Knockout of the Golgi stacking proteins GRASP55 and GRASP65 impairs Golgi structure and function

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ABSTRACT Golgi reassembly stacking protein of 65 kDa (GRASP65) and Golgi reassembly stacking protein of 55 kDa (GRASP55) were originally identified as Golgi stacking proteins; however, subsequent GRASP knockout experiments yielded inconsistent results with respect to the Golgi structure, indicating a limitation of RNAi-based depletion. In this study, we have applied the recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology to knock out GRASP55 and GRASP65, individually or in combination, in HeLa and HEK293 cells. We show that double knockout of GRASP proteins disperses the Golgi stack into single cisternae and tubulovesicular structures, accelerates protein trafficking, and impairs accurate glycosylation of proteins and lipids. These results demonstrate a critical role for GRASPs in maintaining the stacked structure of the Golgi, which is required for accurate posttranslational modifications in the Golgi. Additionally, the GRASP knockout cell lines developed in this study will be useful tools for studying the role of GRASP proteins in other important cellular processes.

INTRODUCTION

The Golgi apparatus is an essential organelle composed of stacks of tightly aligned flattened cisternal membranes, which are often laterally linked into a ribbonlike structure located in the perinuclear region of mammalian cells (Ladinsky et al., 1999). The Golgi resides

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Abbreviations used: BSA, bovine serum albumin; CRISPR, clustered regularly interspaced short palindromic repeats; EM, electron microscope; EndoH, endoglycosidase; FACS, fluorescence-activated cell sorting; GRASP55, Golgi reassembly stacking protein of 55 kDa; GRASP65, Golgi reassembly stacking protein of 65 kDa; MAA, Maackia amurensis lectin; PBS, phosphate-buffered saline; RNAi, RNA interference; sgRNA, single-guide RNA; TGN, trans-Golgi network; VSV-G, vesicular stomatitis virus G protein; WGA, wheat germ agglutinin.

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undergoes a unique disassembly and reassembly process during the cell cycle, which is regulated by phosphorylation of the GRASP proteins (Jesch et al., 2001; Wang et al., 2005a; Xiang and Wang, 2010). On mitotic entry, Cdk1 and several other kinases are activated and phosphorylate GRASP proteins (Vielermeyer et al., 2009; Tang et al., 2012), which impair GRASP oligomerization, resulting in Golgi cisternal unstacking. As cells exit mitosis, GRASP proteins are dephosphorylated after Cdk1 inactivation, enabling GRASPs to oligomerize and Golgi stacks to reform (Wang and Seemann, 2011; Zhang and Wang, 2015). GRASP proteins also interact with other Golgi structural proteins to regulate the morphology of the Golgi. For instance, GRASP65 interacts with GM130 (Nakamura et al., 1995; Barr et al., 1998), while GRASP55 forms a complex with Golgin-45 (Short et al., 2001; Barr, 2005). Both GM130 and Golgin-45 are coiled-coil golgins involved in membrane tethering and Golgi structure formation (Lowe et al., 1998; Lowe et al., 2000; Wong and Munro, 2014). Thus GRASPs and their interacting proteins are essential for Golgi structure formation (Wang and Seemann, 2011; Tang and Wang, 2013).

Further studies in cells using RNA interference (RNAi)-mediated depletion confirmed that knockdown of a single GRASP protein reduced the number of cisternae per stack (Sutterlin et al., 2005; Xiang and Wang, 2010), an effect that was rescued by the expression of exogenous GRASP proteins (Tang et al., 2010b); while simultaneous depletion of both GRASPs resulted in disorganization of the entire stack (Xiang and Wang, 2010). However, GRASP depletion also caused additional effects, and thus GRASPs have been implicated in other cellular processes, including enzyme distribution (Puthenveedu et al., 2006), cargo transport (D’Angelo et al., 2009), unconventional secretion (Gee et al., 2011; Giuliani et al., 2011), cell cycle progression (Sutterlin et al., 2005), apoptosis (Cheng et al., 2010), and cell migration (White et al., 2010). So far it is not clear whether GRASPs possess all of these functions or whether some of the effects are caused by the disruption of the Golgi stacks when GRASPs are depleted. The exact role for GRASP55 and GRASP65, therefore, remains elusive. In this study, we have used the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing technique (Cong et al., 2013; Mali et al., 2013) to create loss-of-function alleles (referred to hereafter as “knockout”) of GRASP55 and GRASP65, single or in combination, to investigate their roles in Golgi structure formation and function.

RESULTS

Generation of GRASP55/65 knockout HeLa and HEK293 cells

To establish GRASP55 and GRASP65 single-knockout cell lines, we designed multiple single-guide RNAs (sgRNAs) targeting exon 1 of GRASP55 (55T1, 55T2, 55T3) and exon 2 of GRASP65 (65T1, 65T2) using the target design software developed by Feng Zhang’s lab at the Massachusetts Institute of Technology (http://crispr.mit.edu/) (Figure 1A). These sgRNAs all target to the coding sequences of the gene immediately downstream of the translation initiation site, and therefore no truncated proteins should be made in the cell. These sgRNAs were cloned into pSpCas9(BB)-2A-GFP(PX458) and pSpCas9(BB)-2A-Puro(PX459) vectors so positive cells could be selected by fluorescence-activated cell sorting (FACS) or by puromycin resistance. This generated a heterogeneous population of cells where ~50% of cells had no detectable levels of the target protein as determined by immunofluorescence microscopy. We then examined the morphology of the Golgi in the mixed populations of cells by immunofluorescence microscopy using GM130 as a Golgi marker. In both HeLa and HEK293 cells, GRASP65-negative cells displayed no significant changes in Golgi morphology compared with cells that expressed GRASP65 (Figure 1B and Supplemental Figure S2A). However,

![FIGURE 1: Construction of GRASP55 and GRASP65 single-knockout cells. (A) sgRNAs designed to target GRASP55 and GRASP65 using CRISPR/Cas9-mediated gene deletion. The translation initiation ATG codon is indicated and referred to as coding sequence 1 (for A); exons are indicated as boxes and introns indicated by a line, with the number of nucleotide at the splicing borders indicated. sgRNAs sequences and relative locations are indicated as lines above the exons of the gene. (B) Immunofluorescence images of cell populations transfected with sgRNAs to GRASP55 (left panels) or GRASP65 (right panels). HeLa cells were transfected with GFP-Cas9 plasmids containing sgRNAs against either GRASP55 or GRASP65 and selected for GFP expression by flow cytometry. The Golgi morphology of GRASP knockout cells was assessed by immunofluorescence microscopy for either GRASP55 or GRASP65 cotained with GM130. Scale bars are 10 µm. (C) Quantification of Golgi morphology of GRASP-positive (arrows) and GRASP-negative (asterisks) cells in B. Blinded determination of the Golgi morphology of 300 cells from each sample were quantified across three biological replicates. Error bars represent SEM (Cheeseman et al., 2012). A Student’s t test was performed to determine statistical significance. *p < 0.05.](image-url)
GRASP55-negative cells exhibited an increased frequency of mild Golgi fragmentation compared with GRASP55-positive cells (Figure 1, B and C, and Supplemental Figures S1 and S2).

**Knockout of a single GRASP protein has minor effects on the Golgi morphology**

We then generated stable clones of GRASP single-knockout cells using three targets of GRASP55 (55T1, 55T2, 55T3) and two targets of GRASP65 (65T1, 65T2) in HeLa and HEK293 cells by plating selected whole populations at low density followed by clonal expansion. Multiple clones for each target were generated; consistent results were obtained in different clones generated by different sgRNAs targeting to the same gene (Supplemental Table S1). Genetic deletion of GRASP55 and GRASP65 was confirmed by genomic sequencing (Supplemental Table S2, A and B). Representative clones for each targeting sgRNA were further characterized.

Western blot analysis of GRASP55 knockout clones demonstrated that GRASP55 depletion was effective; as no GRASP55 signal was detected (Figure 2A and Supplemental Figure S3A). Knockout of GRASP55 significantly increased the level of GRASP65 in HEK293 cells (Supplemental Figure S3, A and B), although this effect was not as obvious in HeLa cells (Figure 2, A and B). GRASP55 deletion also resulted in a significant reduction of Golgin-45 in HeLa cells (Figure 2, A and B, and Supplemental Figure S3, A and B). Deletion of GRASP55 resulted in a minor, but significant, increase in the level of Golgi fragmentation in both HeLa and HEK293 cells, as assessed by immunofluorescence microscopy for GM130 and TGN46 (Figure 2, C–E, and Supplemental Figure S3, C–E). However, colocalization of GM130 and TGN46, as measured by Pearson’s correlation coefficient, remained unchanged in HeLa cells.

Knockout of GRASP65 was also confirmed by Western blotting (Figure 3A and Supplemental Figure S4A). Interestingly, GRASP65 deletion significantly increased the protein level of GRASP55 in HeLa cells (Figure 3A), indicating that a mechanism of compensation might exist between GRASP proteins. GRASP65 deletion also reduced the level of GM130, in particular in HEK293 cells (Figure 3, A and B, and Supplemental Figure S4, A and B), consistent with previous reports (Xiang and Wang, 2010). GRASP65 knockout had no significant effects on Golgi morphology when assessed by immunofluorescence microscopy (Figure 3, C–E, and Supplemental Figure S4, C–E).

**Double deletion of both GRASP55 and GRASP65 results in severe Golgi fragmentation**

Deletion of GRASP55 or GRASP65 individually had only a mild impact, if at all, on the Golgi morphology when assessed by immunofluorescence microscopy. This can be explained by three possible reasons: 1) GRASP55 and GRASP65 play complementary roles in Golgi structure formation; the second GRASP protein can largely maintain the Golgi structure intact when the first one is deleted (Xiang and Wang, 2010); 2) the increased level of the other GRASP protein when its homologue is deleted may provide compensatory effect on Golgi structure formation; and 3) light microscopy does not reach the appropriate resolution to assess Golgi stack formation, and therefore the effect must be assessed by electron microscopy (EM). To address the first two possibilities, we simultaneously deleted both GRASP proteins in HeLa and HEK293 cells. Like the generation of GRASP55/65 single-knockout cells, 65T1 HeLa and HEK293 cells, instead of wild-type cells, were transfected with GRASP55 sgRNA target #2 to generate a double-knockout population and multiple, individual clones were selected (Supplemental Table S1). Deletion of both GRASP55 and GRASP65 was confirmed by Western blotting and sequencing of the genomic DNA (Figure 4A, Supplemental Figure S5A, and Supplemental Table S2, A and B). Further characterization of two representative clones demonstrated that GRASP double-knockout resulted in a significant reduction in the protein levels of both GM130 and Golgin-45 in both HeLa and HEK293 cells (Figure 4, A and B) and HEK293 cells (Supplemental Figure S5, A and B). We then assessed the Golgi morphology by immunofluorescence for GM130 and TGN46. Double knockout of GRASP proteins resulted in a dramatic dispersal of Golgi structures in both cell lines (Figure 2, A and B, and Supplemental Figure S3, A and B). Deletion of GRASP55 resulted in a minor, but significant, increase in the level of Golgi fragmentation in both HeLa and HEK293 cells, as assessed by immunofluorescence microscopy for GM130 and TGN46 (Figure 2, C–E, and Supplemental Figure S3, C–E). However, colocalization of GM130 and TGN46, as measured by Pearson’s correlation coefficient, remained unchanged in HeLa cells. Knockout of GRASP65 was also confirmed by Western blotting (Figure 3A and Supplemental Figure S4A). Interestingly, GRASP65 deletion significantly increased the protein level of GRASP55 in HeLa cells (Figure 3A), indicating that a mechanism of compensation might exist between GRASP proteins. GRASP65 deletion also reduced the level of GM130, in particular in HEK293 cells (Figure 3, A and B, and Supplemental Figure S4, A and B), consistent with previous reports (Xiang and Wang, 2010). GRASP65 knockout had no significant effects on Golgi morphology when assessed by immunofluorescence microscopy (Figure 3, C–E, and Supplemental Figure S4, C–E).

![FIGURE 2: GRASP55 deletion has minor effects on the Golgi structure. (A) Western blots of Golgi proteins in GRASP55 knockout HeLa cells. Wild-type and representative GRASP55 knockout clones from three separate sgRNAs (T1, T2, and T3) were lysed and blotted for GRASP55/65, Golgin-45, and GM130. (B) Quantification of A for the relative levels of GRASP65, Golgin-45, and GM130 in GRASP55 knockout cells. Error bars represent SEM. (C) Immunofluorescence of GRASP55 knockout clones stained for GM130 and TGN46. (D) Colocalization of GM130 and TGN46 (Figure 2, C–E, and Supplemental Figure S3, C–E). However, colocalization of GM130 and TGN46, as measured by Pearson’s correlation coefficient, remained unchanged in HeLa cells. Knockout of GRASP65 was also confirmed by Western blotting (Figure 3A and Supplemental Figure S4A). Interestingly, GRASP65 deletion significantly increased the protein level of GRASP55 in HeLa cells (Figure 3A), indicating that a mechanism of compensation might exist between GRASP proteins. GRASP65 deletion also reduced the level of GM130, in particular in HEK293 cells (Figure 3, A and B, and Supplemental Figure S4, A and B), consistent with previous reports (Xiang and Wang, 2010). GRASP65 knockout had no significant effects on Golgi morphology when assessed by immunofluorescence microscopy (Figure 3, C–E, and Supplemental Figure S4, C–E).](image-url)
the Golgi in 95% of the HeLa cells (and ∼65% in HEK293 cells) and significant decrease in the colocalization between GM130 and TGN46, indicating a disruption of Golgi stack formation (Figure 4, C–E, and Supplemental Figure S5, C–E). Furthermore, adding back of a single GRASP protein in GRASP double-knockout cells was sufficient to rescue the Golgi ribbon-linking defect observed by microscopy (Supplemental Figure S6). Overall this indicates that GRASP proteins play complementary roles in Golgi ribbon linking.

GRASP deletion impairs Golgi stacking

To more closely examine the morphology of the Golgi in GRASP single- and double-knockout cells, we performed AiryScan confocal microscopy, which significantly improves resolution compared with standard confocal microscopy (Huff, 2015). Like conventional confocal microscopy, the morphology of the Golgi in GRASP55 and GRASP65 single-knockout clones exhibited a compact Golgi ribbon and significant colocalization between GM130 and TGN46, similarly to the morphological characteristics of the Golgi in parental cells. However, the Golgi in GRASP double-knockout cells was extremely fragmented and disorganized, with a significant decrease in colocalization between GM130 and TGN46 (Figure 5A). To specify the effects of GRASP deletion on Golgi stacking, we treated WT and GRASP knockout cells with nocodazole, which is known to dissociate the Golgi ribbon into distinct Golgi stacks but does not significantly disrupt Golgi stack formation in interphase cells. On nocodazole treatment, GM130 and TGN46 colocalized in wild-type control cells, as assessed by AiryScan confocal microscopy. Deletion of a single GRASP did not significantly affect colocalization between GM130 and TGN46; however, double deletion of both GRASPs resulted in severe separation between GM130 and TGN46 (Figure 5, B and C), indicating that GRASP double knockouts impair stack formation.

To determine the ultrastructural details of the Golgi in GRASP knockout cells, we performed electron microscopy. As shown in Figure 5, D and E (galleries of images are shown in Supplemental Figure S8, A–D), depletion of a single or both GRASP proteins resulted in a higher frequency of disorganized membranes, such as short and unaligned cisternae, a reduced number of cisternae in the stack, and vesicle accumulation; the effects were more dramatic in GRASP double-knockout cells. Quantitation of the EM images showed that the ratio of disorganized membrane clusters versus distinguishable Golgi stacks in the perinuclear region of the cell was significantly increased in GRASP knockout cells compared with wild-type HeLa cells (3.5 ± 2.7% in wild-type, 43.0 ± 4.4% and 27.0 ± 2.8% in GRASP55 or GRASP65 knockout cells, respectively, and 73.2 ± 10.0% in double-knockout cells) (Figure 5, D and E). Moreover, even the distinguishable Golgi stacks in both single- and double-knockout cells did not seem to be normal, as they often contained swollen and shorter Golgi cisternae that were not properly aligned and a reduced number of cisternae per stack (Figure 5, D–H). If only well-organized Golgi stacks in the cells were quantified, then the number of organized Golgi stacks per cell was significantly reduced in GRASP55 and GRASP65 single-knockout cells (0.41 ± 0.06 and 0.55 ± 0.02, respectively) compared with control cells (2.06 ± 0.11), while almost no well-organized Golgi stacks were visible in GRASP double-knockout cells (Figure 5F).

These results provide compelling evidence that GRASP55/65 are required for Golgi stack formation.

GRASP knockout accelerates protein trafficking but impairs accurate glycosylation of proteins and lipids

Previous studies showed that GRASP depletion by RNAi disrupts Golgi stack formation, which subsequently impairs accurate protein glycosylation and sorting (Xiang et al., 2013). To test the effect of GRASP knockout on protein trafficking, we expressed the
GRASP knockout disrupts Golgi structure

FIGURE 4: Double deletion of GRASP55 and GRASP65 results in Golgi fragmentation. (A) Western blots of Golgi proteins in GRASP55 and GRASP65 double-knockout cells. Wild-type and two representative GRASP55 and GRASP65 double-knockout clones (DKO-C1 and DKO-C2) were analyzed by Western blot for GRASP55, Golgin-45, and GM130. (B) Quantification of the relative levels of Golgin-45, and GM130 in GRASP double-knockout cells in A. Error bars represent SEM. (C) Immunofluorescence microscopy of GRASP55/65 double-knockout cells stained for GM130 and TGN46. The lower three rows are increased magnifications of a single cell’s Golgi. Scale bars are 10 µm. (D) Colocalization of GM130 and TGN46 quantified by the Pearson’s correlation coefficient of z-stacks from GRASP double-knockout clones from C. Error bars represent SEM. (E) Quantification of cells with fragmented Golgi from GRASP double-knockout cells. Wild-type HeLa cells were analyzed by Western blot for GRASP55, Golgin-45, and GM130. (B) Quantification of the relative levels of Golgin-45, and GM130 in GRASP double-knockout cells in A. Error bars represent SEM. A Student’s t test was performed to determine statistical significance. *p < 0.05; **p < 0.01; ***p < 0.001.

DISCUSSION

In this study, we have provided new evidence that GRASP55/65 play essential roles in Golgi structure formation, in particular in stacking. Knockout of a single GRASP protein reduces the number of cisternae in the stack, while double deletion of both GRASPs results in disorganization of the entire Golgi stack. These results are consistent with our previous results that inhibition of GRASP55/65 by microinjection of GRASP65 antibodies (Wang et al., 2003) or by depletion of GRASP proteins by RNAi (Tang et al., 2010b; Xiang and Wang, 2010) both disrupted the Golgi structure (Xiang and Wang, 2010; Xiang et al., 2013). These results demonstrate that GRASP55/65 function as Golgi stacking factors.

A second function for the GRASP proteins in Golgi structure formation is to link the Golgi stacks together to form a ribbon (Puthenveedu et al., 2006; Tang et al., 2016). In this study, however, knocking out a single GRASP protein in either HeLa or HEK293 cells did not cause significant Golgi fragmentation at the level of immunofluorescence. Previously Lee et al. (2014) observed that transient knockdown of both GRASPs did not eliminate Golgi stacking. Regardless of the knockdown efficiency, the Golgi stacks are disorganized and the cisternae are not properly aligned, although the Golgi membranes are still concentrated and closely associated with each other (Lee et al., 2014). We speculate that golgins and GRASPs may have different roles in Golgi structure formation: While golgins and membrane-microtubule interactions bring Golgi membranes closer together (Lee et al., 2013), GRASPs function to link the Golgi stacks together to form a ribbon. This is consistent with our previous results that inhibition of GRASP55/65 by microinjection of GRASP65 antibodies (Wang et al., 2003) or by depletion of GRASP proteins by RNAi (Tang et al., 2010b; Xiang and Wang, 2010) both disrupted the Golgi structure (Xiang and Wang, 2010; Xiang et al., 2013). These results demonstrate that GRASP55/65 function as Golgi stacking factors.

Temperature-sensitive mutant of vesicular stomatitis virus G protein (VSV-G) in cells by viral infection (Xiang et al., 2013). As shown in Figure 6, A and B, single or double deletion of GRASP proteins accelerated VSV-G trafficking indicated by the increased amount of VSV-G protein that is resistant to EndoH treatment by 15 min after the temperature shift.

To test the effect of GRASP deletion on glycosylation of plasma membrane proteins, we performed cell surface staining with fluorescently labeled wheat germ agglutinin (WGA) that binds sialic acid and N-acetylgalactosamine (GlcNAc), and with Maackia amurensis lectin (MAA), which binds α(2,3) sialic acid of glycans. GRASP single-knockout cells displayed a reduction in the intensity of both lectins regardless of the knockdown efficiency, the Golgi stacks are disorganized and the cisternae are not properly aligned, although the Golgi membranes are still concentrated and closely associated with each other (Lee et al., 2014). We speculate that golgins and GRASPs may have different roles in Golgi structure formation: While golgins and membrane-microtubule interactions bring Golgi membranes closer together (Lee et al., 2013), GRASPs function to link the Golgi stacks together to form a ribbon. This is consistent with our previous results that inhibition of GRASP55/65 by microinjection of GRASP65 antibodies (Wang et al., 2003) or by depletion of GRASP proteins by RNAi (Tang et al., 2010b; Xiang and Wang, 2010) both disrupted the Golgi structure (Xiang and Wang, 2010; Xiang et al., 2013). These results demonstrate that GRASP55/65 function as Golgi stacking factors.

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Oligomerization (Tang et al., 2010b; Truschel et al., 2011; Feng et al., 2013), a key property essential for Golgi stacking (Wang et al., 2003, 2005a). The lack of an obvious phenotype in Golgi stacking in the GRASP65 knockout mouse may also be due to the complementation by GRASP55 (Shorter et al., 1999; Lee et al., 2014). Taking the results together, we conclude that a complete knockout of both GRASPs is needed to further evaluate their functions. In this study, we designed multiple sgRNAs targeting to exon 1 of GRASP55 and exon 2 of GRASP65, which are directly together to the pericentriolar region, GRASPs organize them into proper stacks. Consistent with our results, this study showed that knockdown of GRASP55/65 increased CD8 trafficking (Lee et al., 2014). Recently a GRASP65 knockout mouse has been reported, with only limited defects in the structure and function of the Golgi (Veenendaal et al., 2014). One major concern of this knockout mouse is the potential for only partial deletion of the gene, leaving a possibility that an N-terminal 115-amino-acid fragment of GRASP65 may still be translated; this fragment is sufficient for oligomerization (Tang et al., 2010b; Truschel et al., 2011; Feng et al., 2013), a key property essential for Golgi stacking (Wang et al., 2003, 2005a). The lack of an obvious phenotype in Golgi stacking in the GRASP65 knockout mouse may also be due to the complementation by GRASP55 (Shorter et al., 1999; Lee et al., 2014). Taking the results together, we conclude that a complete knockout of both GRASPs is needed to further evaluate their functions. In this study, we designed multiple sgRNAs targeting to exon 1 of GRASP55 and exon 2 of GRASP65, which are directly
GRASP knockout disrupts Golgi structure downstream of the first ATG for translation initiation. Treatment of cells with these sgRNAs resulted in either insertions or deletions that caused a frame shift of the gene with an immediate stop codon, as confirmed by DNA sequencing of individual clones (Supplemental Table S2). This ensures that no functional, truncated proteins are generated in the cell lines. Analysis of these cells with light and electron microscopy demonstrated that double-deletion of both GRASPs completely disrupted Golgi stack formation and ribbon linking. On the basis of these results and previous literature, we conclude that stacking is the primary function of GRASP proteins.

GRASP depletion also accelerates protein trafficking and disrupts glycosylation of cell surface proteins. These results are consistent with our previous study with GRASP depletion by RNAi (Xiang et al., 2013). A plausible explanation for this finding is that when Golgi cisternae are fully stacked, vesicles can form and fuse only at the peripheral area of the cisternae; once the cisternae are unstacked, more membrane area becomes accessible, thereby increasing the rate of vesicle budding and cargo transport through the Golgi (Wang et al., 2008; Wang and Seemann, 2011). In support of this, an in vitro budding assay has consistently demonstrated that unstacking increased the rate of coat protein complex I (COPI) vesicle formation from Golgi membranes (Wang et al., 2008). GRASP proteins have also been implicated in cell cycle control (Sutterlin et al., 2002). In this study, we did not observe significant change in cell growth or apoptosis, similar to GRASP knockdown (Xiang et al., 2013), although more careful characterization of cell growth in these cell lines is needed in the future.

GRASP depletion also altered the glycolipid profile on the cell surface. This could be due to either a shift in the production of GM1 and Gb3 or due to a defect in trafficking. Further characterization of glycolipid trafficking and production in GRASP depleted cells is clearly warranted.

An interesting observation we have made in this study is that when one GRASP protein was deleted, the level of the other GRASP protein often increased. For example, when GRASP65 was deleted, the GRASP55 protein level increased by 1.6-fold (Figure 3). These results reveal an autonomous regulatory mechanism in maintaining the Golgi integrity and function. That is, not only GRASP55 and GRASP65 play complementary roles in Golgi stack formation and function, but also the total amount of GRASP proteins in the cell might...
be also regulated and therefore the total force to hold Golgi cister-
nae into stacks remains consistent. The nature of this mechanism,
including the regulation of GRASP mRNA and protein synthesis
and regulation by cellular metabolic activities, as well as GRASP
targeting and degradation, will be interesting future topics of this
study.

In conclusion, we have generated GRASP55 and GRASP65
single- and double-knockout HeLa and HEK293 cells. Character-
ization of these cell lines demonstrated that GRASP55 or
GRASP65 single-knockout partially impaired Golgi cisternal
stacking, whereas double-knockout of both GRASP proteins dis-
assembled the entire Golgi structure. Furthermore, disassembly
of the Golgi structure not only accelerated protein trafficking but
also impaired accurate glycosylation of cell surface proteins and
lipids. These cell lines provide useful tools to study the mecha-
nism and biological significance of Golgi structure formation and
could potentially be used to study the pathology of diseases in
which the Golgi is defective, such as Alzheimer’s disease
(Nakagomi et al., 2008; Joshi et al., 2014), congenital disorders of
glycosylation (Willett et al., 2013), foot-and-mouth disease (Zhou
et al., 2013), reoxygenation injury (Li et al., 2016), and cancer
(Petrosyan, 2015).

MATERIALS AND METHODS
Reagents, plasmids, and antibodies
All reagents were purchased from Sigma-Aldrich, Invitrogen, Roche,
Calbiochem, and Fisher unless otherwise stated. Antibodies used in
this study include monoclonal antibodies against LAMPI (H4A3;
Developmental Studies Hybridoma Bank [DSHB]), LAMPI (H4B4;
DSHB), integrin β1 (PS5D2, DSHB), integrin α5 (B1G2, DSHB), Shiga
toxin B (A42L, Thermo-Fisher), and β-actin (Sigma); polyclonal anti-
bodies against GRASP55 (ProteinTech), GRASP65 (UT465; Joachim
Seemann, UT Southwestern), GM130 (N73; Joachim Seemann, UT
Southwestern), Golgin-45 (ProteinTech), TGN46 (Bio-Rad), and
GFP (Santa Cruz). Vascular stomatitis virus G protein (VSVG)-GFP
adenovirus was a gift from David Sheff (University of Iowa) and
Heike Fölsch (Northwestern University). The pUC19 plasmid was a
gift from Daniel Klionsky (University of Michigan). pSpCas9(BB)
2A-GFP(PX458), pSpCas9(9B)-2A-Puro (PX459) plasmids are from
Addgene. Other materials used in this study include the following:
tetramethylrhodamine (TRITC)-WGA (EY laboratories), TRITC-
MAA (EY laboratories), fluorescein isothiocyanate (FITC)-cholera
toxin B subunit (C1655, Sigma), Shiga toxin type 1 Subunit B (NR-
860, BEI Resources), and puromycin (Thermo-Fisher).

CRISPR-Cas9 knockout of GRASP genes
Guide RNA sequences targeting the GRASP55 and GRASP65 ge-
etic loci were designed using the MI T Zhang Lab sgRNA Design
Tool (crispr.mit.edu). Duplexed sgRNA oligos purchased from Invit-
rogen were digested and ligated into pSpCas9(BB)-2A-GFP(PX458)
and pSpCas9(9B)-2A-Puro(PX459) to generate GRASP55/65 GFP or
Puro plasmids, respectively. CRISPR knock-out cells were generated
by transfection with GRASP55/65 GFP or Puro plasmids followed by
enrichment of GFP-expressing cells by FACS sorting or by selection
with 1 μg/ml puromycin, respectively. Individual clones were gen-
erated by plating cells at low density and isolating individual colonies.
GRASP knockout was confirmed by Western blotting, immunofluo-
rescence, and DNA sequencing. For sequencing of GRASP55 and
GRASP65, genomic loci were amplified by PCR using the following
primers:

GRASP55 (5′-CCCGGATCCCTCTGTTGTTGATGGTGCTGCT3′, 3′-
GATCCTCTAGAGCAGGCTTCCCCACAG-3′, 3′-CCCAAGCTTCTCCAGGGCCTCCCTCTCA-5′),
GRASP65 (5′-CCCG-

PCR products were cloned into pUC19, and the DNA sequence
of 10–20 clones from each cell line was determined by Sanger Se-
quencing at the University of Michigan DNA Sequencing Core using
M13Rev standard sequencing primer. The sequencing results are
aligned with NCBI Reference Sequences of GRASP55 (NM_015530.4)
and GRASP65 (NC_000003.12).

Immunofluorescence microscopy
Immunofluorescence was performed as described previously (Wang
et al., 2005a; Tang et al., 2010a). Briefly, HeLa and HEK293 cells were
fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline
(PBS) for 15 min followed by quenching with 50 mM NH4Cl in PBS
for 10 min and permeabilization with 0.1% Triton X-100 in PBS for 10
min. Permeabilized cells were blocked with 2% bovine serum albu-
umin (BSA) in PBS for 1 h followed by incubation with primary anti-
odies for 1.5 h, washed with PBS, and incubated with secondary
antibodies for 45 min at room temperature. DNA was stained with
Hoechst (1 μg/ml), and coverslips were mounted with Moviol. Wide-
field fluorescence microscopy was performed on a Zeiss Observer
Z1 using a 63×/1.4 oil objective at a Z-step of 0.5 μm. Standard con-
focus microscopy was performed on a Leica SP5 using a 63×/1.4 oil
objective at a 400-Hz scan rate in a 512 × 512 scan field with a Z-step
of 0.5 μm. Airyscan confocal images were collected using an LSM
880 confocal system outfitted with an Airyscan detector (Carl Zeiss,
Jena, Germany) to optimize resolution of fixed cell preparations.
Briefly, multitrack acquisition was performed with 405-, 488-, and
633-nm wavelengths using a Plan-Apochromat 63×/1.4 oil objective.
Images were scanned with a pixel scaling of 37 nm in XY with a Z-
step of 144 nm. Resulting emission was centered on a 32-element
GaAsP-based spatial detector, and channels were reassigned to a
central GaAsP element to effectively reduce imaging volumes from
1.25 Aiy units to 0.2 Aiy units. This reassignment step was per-
formed via the ZEN software (Carl Zeiss) using a recommended Wie-
er filter parameter for weighing noise in given images. Final images
were assessed against confocal (nonAiryscan) images to ensure arti-
fact-free improvements in resolution and signal to noise.

Electron microscopy
EM was performed as previously described (Tang et al., 2010a).
Briefly, cells were plated in six-well dishes. After 24 h of culture,
cells were fixed with 2.5% glutaraldehyde and then processed for
Epon embedding. Sections of 60 nm were mounted onto Formvar-
coated nickel grids and double contrasted with 2% uranyl acetate
for 5 min and 3% lead citrate for 5 min. Grids were imaged using a
Philips CM100 Biotwin and JEOL JEM-1400 transmission electron
microscope. EM images were taken from the perinuclear region of
the cell where Golgi membranes were normally concentrated.
Golgi stacks and Golgi clusters were identified using morphologi-
cal criteria and quantitated using standard stereological tech-
niques. For HeLa wild-type and GRASP knockout cells, the profiles
had to contain a nuclear profile with an intact nuclear envelope.
A cisterna was defined as a membrane-bound structure in the Golgi
cluster whose length is at least 4 times its width, normally 20–30 nm
in width and longer than 150 nm (Luccoq et al., 1989), and a stack
is the set of flattened, disk-shaped cisternae resembling a stack of
plates. Multiple unstacked cisternae and vesicles were counted as
disorganized membrane clusters, whereas stacked structures with
two or more cisternae were counted as distinguishable Golgi stacks
and only stacked structures with well aligned, smooth, normal-
length cisternae were counted as well-organized Golgi stacks.
Lectin staining
Lectin staining was performed as previously described (Willett et al., 2013). In short, cells were grown on coverslips to 70% confluence then incubated with ice-cold PBS for 15 min followed by fixation with 1% PFA for 15 min and quenching with 50 mM NH₄Cl for 10 min. Cells were then washed three times with PBS and blocked in 1% BSA in PBS for 30 min at room temperature. After blocking, cells were incubated with fluoresceine-conjugated lectins for 30 min at room temperature followed by three 10-min PBS washes and mounted onto glass slides. The following lectins (and working concentrations) were used: TRITC-WGA (2 μg/ml) and TRITC-MAA (20 μg/ml).

Fluorescence-activated cell sorting
Flow cytometry analysis for lectin staining was performed as previously described (Bailey Blackburn et al., 2016). In short, cells were grown to 70% confluency and then incubated with ice-cold PBS for 15 min followed by treatment with 20 mM EDTA for 5 min. Cells were collected after treatment with 20 mM EDTA for 5 min and resuspended in 0.1% BSA/PBS. After blocking on ice for 30 min, cells were incubated with fluoresceine-conjugated lectins in 0.1% BSA with indicated concentration for 30 min while shaking on ice followed by washing with ice-cold PBS. Cells were then fixed with 1% PFA for 15 min and quenched with 50 mM NH₄Cl for 10 min before submitted for flow cytometry. Cells were analyzed using the LSRFortessa (BD Biosciences) flow cytometer gated for correct cell size versus complexity and fluorescence intensity. Analysis was done using FCS Express 6 software. Fluorescence intensity was quantified from 10,000 cells from three replicates. Statistical significance was determined by performing a two-tailed Student’s t test.

VSV-G trafficking assay
VSV-G trafficking assay was performed as previously described (Xiang et al., 2013). Briefly, cells were plated in 3.5-cm dishes and cultured overnight. Subsequently the medium was removed and serum-free medium was added that contained VSV-G-ts045-GFP adenovirus. Following a 2 h incubation with the virus, the VSV-G–containing medium was removed, and cells were grown in full medium at 40.5°C for 16 h. Cells were then treated with 0.1 mM cycloheximide for 1 h prior to shifting the temperature to 32°C. Cells were harvested at the indicated time points, and an Endo-H assay was performed.

Shiga toxin and cholera toxin binding assay
Shiga toxin and cholera toxin assay were performed as previously described (Selyunin and Mukhopadhyay, 2015). In short, cells were plated on coverslips and cultured overnight. Cells were then washed three times with cold PBS and incubated with 4 μg/ml purified Shiga toxin 1B subunit in cold medium for 30 min on ice. After being washed three times with ice-cold PBS, cells were fixed with 4% PFA and quenched with 50 mM NH₄Cl, followed by subsequent incubation with an anti-Shiga toxin B primary antibody for 1.5 h and secondary antibody for 1.5 h at room temperature. DNA was stained with Hoechst. Coverslips were mounted with Moviol. Images were taken on a Leica SP5 confocal microscope.

For cholera toxin binding, cells were plated onto coverslips and cultured overnight. Cells were washed three times with ice-cold PBS and incubated with 4 μg/ml FITC-conjugate cholera toxin B subunit in cold media for 30 min on ice. Cells were then washed three times with ice-cold PBS and fixed with 4% PFA, quenched with 50 mM NH₄Cl, and stained for DNA with Hoechst. Images were taken on a Leica SP5 confocal microscope.

For the addback experiment, GRASP double-knockout cells were transfected with GRASP55-mCherry and either GRASP65-myc (cholera toxin assay) or GRASP65-GFP (Shiga toxin assay). Cells were processed as previously described but with minor alterations to the cholera toxin assay protocol to enable visualization of exogenously expressed GRASP proteins and toxin labeling. Briefly, after toxin binding and fixation, cells were subject to permeabilization with 0.1% Triton X-100 in PBS and blocking in 1% BSA in PBS followed by immunofluorescence using a myc antibody.

Quantification and statistics
In all figures, the quantification results are expressed as the mean ± SEM from three independent experiments, unless otherwise stated. The statistical significance of the results was assessed using a two-tailed Student’s t test. For quantitation of VSV-G trafficking, a one-tailed Student’s t test was used to determine statistical significance; *p < 0.05; **p < 0.01; ***p < 0.001 in all figures.

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