting (Fig. 3, C and F) showed that depletion of both proteins was efficient (~95%). We also noticed that the level of GM130 was reduced when both GRASPs were depleted, whereas Gos28 was unaffected (Fig. 3 C). Using Gos28 (Fig. 3, A and B) and GM130 (see Fig. S4 K) as Golgi markers, simultaneous depletion of both GRASP proteins had only minor effects on organization and localization of the Golgi ribbon at the level of fluorescence microscopy, much as was seen with depletion of GRASP55 alone.

Strikingly, however, EM analysis showed that simultaneous depletion of GRASP55 and GRASP65 led to complete disassembly of the Golgi stacks (Fig. 3, D and E), an effect much



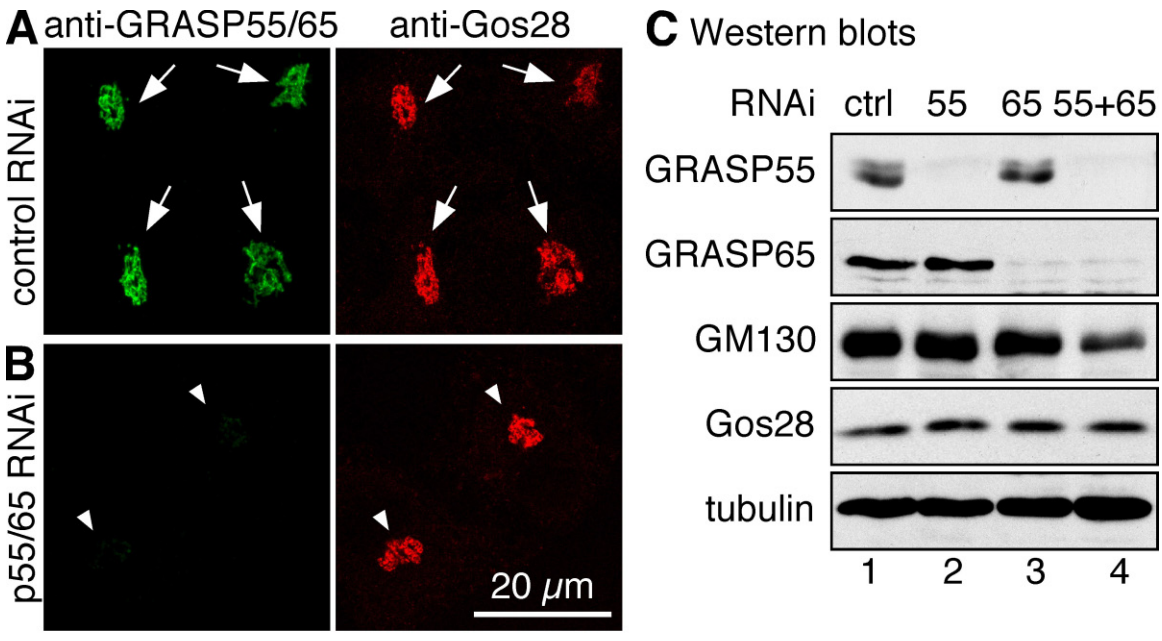
RNAi	marker	intact	mild frag	scattered
control	GM130	97.4±0.1	1.2±0.1	1.3±0.2
	GRASP65	97.9±0.4	0.9±0.2	1.1±0.2
	Gos28	96.3±1.1	2.1±0.7	1.6±0.4
GRASP55	GM130	74.2±3.7	24.2±3.4	1.6±0.4
	GRASP65	75.2±2.3	23.2±2.3	1.6±0.1
	Gos28	76.3±1.5	21.9±1.8	1.6±0.4

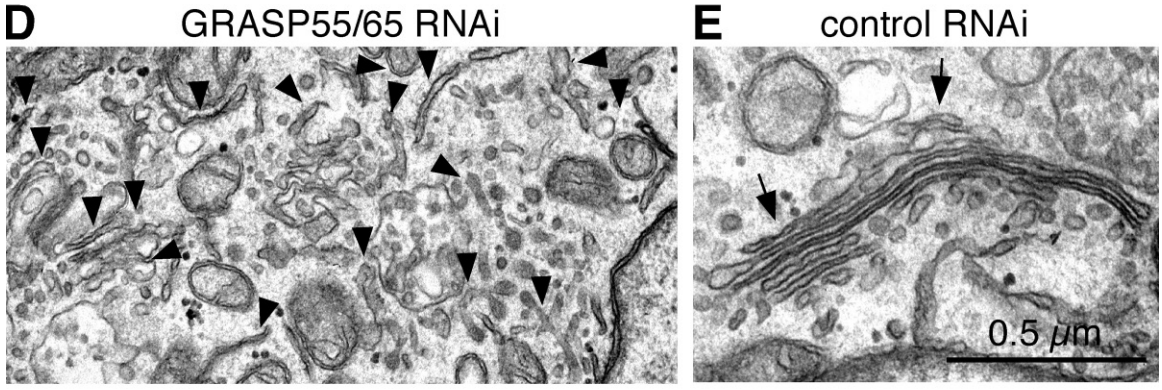
Figure 2. **Effect of GRASP55 depletion on Golgi ribbon linking.** (A–C) Enlarged confocal fluorescence images of the Golgi with classified morphology in GRASP55 knockdown cells stained for GM130. Bar, 10 µm. mild frag., mildly fragmented. (D) Quantitation (mean ± SEM) of classified Golgi morphology in HeLa cells treated with control or GRASP55 siRNA from three independent experiments. GM130, GRASP65, and Gos28 were used as Golgi markers and 300 cells were counted. (E and F) FRAP analysis of ManII-GFP–expressing HeLa cells transfected with indicated siRNAs. The indicated regions (arrows) were photobleached with laser pulses and fluorescence recovery was recorded. Representative images at indicated times are shown. Bar, 5 µm. (G) Quantitation of FRAP results. Fluorescence recovery was represented by the ratio of GFP fluorescence intensity in the bleached area to that of the entire Golgi. Normalization was set between the values before bleaching and the first time point after bleaching. Results represent mean ± SEM from two independent experiments, with more than 17 cells quantified in each case.

more dramatic than depletion of GRASP55 or GRASP65 alone (Fig. 3 F). The Golgi membranes were transformed into single cisternae (Fig. 3 D, arrowheads) and tubulovesicular or dilated structures. There were no detectable normal Golgi stacks in most (>80%) cells that were examined. The effects of GRASP55/65 double depletion could be partially rescued by expression of GRASP55 (or its mutants) or GRASP65, as described later (see Fig. 8). These data provide strong evidence that GRASP55 and GRASP65 play complementary and essential roles in Golgi cisternal stacking.

GRASP55 is phosphorylated by MAP kinase during mitosis

We next addressed the molecular mechanisms of GRASP55 function in Golgi stacking. We hypothesized that, as in the case of GRASP65, cell cycle–regulated GRASP55 phosphorylation may be essential for Golgi disassembly during mitosis and reassembly in telophase and cytokinesis. Phosphorylation of GRASP55 in the cell cycle was first confirmed by γ-[^{32}P]ATP labeling (Fig. S3 A). We then developed a band-shift assay to analyze GRASP55 phosphorylation. When Golgi membranes were treated with mitotic cytosol (MC), GRASP55 exhibited a sharp decrease in mobility on SDS-PAGE. This shift was caused by phosphorylation, because treatment of mitotic Golgi fragments (MGFs) with calf intestine alkaline phosphatase (CIP), a nonspecific protein phosphatase, largely restored the original SDS-PAGE mobility. The mobility shift was abolished by adding β-glycerophosphate (β-GP), a general phosphatase inhibitor (Fig. 4 B). Unlike GRASP65 (Lin et al., 2000; Wang et al., 2003), GRASP55 was not phosphorylated by plk and was only slightly phosphorylated by cdc2 (Fig. 4 C). Treatment of Golgi membranes with a combination of mitogen-activated protein kinase ERK2 and constitutively active MEK1 (mitogen-activated protein kinase kinase 1) shifted GRASP55 to a similar extent as MC, consistent with previous reports that GRASP55 is phosphorylated by ERK2 (Jesch et al., 2001). Phosphorylation of GRASP55 by ERK2 was confirmed using both recombinant GRASP55 and endogenous GRASP55 in cell





F Quantitation of knockdown efficiency and effects on Golgi stacking

RNAi	control	GRASP55	GRASP65	GRASP55 GRASP65
GRASP55 KD efficiency	—	98.4 ± 0.3%	—	95.5 ± 0.8%
GRASP65 KD efficiency	—	—	96.2 ± 2.9%	94.8 ± 4.1%
cisternal number/stack	5.7 ± 0.2	3.7 ± 0.1 *p<0.005*	3.8 ± 0.1 *p<0.005*	*

Figure 3. **Depletion of both GRASP55 and GRASP65 leads to disassembly of the entire Golgi stack.** (A and B) Confocal fluorescence images of GRASP55 and GRASP65 double-knockdown cells. HeLa cells were transfected with a mixture of GRASP55 and GRASP65 siRNA (B) or with control siRNA (A). Cells were fixed and immunostained for both GRASP55 and GRASP65 (anti-GRASP55/65), and for Gos28. (C) Immunoblots of HeLa cells transfected with indicated siRNA. (D and E) Representative EM micrographs of cells transfected with indicated siRNAs. Arrowheads in D indicate single cisternae and Golgi fragments. Arrows in E indicate normal Golgi stacks. (F) Quantitation (mean ± SEM) of GRASP55 and GRASP65 knockdown efficiency and effects on Golgi stacking from three independent experiments. Statistical significance was assessed by comparison with the control siRNA cells. *, double knockdown cells were not quantified due to the complete disassembly of the Golgi stacks.

lysates (Fig. S3, B–D). In addition, GRASP55 phosphorylation was inhibited by the MEK1 inhibitor U0126 and was reversed when MGFs were treated with interphase cytosol (Fig. S3, C–E). These data suggest that GRASP55 is subjected to phosphorylation and dephosphorylation during the cell cycle.

The phosphorylation sites on GRASP55 are located in the C-terminal SPR domain

GRASP55 contains an N-terminal GRASP domain (aa 1–212) and a C-terminal SPR domain (aa 213–454; Fig. 4 A). To identify the phosphorylation sites, recombinant GRASP55 fragments were treated with mitotic cytosol (MC) or interphase cytosol (IC)

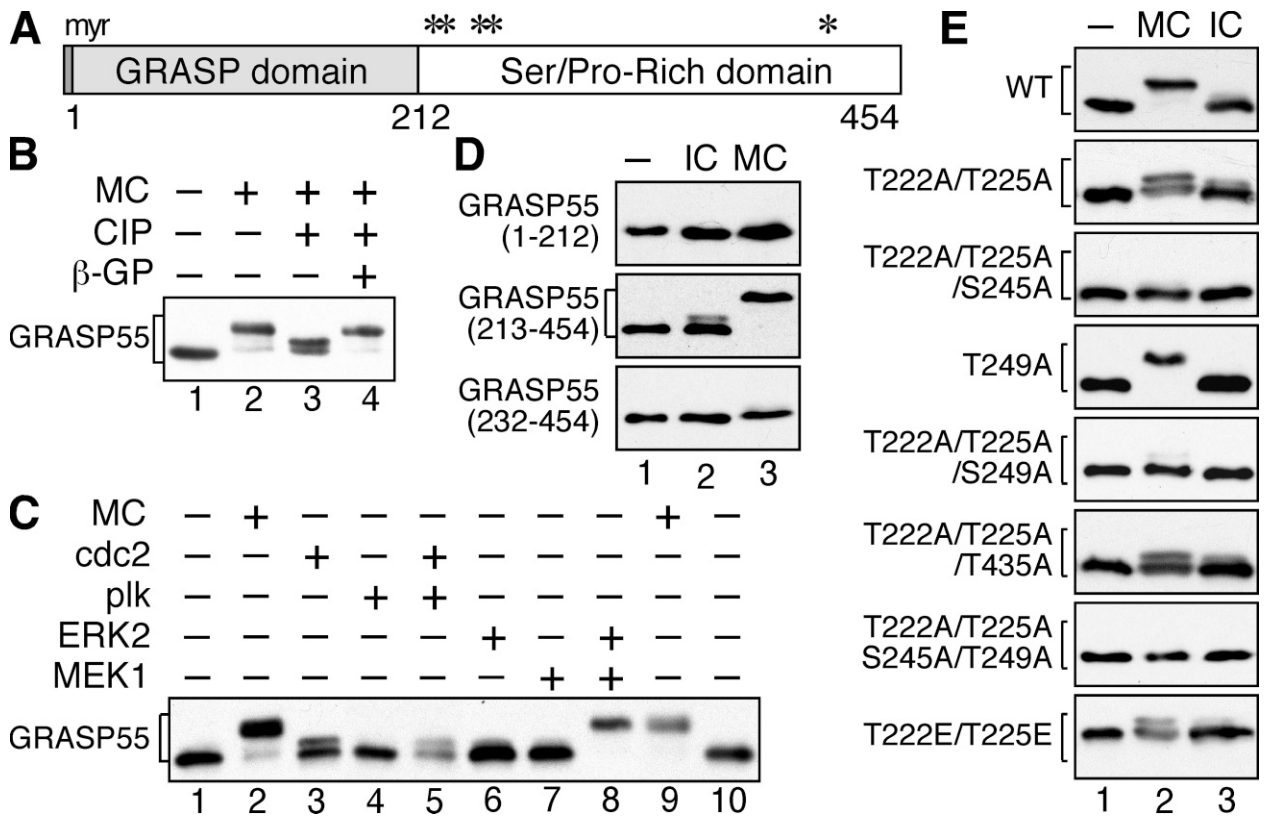


Figure 4. **GRASP55 is phosphorylated during mitosis.** (A) GRASP55 schematic with phosphorylation sites indicated. Predicted phosphorylation sites (T222, T225, S245, T249, and T435) are indicated by asterisks. myr: myristoylated N-terminal glycine. (B) Detection of GRASP55 phosphorylation using a band-shift assay. Golgi membranes were either treated with buffer (lane 1) or mitotic cytosol (MC, lanes 2–4). Membranes were reisolated and further treated with calf intestine alkaline phosphatase (CIP) in the absence (lane 3) or presence (lane 4) of a general phosphatase inhibitor, β-glycerophosphate (β-GP). Note the increase in molecular weight of GRASP55 under mitotic conditions was reversed by CIP. (C) GRASP55 phosphorylation by ERK2. Golgi membranes were incubated with indicated proteins followed by Western blotting. Note that ERK2/MEK1 phosphorylated GRASP55 to a similar extent as MC. (D) The mitotic phosphorylation sites of GRASP55 are in the SPR domain. GST-tagged GRASP55 constructs were incubated with buffer (lane 1), or interphase (IC, lane 2) or mitotic (MC, lane 3) cytosol and analyzed by immunoblotting. Note that the SPR domain (213–454), but not the GRASP domain (1–212), exhibited a band shift after MC treatment, whereas deletion of aa 213–231 largely abolished the phosphorylation. (E) Mapping the phosphorylation sites of GRASP55. Potential sites (asterisks in A) in His-GRASP55 were mutated to alanines or glutamates and the purified proteins were analyzed as in D.

and analyzed by the band-shift assay. As shown in Fig. 4 D, no mobility shift was observed for the GRASP domain when treated with MC, whereas the SPR domain exhibited a sharp band shift. In addition, deletion of a portion of the SPR domain, aa 213–231, significantly reduced the molecular weight shift (Fig. 4 D), indicating that this segment contains a majority of the phosphorylation sites, or that it may function as a prerequisite for phosphorylation of other sites.

To map more precisely the phosphorylation sites in the SPR domain, potential phosphorylation sites T222, T225, S245, T249, and T435, some of which were previously identified (Jesch et al., 2001; Duran et al., 2008), were mutated to alanines, either individually or in combination. As shown in Fig. 4 E, phosphorylation of GRASP55 by mitotic cytosol was largely abolished when T222 and T225 were mutated, and further mutation of S245 or T249 almost completely abolished the phosphorylation. Mutation of T435 had no detectable effect on GRASP55 phosphorylation. These data suggest that S245, T249, and particularly T222 and T225, are the key phosphorylation sites on GRASP55 during mitosis. As shown below, a T222A–T225A–S245A mutant was constructed to probe GRASP55 function in vitro and in vivo. In addition, T222 and T225 were also mutated to glutamates to mimic the phosphorylated state of the protein (Fig. 4 E).

GRASP55 forms higher order oligomers in a cell cycle–dependent manner

In analogy to GRASP65, we hypothesized that the stacking activity of GRASP55 might involve dimerization and oligomerization (Wang et al., 2003, 2005). To test whether GRASP55 interacts with itself, MBP-GRASP55 and His-GRASP55 recombinants were separately purified, mixed, and incubated with either buffer or mitotic cytosol. Protein complexes were isolated using nickel beads for His-GRASP55 or amylose beads for MBP-GRASP55, and analyzed by SDS-PAGE and immunoblotting for GRASP55. As shown in Fig. 5 A, the two tagged forms copurified under control conditions, but this was abolished by treatment with mitotic cytosol, regardless of the beads that were used, indicating that GRASP55 interacts with itself and that this interaction is abolished by mitotic cytosol.

Oligomerization of GRASP55 was also analyzed by electrophoresis on nondenaturing gels. As shown in Fig. 5 B, His-GRASP55 exhibited four bands, with apparent molecular

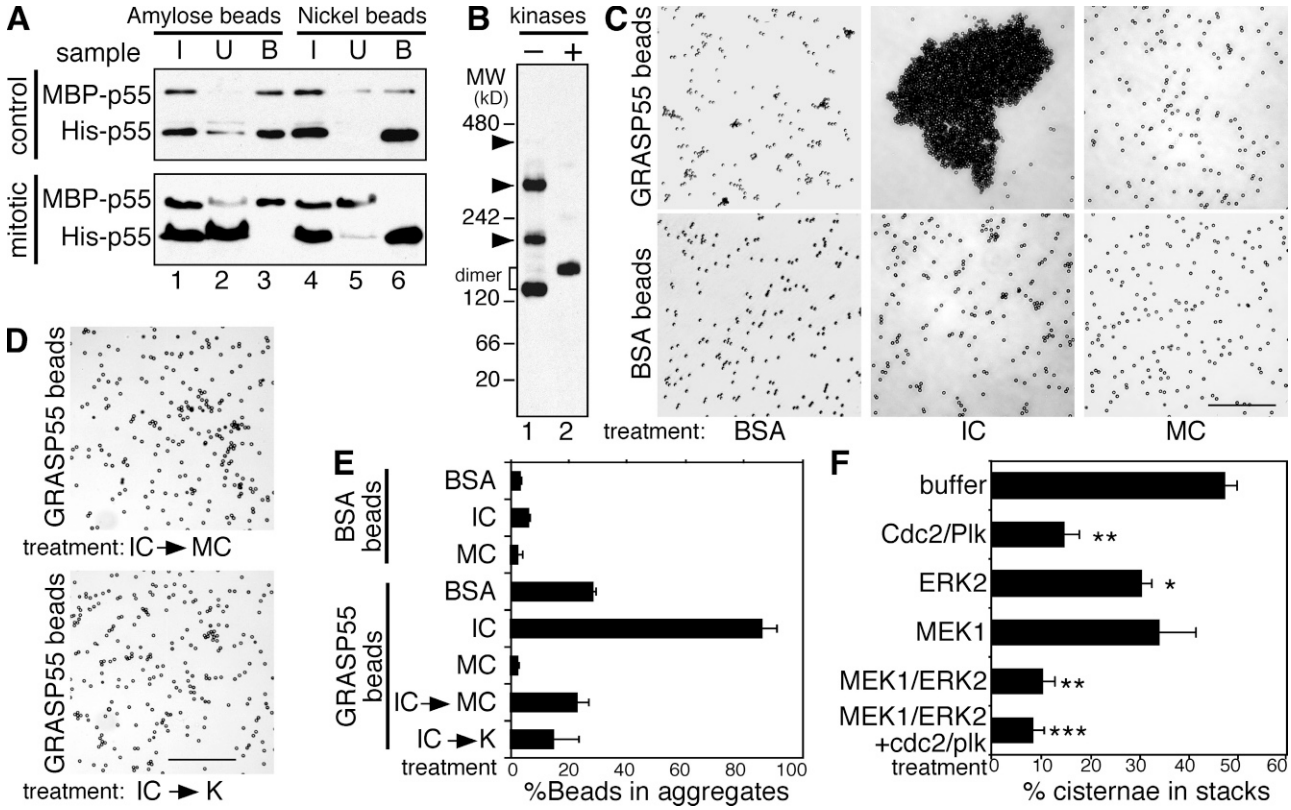


Figure 5. **Cell cycle–regulated GRASP55 oligomerization is sufficient to link adjacent surfaces.** (A) Co-purification of MBP-GRASP55 and His-GRASP55. Differently tagged proteins were separately purified, mixed, and incubated in the presence of buffer (control) or mitotic cytosol (mitotic). The protein complex was isolated using either amylose (lanes 1–3) or nickel beads (lanes 4–6). Equal proportions of input (I), unbound (U), or bound (B) fractions were analyzed by immunoblotting for GRASP55. Note the copurification of the two proteins under control but not mitotic conditions. (B) Analysis of GRASP55 oligomerization by nondenaturing gels. His-GRASP55 was incubated in the absence (−, lane 1) or presence (+, lane 2) of ERK2/MEK1 kinases followed by nondenaturing electrophoresis and Western blotting. Molecular weight standards are indicated on the left. Note that the higher molecular weight bands in lane 1 (arrowheads) diminished after kinase treatment. (C) Aggregation of GRASP55-coated beads. Purified His-GRASP55 or BSA was covalently coupled to the surface of magnetic Dynal beads and incubated with BSA, interphase cytosol (IC), or mitotic cytosol (MC). After incubation the beads were placed on glass slides and random fields were photographed. A representative image of each condition is shown. Note that GRASP55-coated beads aggregated slightly in the presence of BSA; this was enhanced by IC, but inhibited by MC. (D) As in C, except that GRASP55 beads were first aggregated using IC and then treated with either MC (IC→MC) or with purified ERK2/MEK1 kinases (IC→K). Note that aggregates were reversibly disassembled by MC and kinases. Bar, 100 µm. (E) Quantitation (mean ± SEM) of C and D; $n = 3$. (F) Treatment of Golgi membranes with purified kinases leads to cisternal membrane unstacking. Purified Golgi stacks were treated with either buffer or indicated kinases, and analyzed by EM. Shown are the quantitation results from a representative experiment. Statistical significance was assessed by comparison of kinase treatment with buffer treatment. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

weights of 130, 194, 288, and 418 kD (lane 1, arrowheads). These bands were more visible after longer exposure (Fig. S3 F). Because the apparent molecular weight of the most rapidly migrating band was twice that of GRASP55 on denaturing gels (64 kD), this result suggested that GRASP55 formed both homodimers and higher oligomers. After kinase treatment, His-GRASP55 exhibited only one major band (139 kD), most likely representing the phosphorylated dimer. All of these bands were shown by analysis on second dimensional denaturing gels to correspond to GRASP55 rather than contaminants (unpublished data). Similar experiments showed that the endogenous GRASP55 in Golgi membranes formed oligomers under interphase conditions, but remained as dimers when the Golgi membranes were treated with ERK2/MEK1 or mitotic cytosol (Fig. S3). Further analysis by gel filtration and protein cross-linking also confirmed this conclusion (unpublished data). Collectively, these results demonstrate conclusively that GRASP55 forms phosphorylation-regulated oligomers.

Trans-oligomerization of GRASP55 is sufficient to link surfaces together

We then used an established bead assay (Wang et al., 2003) to test whether GRASP55 oligomers could link adjacent Golgi cisternae. Purified GRASP55 or bovine serum albumin (BSA) was covalently linked to the surface of magnetic Dynal beads. After treatment with BSA solution (control), IC, or MC, beads were placed on glass slides and observed under bright-field illumination. As shown in Fig. 5 C, GRASP55-coated beads aggregated after BSA treatment (27.7 ± 1.5%). Aggregation was enhanced upon IC treatment (85.7 ± 5.5%), but inhibited by MC (2.4 ± 1.4%). In contrast, BSA-coated beads did not aggregate regardless of the treatment (Fig. 5, C and E). To test whether the aggregation was regulated by phosphorylation, we sequentially treated GRASP55-coated beads with IC followed by MC (IC→MC) or purified ERK2/MEK1 kinases (IC→K). Both treatments led to disaggregation of the beads (22.6 ± 8.3% and 14.1 ± 12.9%, respectively, Fig. 5, D and E).

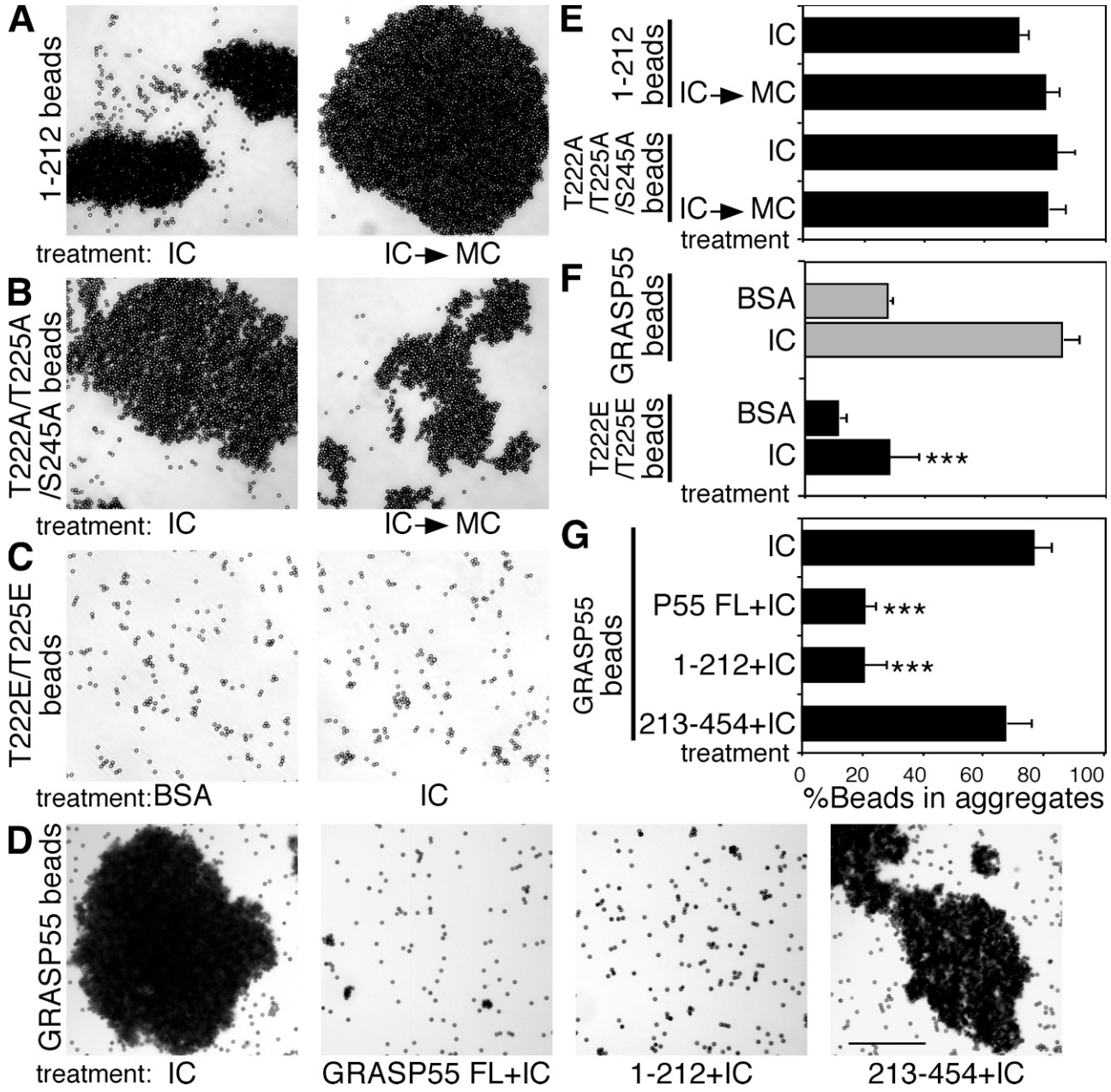


Figure 6. **Mapping the domain structure of GRASP55 for oligomerization and mitotic regulation.** (A) Purified His-tagged GRASP domain (1–212) was coupled to Dynal beads and sequentially incubated with interphase cytosol (IC) and mitotic cytosol (IC→MC). Note that aggregates of beads formed by IC treatment were not dispersed by MC. (B) As in A, using the T222A–T225A–S245A mutant. Beads aggregated under both conditions. (C) Beads coated with the T222E–T225E mutant were incubated with BSA or IC. Note that beads did not aggregate after IC treatment. (D) Wild-type GRASP55-coupled beads were incubated with IC in the presence of soluble full-length (FL) GRASP55, the GRASP domain (1–212), or the SPR domain (213–454) recombinants. Bar, 100 µm. Note that the aggregation was suppressed by the soluble full-length protein and the GRASP domain, but not by the SPR domain. (E) Quantitation of A and B from three independent experiments (mean ± SEM). (F) Quantitation of C. Aggregation of the beads coupled with the T222E–T225E mutant was significantly reduced compared with those coated with wild-type GRASP55 when treated with IC. (G) Quantitation of D. **, $P \leq 0.01$; ***, $P \leq 0.001$.

To determine the effect of GRASP55 phosphorylation on stacking of Golgi membranes, we treated purified Golgi stacks with these kinases (Fig. 5 F) and quantified the EM images. 47.4 ± 2.6% of the cisternae were found in stacks when Golgi membranes were treated with buffer alone. Treatment with either ERK2 or MEK1 slightly reduced the percentage of cisternae in stacks (30.6 ± 2.2% for ERK2; 33.1 ± 7.5% for MEK1). This reduction may result from the weak kinase activity of wild-type ERK2, or the activation of membrane-bound ERK kinase by the addition of constitutively active MEK1 (Colanzi et al., 2000; Cha and Shapiro, 2001). When Golgi membranes were incubated with ERK2 and MEK1 together, the percentage of cisternae in stacks dropped to 10.6 ± 2.5%, a level similar to cdc2/plk treatment (14.8 ± 3.0%) that resulted in GRASP65 phosphorylation (Wang et al., 2003). When the four kinases were all included in the reaction, the percentage of cisternae in stacks was reduced to 8.6 ± 2.3% (Fig. 5 F). It is necessary to note that the effects of ERK2/MEK1 treatment may also be partially due to GRASP65

phosphorylation, as ERK2 also phosphorylates GRASP65, at least on S277 (Yoshimura et al., 2005; Bisel et al., 2008). Nevertheless, these results show that phosphorylation of Golgi proteins such as GRASP55 by ERK2/MEK1 leads to Golgi membrane unstacking.

Mapping the domain structure of GRASP55 for oligomerization and mitotic regulation

To map the domain of GRASP55 required for oligomerization, purified GRASP domain (aa 1–212, His tagged) was coupled to the surface of Dynal magnetic beads and sequentially treated with IC and MC. These beads aggregated extensively upon IC treatment (70.6 ± 3.2%), but the aggregates were not dispersed by MC (79.4 ± 4.7%; Fig. 6, A and E). Further experiments using gel filtration confirmed that the GRASP domain alone could form oligomers (unpublished data). In addition, when a soluble form of the GRASP domain or full-length GRASP55 (FL-GRASP55) was added into the reaction, it inhibited aggregation of beads coated with full-length GRASP55. The percentage of aggregated beads dropped from 76.3 ± 6.1% to 20.6 ± 3.6% with FL-GRASP55 and to 20.5 ± 7.6% with the GRASP domain. In contrast, the SPR domain (aa 213–454) had no significant effect (67.5 ± 8.0%; Fig. 6, D and G). These results demonstrate that the GRASP domain alone is sufficient for oligomerization and that its oligomerization is not mitotically regulated.

We then determined the phosphorylation sites that regulate GRASP55 oligomerization. Beads coated with the nonphosphorylatable T222A–T225A–S245A mutant formed aggregates upon IC treatment (83.5 ± 6.0%), and the aggregates largely remained after further treatment with MC (80.3 ± 5.7%; Fig. 6, B and E). In contrast, beads coated with the T222E–T225E mutant aggregated slightly when treated with IC (28 ± 10.2%), a level significantly reduced compared with beads coated with wild-type GRASP55 (85.7 ± 5.5%; Fig. 6, C and F). Collectively, these data demonstrate that the GRASP domain of GRASP55 is both necessary and sufficient for oligomerization, whereas the SPR domain plays a role in mitotic regulation.

Expression of nonphosphorylatable GRASP55 mutants enhances Golgi stack formation in interphase cells

To test the function of GRASP55 in vivo, we generated HeLa cell lines that stably expressed various rat GRASP55 constructs using an inducible promoter (Heusser et al., 2006). Western blot analysis showed that each of the constructs was expressed at relatively equivalent levels upon doxycycline induction (Fig. 7 H). Fluorescence microscopy showed that the GFP-tagged wild-type GRASP55, the GRASP domain, and the T222A–T225A–S245A and the T222E–T225E mutants localized to the Golgi in interphase cells, as shown by costaining for GRASP65 (Fig. S4, A–F) and GM130 (not depicted). The SPR domain lacked a Golgi localization signal and was largely cytoplasmic. Expression of GRASP55 and its mutants did not significantly affect the overall organization of the Golgi as judged by fluorescence microscopy.

When examined by EM, however, alignment of the cisternal membranes was improved in cells expressing the GRASP domain or the nonphosphorylatable T222A–T225A–S245A mutant compared with cells expressing GFP alone (Fig. 7, C and D vs. A). The number of cisternae per stack increased from 5.7 ± 0.2 in cells expressing GFP to 6.9 ± 0.1 and 6.7 ± 0.1 in cells expressing the GRASP domain or the T222A–T225A–S245A mutant, respectively (Fig. 7 G). Expression of wild-type GRASP55 (5.7 ± 0.1), the T222E–T2225E mutant (5.5 ± 0.1), or the SPR domain (5.5 ± 0.1) did not significantly affect the number of cisternae per stack.

Rescue of the Golgi structure in GRASP55- and GRASP65-depleted cells by expression of wild-type or mutant GRASP55 and GRASP65

Further experiments showed that Golgi structure in GRASP55-depleted cells could be rescued by expressing GRASP55 or its mutants (Fig. S5; Fig. S2, C–F). Expression of the GRASP domain or the T222A–T225A–S245A mutant increased the average number of cisternae per stack to 6.0 ± 0.1 and 6.1 ± 0.1, respectively, slightly higher than expression of wild-type GRASP55 (5.6 ± 0.1).

To understand whether GRASP55 and GRASP65 have redundant functions in Golgi stacking, we tested whether expression of a single GRASP protein (or its mutant) could rescue Golgi structure in cells with both GRASPs depleted. By fluorescence microscopy, all the exogenous GRASP constructs were localized at the Golgi in the absence of endogenous proteins (Fig. S4, K–O). When examined by EM, Golgi stacks were observed in most cells expressing exogenous GRASP proteins, but not in cells expressing GFP. The number of cisternae per stack was 4.7 ± 0.2 and 4.5 ± 0.2 in cells expressing wild-type GRASP55 and GRASP65, respectively (Fig. 8; Fig. S2, G–J), suggesting that expression of a single GRASP protein can only partially rescue the phenotype caused by the depletion of both endogenous GRASP proteins. Expression of the GRASP domain (5.2 ± 0.2) and the T222A–T225A–S245A mutant (4.9 ± 0.2) of GRASP55 rescued Golgi stacking to a greater extent than expression of the wild-type protein, but rescue was still incomplete. These results indicate that the GRASP proteins have complementary functions in Golgi stacking in mammalian cells.

Expression of nonphosphorylatable GRASP55 mutants inhibits mitotic Golgi fragmentation

Because the nonphosphorylatable GRASP55 mutants form oligomers that are resistant to mitotic regulation, these mutants were expected to inhibit Golgi disassembly at the onset of mitosis. To test this, Golgi structure in mitotic cells was analyzed. By confocal microscopy, exogenous GRASP55 colocalized with GRASP65 (Fig. 9, B–E) and other Golgi markers, such as GM130 (not depicted), on the mitotic clusters, whereas GFP alone (Fig. 9 A) was evenly distributed throughout cells. Interestingly, mitotic cells expressing the GRASP domain or the T222A–T225A–S245A mutant increased in number and size of mitotic clusters compared with those expressing wild-type GRASP55, the SPR domain, or GFP alone (Fig. 9, A–D). Notably, the Golgi in cells expressing the T222E–T225E mutant

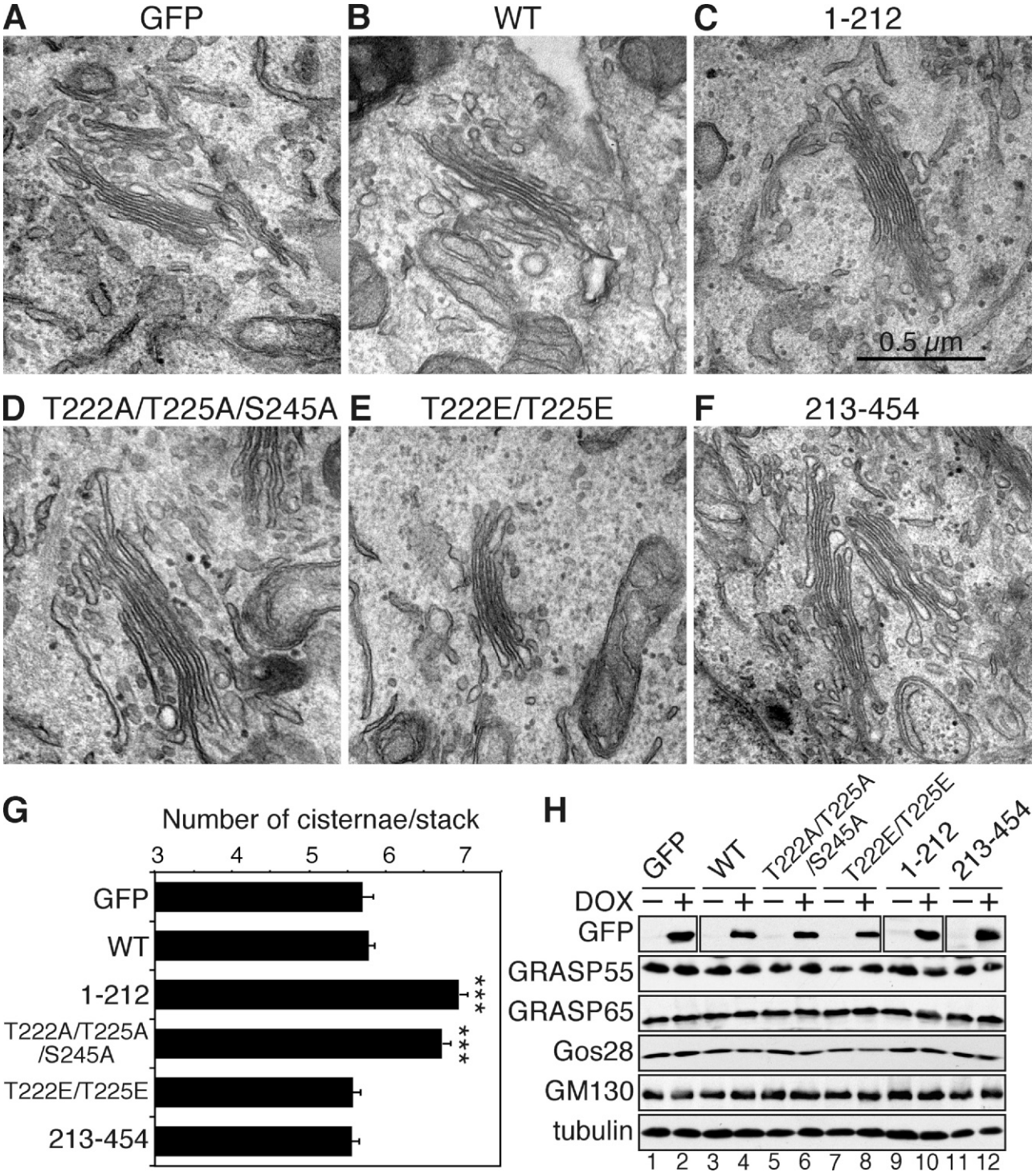


Figure 7. **Overexpression of nonregulatable GRASP55 mutants enhances Golgi stack formation in interphase cells.** (A–F) Representative EM images of interphase cells expressing indicated GRASP55 constructs. Bar, 0.5 µm. Note that the Golgi structures in cells expressing the GRASP domain (C) and the T222A–T225A–S245A mutant (D) are better organized than those in the GFP cell line (A). (G) Quantitation (mean ± SEM) of 20 cells from conditions indicated in A–F. Statistical significance was assessed by comparison to the GFP cell line. ***, $P < 0.001$. (H) Cells in A–F were lysed in SDS buffer and analyzed by Western blotting.

exhibited more extensive fragmentation than that in GFP-expressing cells (Fig. 9 E).

When observed by EM, a considerable amount of Golgi membranes in cells expressing the GRASP domain or the T222A–T225A–S245A mutant remained as Golgi remnants (e.g., mini stacks, short cisternae, and tubular structures; Fig. 9, F–I). Some membranes were vesiculated, but these vesicles were presumably held together by nonphosphorylatable GRASP55 proteins, which remain oligomeric during mitosis. Quantitation of EM images showed that the number of Golgi clusters in each profile for mitotic cells expressing the GRASP domain (7.5 ± 0.9) and the T222A–T225A–S245A mutant (6.9 ± 0.8) was at least

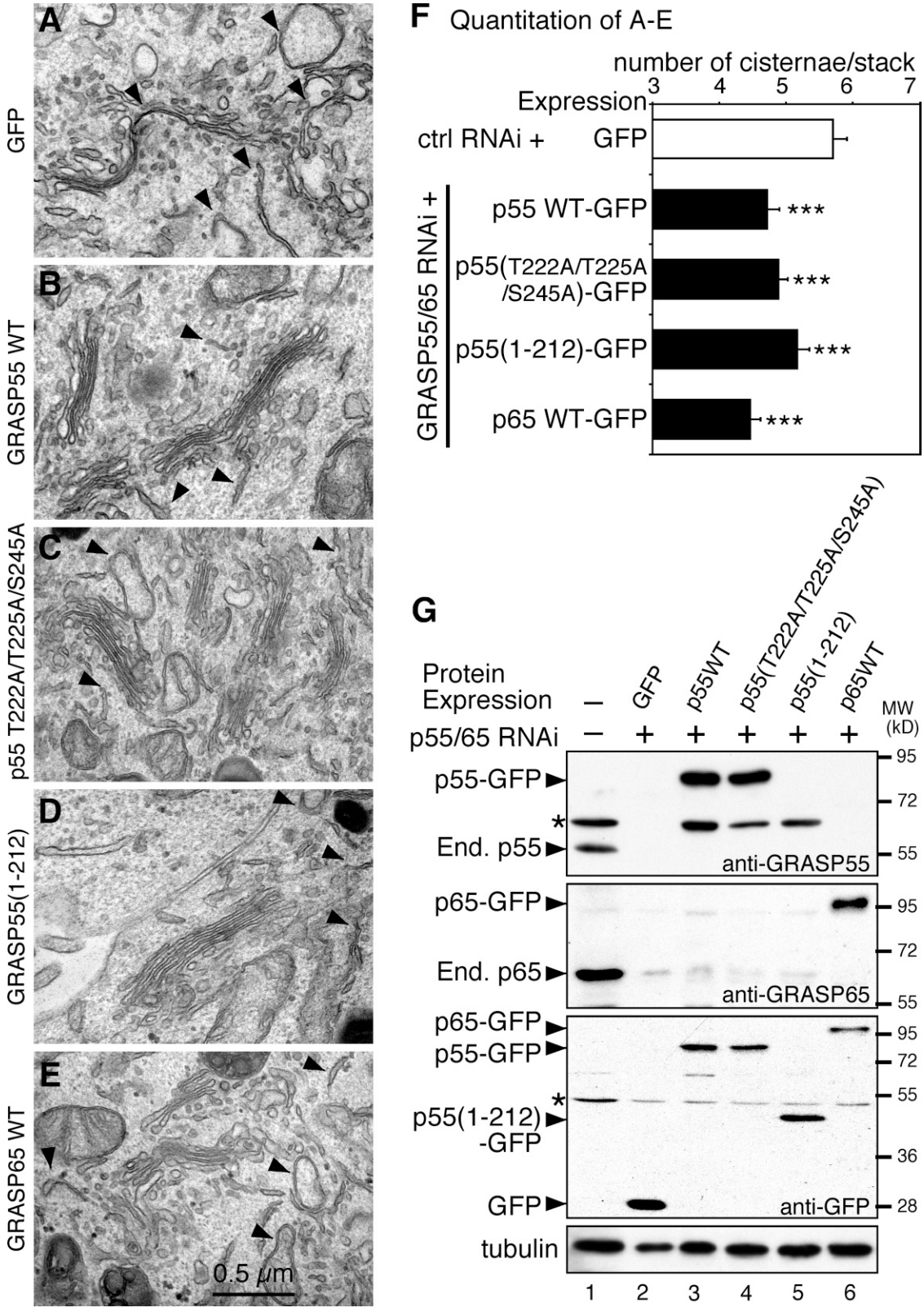


Figure 8. **Rescue of Golgi structure by expression of exogenous GRASP55 and GRASP65 in cells with both GRASPs depleted.** (A–E) EM images of HeLa cell lines transfected with a mixture of GRASP55 and GRASP65 siRNAs and induced to express indicated protein. Arrowheads indicate unstacked cisternae. (F) Quantitation of 20 cells in B–E. Statistical significance was assessed by comparison to the GFP cell line treated with control siRNA. ***, $P < 0.001$. (G) Western blots of cells in A–E. "End. p55", endogenous p55; "End. p65", endogenous GRASP65. Asterisk indicates a nonspecific band.

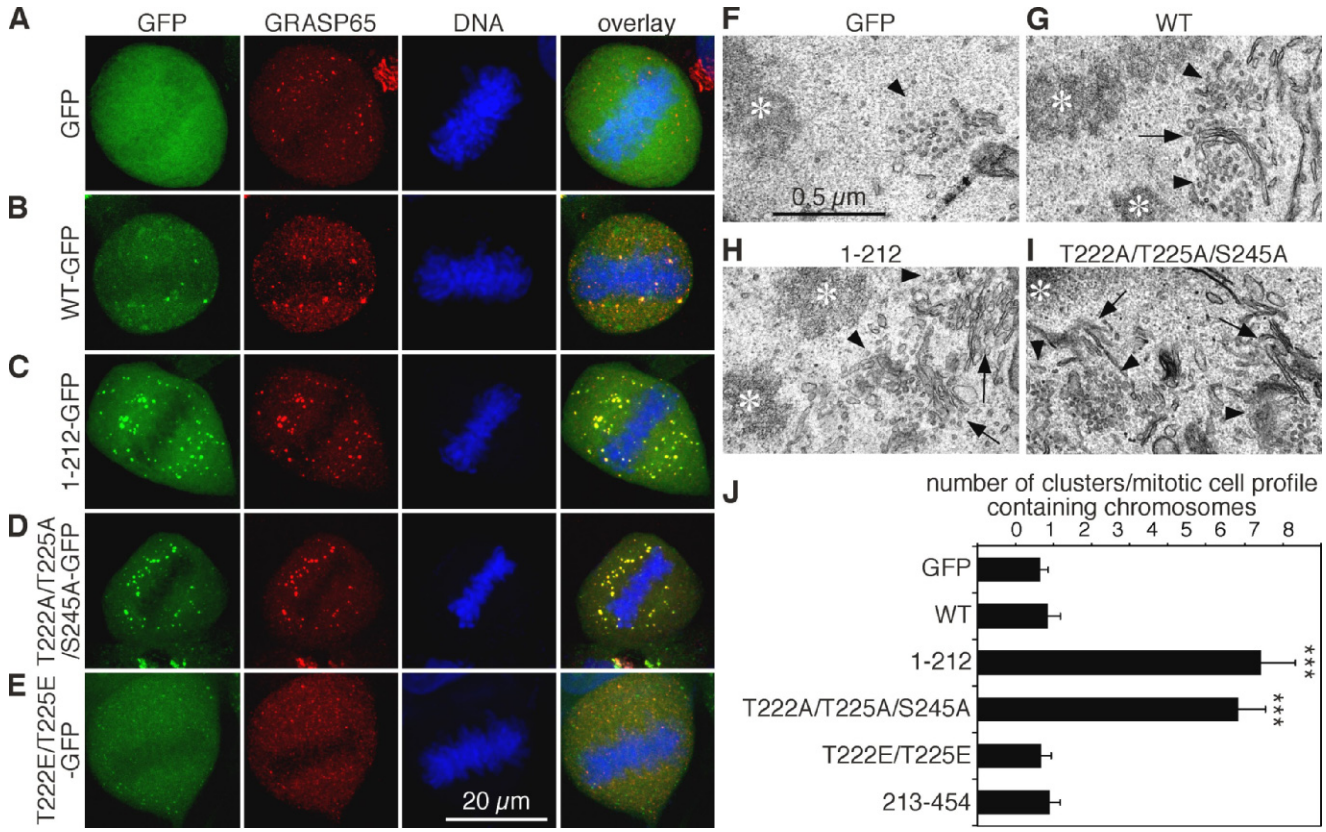


Figure 9. **Expression of nonphosphorylatable GRASP55 mutants inhibits mitotic Golgi disassembly.** (A–E) Confocal images of mitotic cells of indicated HeLa cell lines stained for DNA and GRASP65. Note that cells expressing the GRASP domain (C) and the T222A–T225A–S245A mutant (D) had more mitotic clusters, whereas Golgi fragmentation in cells expressing the T222E–T225E mutant (E) was more complete compared with that in GFP-expressing cells (A). Bar, 20 µm. (F–I) Representative EM images of mitotic cells expressing indicated GRASP55 constructs. Mitotic cells were collected by shake-off after release from a double-thymidine block and processed for EM. Bar, 0.5 µm. Note the remaining Golgi cisternal membranes or stacks (arrows) and vesicle clusters (arrowheads) in the GRASP domain–expressing (H) and the T222A–T225A–S245A mutant–expressing (I) cells. Asterisks indicate condensed chromosomes. (J) Quantitation of the number of Golgi clusters in mitotic cells expressing indicated proteins. 20 cells were examined in each case. Results are expressed as the mean ± SEM. Statistical significance was assessed by comparison to the GFP cell line. ***, $P < 0.001$.

three times greater than the number in cells expressing wild-type GRASP55 (1.9 ± 0.3), the T222E–T225E mutant (1.7 ± 0.3), the SPR domain (1.9 ± 0.3), or GFP (1.6 ± 0.2) (Fig. 9 J). These results substantiate the in vitro data (Fig. 6), suggesting that the lack of mitotic regulation of GRASP55 prevents the breakdown and dispersal of Golgi membranes during mitosis. Collectively, these results provide strong evidence that GRASP55 functions together with GRASP65 to link Golgi membranes into stacks and outline a molecular mechanism for GRASP55 function.

Discussion

The role of GRASP55 in Golgi stack formation was first indicated by an in vitro Golgi reconstitution assay (Shorter et al., 1999). However, siRNA-based depletion experiments in other studies led to inconsistent conclusions (Duran et al., 2008; Feinstein and Linstedt, 2008). To address these discrepancies, we performed systematic EM analysis of GRASP55-depeleted cells. Our results showed that GRASP55 knockdown significantly reduced the number of cisternae per stack and that this effect was rescued by the expression of exogenous rat GRASP55 or its mutants. When GRASP55 was depleted, GRASP65 expression and Golgi targeting were not affected, and vice versa, indicating that the two homologues might function and target to the Golgi independently. Strikingly, simultaneous depletion of both GRASP proteins led to the disassembly of the entire Golgi stack, an effect that was partially rescued by the expression of GRASP55 or GRASP65. Given that GRASP55 and GRASP65 localize to different parts of the Golgi stack, these results strongly suggest that GRASP55 and GRASP65 play complementary and essential functions in Golgi stacking.

It has been reported that GRASP55 depletion results in scattered Golgi and therefore that GRASP55 plays a role in Golgi ribbon linking (Feinstein and Linstedt, 2008). In our study, however, the majority of the cells with depletion of GRASP55 contained intact Golgi as judged by fluorescence microscopy; only 22–24% cells had partially fragmented Golgi. The heterogeneity of Golgi morphology may account for the inconsistent observation made by the other studies (Duran et al., 2008; Feinstein and Linstedt, 2008). FRAP analysis confirmed that the Golgi ribbon was affected by GRASP55 depletion, suggesting that either GRASP55 is directly involved in Golgi ribbon linking, or the observed effect is caused by Golgi unstacking. The mechanism for Golgi ribbon formation is so far unclear, but disruption of many factors can potentially affect the Golgi ribbon. For example, depletion of the cargo receptors Surf4 and

ERGIG53, p25, golgin 84, or the microtubule-binding protein CLASP all lead to Golgi fragmentation (Diao et al., 2003; Mitrovic et al., 2008; Miller et al., 2009). In addition, it has been shown that Golgi disassembly affects protein trafficking (Wang, 2008), which may indirectly affect ribbon formation. Therefore, whether GRASP55 plays a direct role in Golgi ribbon formation in addition to stacking requires further investigation.

Further biochemical experiments demonstrated that GRASP55 formed oligomers in a mitotically regulated fashion, and that these oligomers are sufficient to link Golgi membranes together, a mechanism shared with GRASP65 (Wang et al., 2003, 2005). GRASP65 is phosphorylated by cdc2 and plk during mitosis, whereas GRASP55 is mainly phosphorylated by MAPK. This difference may allow for accurate regulation of the alternating oligomeric status of the two proteins to fulfill physiological functions. For example, it has been shown that expression of an S277A mutant of GRASP65 in tissue culture cells inhibited Golgi orientation toward the leading edge in a wound-healing assay (Bisel et al., 2008). A similar mechanism may exist for GRASP55 to allow cells to react to different conditions. Indeed, phosphorylation sites on GRASP55 responsible for mitotic regulation were identified in this and previous studies, and phosphorylation at these sites is required for mitotic Golgi disassembly and cell cycle progression (Jesch et al., 2001; Duran et al., 2008).

When expressed in cells, the nonphosphorylatable mutants of GRASP55 (i.e., the GRASP domain and the T222A–T225A–S245A mutant) enhanced Golgi stacking in interphase cells and inhibited Golgi unstacking and disassembly at the onset of mitosis, consistent with the biochemical and knockdown results. Mitotic disassembly of the Golgi in those cells was clearly not complete, as Golgi remnants (short cisternae and stacks) were often seen in mitotic cells by EM. In addition, the Golgi remnants in those cells were clustered, presumably reflecting tethering by the exogenous GRASP55 mutant proteins. Overall Golgi disassembly was not blocked, consistent with a previous report (Feinstein and Linstedt, 2008), and possibly due to the fact that other Golgi structural proteins such as GRASP65 still responded to mitotic regulation. Collectively, the results in this study demonstrate that GRASP55, like GRASP65, plays a critical role in Golgi stacking and that the two proteins function collaboratively to maintain cisternal membrane stacking through a common mechanism, by forming mitotically regulated trans-oligomers. Future studies will determine how differential targeting of the two GRASP proteins leads to the establishment of the polarized structure of the Golgi stack.

Important unanswered questions remain concerning the mechanism of Golgi structure formation. For example, it is unclear why the budding yeast *Saccharomyces cerevisiae* has a GRASP homologue but lacks Golgi stacks, whereas plants have Golgi stacks but no GRASP proteins. First, different organisms may have evolved different mechanisms for Golgi structure formation. Because the organization of the secretory pathway in plant differs from that in mammalian cells (e.g., there is no ERGIC, no Golgi ribbon, and no mitotic Golgi fragmentation), it is possible that an alternative mechanism for Golgi stacking may have developed (Struck et al., 2008). Alternatively, plant cells may have evolved highly divergent and as-yet undiscovered GRASP-related proteins. Indeed, plant golgins have been found only in the last a few years (Hawes, 2005; Latijnhouwers et al., 2005; Matheson et al., 2007). A second possibility is that GRASP has gained different functions during evolution. In addition to stacking, other functions have been reported for GRASP55, GRASP65, and their homologues, including roles in protein trafficking in budding yeast (Behnia et al., 2007) and mammals (Kuo et al., 2000; Barr et al., 2001; Short et al., 2001; D'Angelo et al., 2009), and unconventional secretion in *Dictyostelium* (Kinseth et al., 2007) and noncanonical secretion in *Drosophila* (Schotman et al., 2008) and Golgi ribbon linking (Puthenveedu et al., 2006; Feinstein and Linstedt, 2008; Sengupta et al., 2009), as well as cell cycle regulation (Sütterlin et al., 2002, 2005; Yoshimura et al., 2005; Duran et al., 2008) in mammals. The sole GRASP protein in the budding yeast, Grh1p, lacks the first PDZ domain that mediates the oligomerization of mammalian GRASP (Shorter et al., 1999; Behnia et al., 2007; Sengupta et al., 2009). Therefore, it is possible that GRASPs may have gained different functions in yeast and animal cells. Third, the proposed functions are not mutually exclusive; some of these functions may be related to Golgi stack formation. For example, GRASP oligomerization may mediate either stacking or ribbon linking depending on whether it is localized on the flat region or on the rims of the Golgi cisternae. Furthermore, the Golgi in budding yeast forms stacks under special conditions (Rambourg et al., 1993), and the role of the yeast GRASP homologue has not been tested under such conditions. These questions could be answered only through further experiments. For example, it will be interesting to test whether the Golgi structure in GRASP55/65-depleted HeLa cells could be rescued by the expression of yeast or fly GRASP proteins.

Materials and methods

Reagents

All reagents were from Sigma-Aldrich, Roche, or EMD, unless otherwise stated. The following antibodies were used: monoclonal antibodies against α-actin (Sigma-Aldrich), Gos28 and GM130 (BD Transduction Laboratories), GRASP65 (G. Warren, Max F. Perutz Laboratories, Vienna, Austria), and α-tubulin (K. Gull, University of Oxford, Oxford, UK); polyclonal antibodies against ERK2 (Millipore), GFP (J. Seemann, University of Texas Southwestern Medical Center, Dallas, TX), GM130 (N73, J. Seemann), human GRASP55 (Proteintech Group, Inc.), rat GRASP55 (Susan, against rat GARSP55 aa 1–212; Rich, against rat GRASP55 aa 232–454; both from J. Seemann), human GRASP65 (J. Seemann), rat GRASP65 (Wang et al., 2005), α-ManII (K. Moremen, University of Georgia, Athens, GA), and MEK1 (Millipore).

Preparation of kinases, cytosol, Golgi membranes, and GRASP55 fusion proteins

Golgi membranes (Wang et al., 2006), constitutively active cdc2 and plk (Wang et al., 2003), and interphase (IC) and mitotic (MC) cytosol (Rabouille et al., 1995) were prepared as described previously. cDNA constructs for wild-type ERK2 and constitutively active MEK1 (S218E/S222D/ΔN3) were provided by K. Guan (University of California, San Diego, La Jolla, CA). GRASP55 cDNAs (provided by J. Seemann; GenBank accession no. NM_001007720.1) were cloned into pMAL-c2X (New England Biolabs, Inc.), pET30a, pET23a (EMD), or pGEX (GE Healthcare) vectors by PCR or restriction digestion and confirmed by DNA sequencing. GRASP55 point mutations were introduced using the QuikChange mutagenesis kit (Agilent Technologies) and confirmed by DNA sequencing. Proteins were expressed in BL21-CodonPlus(DE3)RILP bacteria and purified on amylose (New England Biolabs, Inc.), nickel (QIAGEN), or glutathione Sepharose (GE Healthcare) beads.

Phosphorylation assay
5 µg of purified Golgi membranes, or 500 ng of recombinant GRASP55, were mixed with 500 µg of mitotic HeLa cell cytosol or with 1 µg ERK2 and 1 µg MEK1, and an ATP-regenerating system (10 mM creatine phosphate, 0.1 mM ATP, 20 µg/ml creatine kinase, and 20 µg/ml cytochalasin B) was added in MEB buffer (50 mM Tris-HCl, pH 7.4, 0.2 M sucrose, 50 mM KCl, 20 mM β-glycerophosphate, 15 mM EGTA, 10 mM $MgCl_2$, 2 mM ATP, 1 mM GTP, 1 mM glutathione, and protease inhibitors) for a final volume of 50 µl. In some reactions, MEK inhibitor U0126 (200 µM; Promega) was included. Similar reactions were performed with interphase cytosol in KHM buffer (20 mM 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) to determine GRASP55 dephosphorylation. Phosphorylation of GRASP55 was analyzed by a band-shift assay. To ensure that the band-shift was caused by phosphorylation, 5 µg of mitotic cytosol-treated Golgi membranes were further treated with 20 U of CIP (Invitrogen) in the absence or presence of a phosphatase inhibitor, β-glycerophosphate (50 mM) for 60 min at 37°C; the membranes were then analyzed by immunoblotting. For γ-[^{32}P] ATP labeling, 0.5 µCi/µl of γ-[^{32}P] ATP and 5 µM ATP were used. After a 60-min incubation at 37°C, the membranes were pelleted through 0.4 M sucrose at 55,000 rpm for 30 min in a rotor (model TLA55; Beckman Coulter). Samples were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography.

GRASP55 dimerization and oligomerization
To measure GRASP55 oligomerization, recombinant MBP-GRASP55 and His-GRASP55 were separately expressed in bacteria and purified, and mixed followed by treatment with mitotic cytosol or buffer as described for the phosphorylation assay. The protein complex was isolated using amylose beads or nickel beads and analyzed by immunoblotting for GRASP55. For nondenaturing electrophoresis, purified His-GRASP55 was incubated with purified ERK2 and MEK1, or buffer, at 37°C for 60 min. Nondenatured samples were loaded onto a 4–12% NuPAGE Novex Bis-Tris mini gel (Invitrogen) (Wittig et al., 2006). After electrophoresis, the gel was soaked with 0.1% SDS followed by immunoblotting.

Golgi disassembly assay and aggregation of GRASP55-coated beads
Purified rat liver Golgi membranes were treated with kinases at 37°C for 20 min. For a reaction using 20 µg Golgi membranes, the amounts of the kinases used were equivalent to the activity found in 2 mg mitotic cytosol. Normally, ∼20 µg cdc2 and 30 µg plk were used, based on the quality of preparation. For MEK1 and ERK2 activity, we have determined the protein amount in the cytosol by Western blotting using purified kinase as the standard. Normally there are ∼4 µg MEK1 and 2 µg ERK2 in 2 mg interphase or mitotic cytosol. Accordingly, we used 4 µg MEK1 and 2 µg ERK2 to treat 20 µg purified Golgi membranes. After the treatment, the membranes were collected by centrifugation and processed for EM. At least 10 random regions were photographed for each condition and membrane profiles were quantified using the intersection method (Rabouille et al., 1995).

His-tagged, full-length GRASP55, the GRASP domain, or BSA (Invitrogen) were cleared by centrifugation and cross-linked to Dynal beads (M500; Invitrogen) following the manufacturer's instructions (Wang et al., 2003, 2005). The beads (8×10^6) were incubated for 60 min at 37°C with 0.5 mg (25 µl) cytosol or kinase mixture (ERK2/MEK1, 2 µg each) in the presence of 4 µg/ml nocodazole in a 50-µl reaction system and observed under a bright-field microscope. In some experiments, beads were sequentially treated with interphase and mitotic cytosol or kinases (Wang et al., 2003, 2005). For quantitation of the beads, 15 random phase–contrast digital images of each reaction were captured with a 20x lens and a camera (Spot Slider2; Diagnostic Instruments, Inc.). Images were analyzed using MATLAB 7.4 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 ≥6 beads. For large aggregates, only visible surface beads were counted; therefore, the number of beads in these aggregates was underestimated. Results were expressed as the mean ± SEM from three independent experiments; statistical significance was assessed using Student's *t* test.

Cell culture, transfection, and microscopy
HeLa cell transfections were performed using published siRNAs for human GRASP55 (AACTGTCGAGAAGTGATTATT; Applied Biosystems) (Feinstein and Linstedt, 2008) and GRASP65 (CCTGAAGGCACTACTGAAAGCCAAT, Invitrogen) (Sütterlin et al., 2005). Lipofectamine RNAiMAX was used according to the manufacturer's recommendations (Invitrogen). Control nonspecific siRNAs were purchased from Applied Biosystems. Assays were performed 96 h after transfection. To establish stable cell lines, GFP cDNA was first cloned into pRevTRE2 vector using HindIII and NotI sites. GRASP55 cDNA was then inserted at the N terminus of GFP using BamHI and HindIII sites. To generate retroviral particles, HEK293 cells were transfected with pRevTRE2-rGRASP55-GFP and viral DNA pV Pack GP and pV Pack Eco, using Lipofectamine 2000. 36 h after transfection, the virus supernatant was collected and filtered via MILLEX GV (0.45 µm; Millipore) and the undiluted supernatants were used to infect rtTA HeLa m2 cells, a tet-on cell line (Heusser et al., 2006). Infected cells were selected with 500 µg/ml hygromycin for 2 wk, and GRASP55-GFP–positive cells were sorted by flow cytometry. The expression of GRASP55-GFP was induced with 1 µg/ml doxycycline for 48 h, and protein expression was determined by immunoblotting for GRASP55 and/or GFP. For the gene replacement experiment, GRASP55- or GFP-expressing cell lines were transfected with siRNA for 48 h followed by induction with doxycycline for 48 h. Immunofluorescence microscopy and collection of mitotic cells were described previously (Xiang et al., 2007). Pictures were taken with a confocal laser-scanning microscope (model SP5; Leica) using a 100x oil lens. Each image was a maximum projection from a z-stack. Pictures were assembled in Adobe Photoshop.

For EM analysis, Golgi stacks and clusters were identified using morphological criteria and quantified using standard stereological techniques (Wang et al., 2005). For interphase cells, the profiles had to contain a nuclear profile with an intact nuclear envelope; only stacked structures with two or more cisternae were counted. For mitotic cells, the profile had to contain one or more profiles of condensed chromosomes lacking a nuclear envelope. Quite often, multiple condensed chromosomes were aligned at the center of the cell. A low magnification (normally 1600x) image and a series of higher magnification images were taken to cover the entire cell. More than 20 cells were quantified for each reaction.

Fluorescence recovery after photobleaching (FRAP)
HeLa cells were first transfected with siRNA. After 48 h, the cells were trypsinized and plated on chambered coverglasses (Laboratory-Tek II; Thermo Fisher Scientific). After 24 h incubation, cells were transfected with a ManII-GFP cDNA using Lipofectamine 2000 and further cultured for 24 h until they were used for FRAP analysis. FRAP was performed using a laser-scanning confocal microscope (FluoView FV500/IX; Olympus) equipped with an environmental control system set to 37°C and 5% CO_2. Cells were cultured in phenol red–free Opti-MEM (Invitrogen) with 10% FBS and visualized using the argon laser line (488 nm) with a 60x, 1.0 NA water immersion objective. Images were taken every 10 s for 30 s (2% laser power) and the selected area of the Golgi was bleached using 15 laser pulses of the 488-nm laser line at maximal intensity. Fluorescence images were then recorded every 10 s for 480 s. After background correction, the recovery of the fluorescence was calculated as the ratio of the average intensity of the bleached region to that of the entire Golgi. Normalization was set between the values before bleaching and the first time point after bleaching. More than 17 cells were quantified in each experiment. The average of two independent experiments is shown (Fig. 2).

Online supplemental material
Fig. S1 shows the time course of GRASP55 and GRASP65 depletion in HeLa cells. Fig. S2 shows the bar chart distribution of the number of cisternae per stack after different treatments. Fig. S3 demonstrates GRASP55 phosphorylation by MAPK in mitotic cells and oligomerization of GRASP55 in rat liver Golgi. Fig. S4 illustrates fluorescence images of wild-type or GRASP55/65-depleted HeLa cells expressing GRASP proteins or mutants. Fig. S5 demonstrates that the Golgi structure in GRASP55-depleted cells can be rescued by expression of exogenous GRASP55 or its mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200907132/DC1.

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