FEBS Letters

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RESEARCH LETTER

Protein sorting upon exit from the endoplasmic reticulum dominates Golgi biogenesis in budding yeast

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(Received 30 November 2023, revised 15 January 2024, accepted 26 January 2024, available online 23 February 2024)

doi:10.1002/1873-3468.14830

Edited by Lukas Alfons Huber

Cells sense and control the number and quality of their organelles, but the underlying mechanisms of this regulation are not understood. Our recent research in the yeast *Saccharomyces cerevisiae* has shown that long acyl chain ceramides in the endoplasmic reticulum (ER) membrane and the lipid moiety of glycosylphosphatidylinositol (GPI) anchor determine the sorting of GPI-anchored proteins in the ER. Here, we show that a mutant strain, which produces shorter ceramides than the wild-type strain, displays a different count of Golgi cisternae. Moreover, deletions of proteins that remodel the lipid portion of GPI anchors resulted in an abnormal number of Golgi cisternae. Thus, our study reveals that protein sorting in the ER plays a critical role in maintaining Golgi biogenesis.

Keywords: ceramide; endoplasmic reticulum; Golgi biogenesis; GPI; lipid remodeling; protein sorting

Cells meticulously regulate the abundance of their organelles, yet the fundamental question concerning the precise mechanisms governing organelle number control remains largely unexplored [1,2]. Variation in organelle number is determined by the balance between *de novo* synthesis from pre-existing membrane sources, organelle division and fusion, and distribution and degradation by cell division and autophagy, respectively.

The Golgi apparatus, as the central trafficking hub of the cell, orchestrates the transport of membrane and secretory proteins in the exocytic and endocytic pathways and mediates post-translational modifications and sorting of proteins and lipids [3]. The Golgi is structurally unique, and in most fungi, plants, and invertebrates, is composed of stacks of flattened cisternae. The Golgi stack is composed of biochemically distinct sub-compartments, with

Abbreviations
COPII, coat protein complex II; ER, endoplasmic reticulum; ERESs, ER exit sites; GPI, glycosylphosphatidylinositol; GPI-APs, GPI-anchored proteins; SD, synthetic defined minimal; TGN, trans-Golgi network.

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cargo molecules transported from the ER residing sequentially in cis, medial, and trans cisternae and exiting via the trans Golgi network (TGN) [4,5]. Although the functional significance of stacking remains largely unknown, one study suggested that Golgi stacking serves as a quality control mechanism for cargo modification and sorting by reducing the rate of transport. This model has been proposed based on the observation that destruction of the Golgi stack accelerates protein trafficking in the Golgi, but impairs N-glycosylation and sorting of proteins [6]. In contrast, in the budding yeast *Saccharomyces cerevisiae*, there is no stacked Golgi structure, and individual cisternae are dispersed throughout the cytoplasm [7,8]. This feature offered an opportunity to test the cisternal maturation model, and it was demonstrated that each Golgi cisterna in *S. cerevisiae* matures from cis to trans [9,10]. Based on the finding, the number of individual Golgi cisternae would depend on maturation processes with disappearance and appearance.

The Golgi apparatus relies on a supply of lipids and proteins, which are received from coat protein complex II (COPII) vesicles that bud from specialized domains of the endoplasmic reticulum (ER) membrane known as ER exit sites (ERESs) and mediate the vesicular transport of cargo from ER to Golgi [11]. The ER membrane of *S. cerevisiae* is also known to serve as a site for protein sorting [12,13]. Glycosylphosphatidylinositol (GPI)-anchored proteins are sorted to selective ERESs that differ from those for non-GPI-anchored membrane proteins [14–16]. While the physiological significance of this sorting remains to be explored, it is conceivable that the specific allocation of GPI-anchored proteins to designated ERESs may play an important role in the efficient transport to the plasma membrane. If so, mutations that cause perturbations in sorting processes may impact the biogenesis of the Golgi cisternae, which are crucial transit sites in vesicular transport to the plasma membrane.

In this study, we took advantage of the unstacked and dispersed nature of Golgi/TGN cisternae in the budding yeast *S. cerevisiae* to assess the role of protein sorting in Golgi biogenesis. We used mutant strains defective in GPI-anchored protein sorting and showed that the mutants affect the number of Golgi cisternae. Our data provide insight into the physiological role of cargo sorting on Golgi biogenesis.

Materials and methods

Yeast strains, plasmids, and media

Strains of *S. cerevisiae* used for this study are listed in Table S1. RH6095 and RH6979 yeast strains were the kind gifts from Dr. H. Riezman. Other deletion strains were created by the PCR-based one-step gene replacement method. pRS316 (p*TDH3*)-*EGFP-RER1* (CEN, *URA3*), pRS316(p*TDH3*)-*EGFP-GOS1* (CEN, *URA3*), and pRS316(p*ADH1*)-*SEC7-EGFP* (CEN, *URA3*) plasmids were kindly provided by Dr. A. Nakano. Yeast strains carrying plasmids were maintained in synthetic defined minimal (SD) media containing 2% glucose (2% glucose and 0.67% yeast nitrogen base) supplemented with the appropriate amino acids and bases.

Fluorescence microscopy

To assess the numbers of Golgi cisternae, cells expressing GFP-Rer1, GFP-Gos1, or Sec7-GFP were grown in SD medium lacking uracil and visualized by fluorescence microscopy. An appropriate filter setting was used to visualize GFP. The imaged micrographs were analyzed by the IMAGE J open source software (developed at the National Institutes of Health, MD), and the number of fluorescent punctae per cell was counted.

Statistical analysis

Statistical analysis was performed using Tukey–Kramer multiple comparison test. For all tests, P values are classified; $*P < 0.05$ and $***P < 0.001$ and ns – not significant, > 0.05.

Modeling and simulation

Modeling and simulation were conducted using CELLDESIGNER 4.4.2 (https://www.celldesigner.org/). The transport diagram (shown in Fig. S1A) was constructed as per the model depicted in Fig. 4, and mass action kinetics were applied to each edge. Rate constants for mass action, designated as k1 through k8, were assigned. For simulations of mutants unable to form the ERES for GPI-anchored proteins, k5 was set to 0. Simulations for normal transport from transport vesicles to Ld domain, and for traffic jams caused by incomplete formation of Ld and Lo domains, were conducted with k4 set at 0.1 and 0.05, respectively. The steady-state number of vesicles obtained from the simulation was graphed in Fig. S1B. The model file was attached as 'Transport_model.xml'.

Results and Discussion

The number of all Golgi cisternae increases throughout the cell cycle progression

In the budding yeast *S. cerevisiae*, where Golgi cisternae are not stacked but dispersed, the markers for individual Golgi compartment are well established [10]. To determine the number of Golgi, we first used a fluorescently tagged GFP-Rer1 as a marker for cis-

Golgi [17] and quantified the number of fluorescent punctae per cell by fluorescence microscopy. In vegetatively growing wild-type cells, the mean value of the number of cis-Golgi per cell was found to be 24 (Fig. 1A). To investigate if the number of Golgi per cell varies between Golgi cisternae at different maturation levels, we next expressed GFP-Gos1 and Sec7-GFP, markers for medial-Golgi and trans-Golgi/TGN, respectively [10], and measured the numbers of their punctae per cell. We found that the average number of cis-Golgi per cell is similar to that of medial-Golgi, but they are greater than the average number of trans-Golgi/TGN. This suggests that maturation process from the medial-Golgi to the trans-Golgi/TGN is a rate-limiting step for Golgi biogenesis.

If the numbers of Golgi compartments in the mother cell are constant, the numbers of Golgi in the budding cell must be increased during cell cycle progression because Golgi compartments are inherited by daughter cells either through the formation of new Golgi cisternae by membranes exported from the ER or the input of existing Golgi compartments [7,18]. To test this prediction, we divided living wild-type cells proceeding through the cell cycle into three types; (1) cells without budding (G1 phase), (2) cells with budding a small daughter cell (S and G2/M phases), and (3) cells with the daughter and mother cells of approximately the same size (anaphase/telophase), and analyzed the number of Golgi in each cell. As expected, the number of cis-Golgi in cells at G1 phase was lower than that at S-G2/M phase, and the number in S-G2/M phase cells was lower than that in anaphase/telophase cells (Fig. 1B). Similar patterns were observed for medial-Golgi and trans-Golgi/TGN compartments (Fig. 1C,D). These results indicate that number of Golgi cisternae increases throughout the cell cycle progression. Notably, the average number of cis-Golgi in anaphase/telophase cells was 34, and dividing that value by two yielded a value close to the average number of cis-Golgi in G1 phase, 15. Similar results were also obtained for medial-Golgi and trans-Golgi/TGN. Thus, these results suggest that cells attempt to distribute a certain number of Golgi cisternae or maintain Golgi-to-cell volume ratio.

Sorting of GPI-anchored proteins into selective ERESs is required for maintaining the number of Golgi

Proteins and lipids required for maintenance of cis-Golgi are supplied by COPII vesicles. The ER serves not only as the site of COPII vesicle formation but also in budding yeast as the site of protein sorting. It has been known that GPI-anchored proteins and non-GPI-anchored proteins prior to budding are incorporated into different ERES, leading to the delivery of these proteins to the Golgi apparatus in distinct ER-derived COPII vesicles [12,14–16]. However, it remains unknown if this sorting is linked to Golgi biogenesis. To test whether defects in sorting of GPI-anchored proteins have an impact on number of Golgi, we first used an engineered GhLag1 strain, in which endogenous ceramide synthases were replaced by a cotton gene GhLag1, resulting in a cell producing C18 rather than C26 ceramides [19]. In the GhLag1 mutant cells, a GPI-anchored protein with a C26 ceramide moiety Gas1 fails to form clusters and cannot be sorted into selective ERESs [15]. We showed that in this mutant cell at G1 phase level, the numbers of cis-Golgi and medial-Golgi decreased, but the number of trans-Golgi/TGN was not affected (Fig. 2).

This result led us to investigate the impact of deleting other genes involved in protein sorting on Golgi biogenesis. The short unsaturated 2-acyl chain of diacylglycerol of the GPI lipid moiety is replaced by a very long-chain saturated fatty acid (C26:0), and this replacement correlates with the association of GPI-anchored proteins with lipid rafts and their sorting during ER exit [20–22]. As Gup1 acts as an enzyme that adds C26:0 fatty acid to the sn2 position of the GPI-lipid [23], we analyzed whether deletion of Gup1 affects the number of Golgi cisternae. Like GhLag1, the *gup1*Δ expressing Golgi markers exhibited decreased numbers of cis-Golgi and medial-Golgi, while it showed approximately the same number of trans-Golgi/TGN (Fig. 3). Similar results were also obtained with a deletion strain lacking Per1, an enzyme that functions upstream of Gup1 to remove the short unsaturated 2-acyl chain [24]. Thus, these results suggest that sorting of GPI-anchored proteins is required for maintaining the number of Golgi.

To date, it is known that GPI-anchored proteins and non-GPI-anchored proteins are sorted in the ER and transported to the cell surface via the Golgi apparatus in *S. cerevisiae* [12], but the physiological significances of protein sorting remain unclear. Our data show that mutant strains defective in the sorting of GPI-anchored proteins have decreased numbers of cis-Golgi and medial-Golgi. Since GPI-anchored proteins are selectively incorporated into specific COPII vesicles [14–16], the diminished number of formed COPII vesicles resulting from the disrupted sorting process may significantly impact the abundance of cis-Golgi. In connection with this, traffic from the ER to the cis-Golgi in *S. cerevisiae* has been considered to operate in a way that cis-Golgi approaches and contacts the ERES, and concomitantly

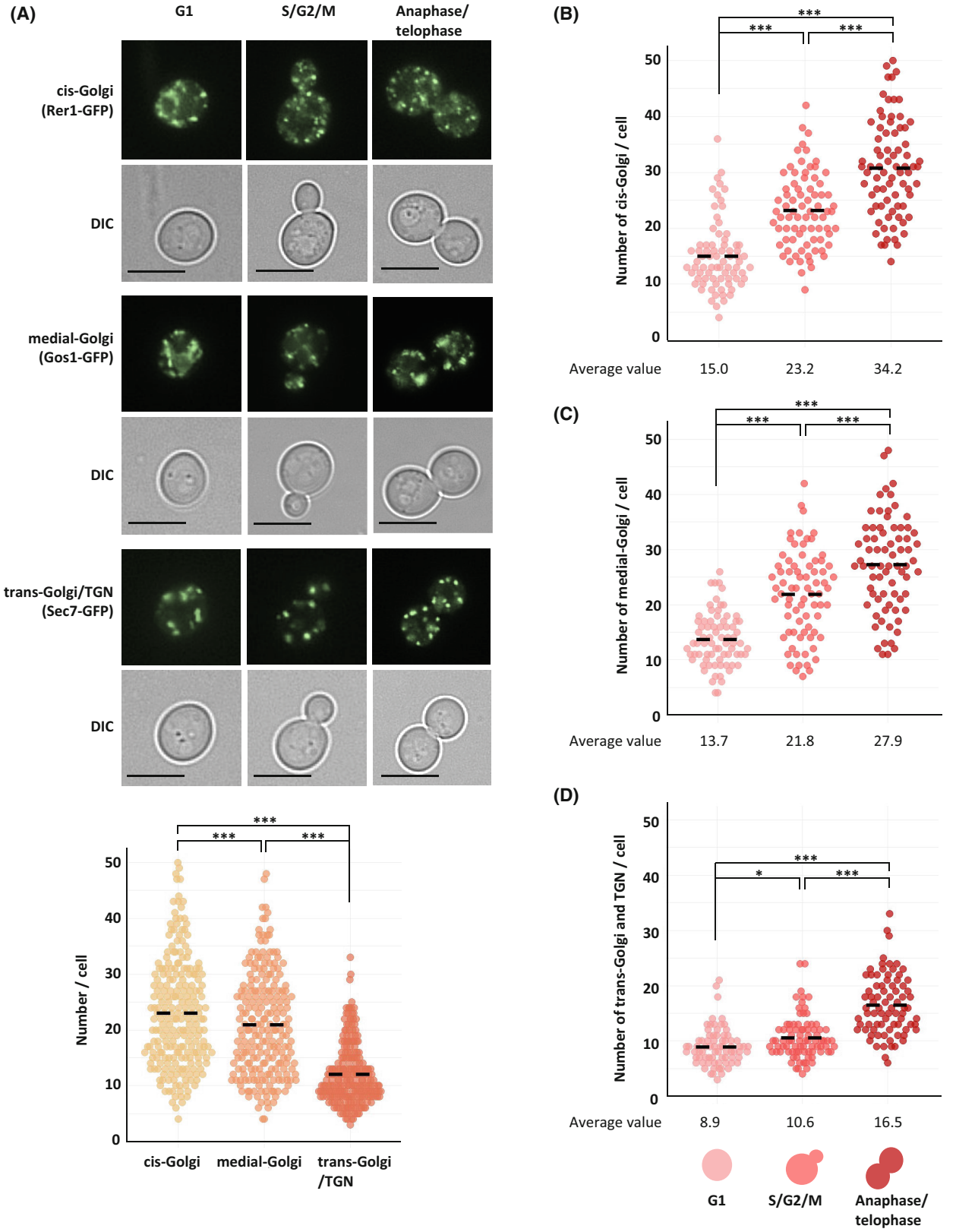

Fig. 1. The number of Golgi cisternae is increased throughout the cell cycle. (A) Wild-type (RH6095) cells expressing GFP-Rer1 (cis-Golgi), GFP-Gos1 (medial-Golgi), or Sec7-GFP (trans-Golgi/TGN) were inoculated in SD medium lacking uracil at 25 °C for 24 h and visualized by fluorescence microscopy. Scale bar, 5 μm. The number of fluorescent punctae per cell was measured. At least 200 cells were evaluated for each sample per experiment, and data represent one of three reproducible and independent experiments. Tukey's test: $*P \leq 0.05$, $***P \leq 0.001$, ns – not significant. (B–D) After dividing the cells into three categories; G1 phase (G1), S to G2/M phase (S-G2/M), and anaphase to telophase (A/T), the number of fluorescent punctae of cis-Golgi (B), medial-Golgi (C), or trans-Golgi/TGN (D) per cell at their different cell cycle stages was measured and more than 75 cells were evaluated for each sample per experiment.

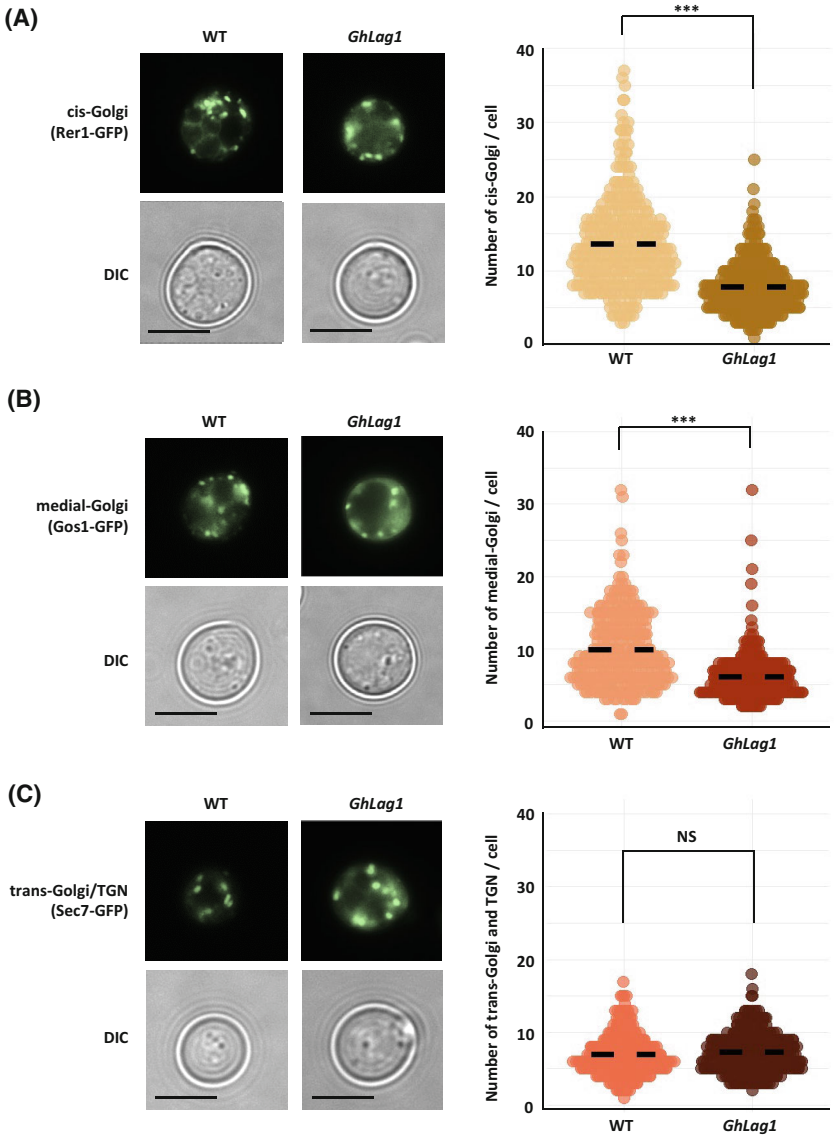

Fig. 2. Ceramide acyl chain length plays an important role in maintaining the number of Golgi. (A–C) Wild-type (RH6095) and GhLag1 (RH6979) strains expressing Golgi markers (A, cis-Golgi; B, medial-Golgi; C, trans-Golgi/TGN) were inoculated in SD medium lacking uracil, visualized by fluorescence microscopy, and the number of fluorescent punctae per cell at G1 phase was measured and more than 300 cells were evaluated for each sample per experiment as described in Fig. 1. Scale bar, 5 μm.

with the collapse of COPII coats, fuse with COPII vesicles at the ERES and then captures cargo [11,25]. As cis-Golgi matures into medial-Golgi, a decreased number of cis-Golgi will naturally result in a reduction in the number of medial-Golgi. It is also possible that a stimulated maturation rate from cis-Golgi to trans-

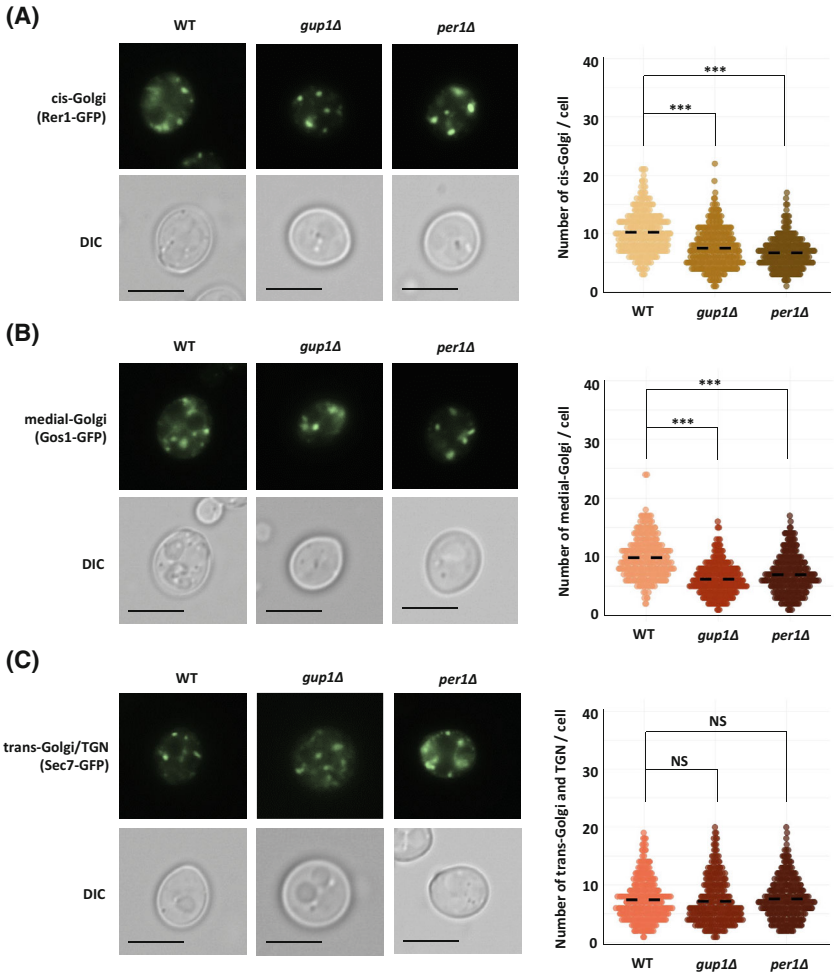

Fig. 3. Lipid remodeling of GPI-anchored proteins is required for Golgi biogenesis. (A–C) Wild-type (FKY4602), *per1*Δ(FKY5375), and *gup1*Δ (FKY4881) strains expressing Golgi markers (A, cis-Golgi; B, medial-Golgi; C, trans-Golgi/TGN) were inoculated in SD medium lacking uracil, visualized by fluorescence microscopy, and the number of fluorescent punctae per cell at G1 phase was measured and more than 300 cells were evaluated for each sample per experiment as described in Fig. 1. Scale bar, 5 μm.

Golgi is responsible for the decreased number of cis- and medial-Golgi. Remarkably, we found that the number of trans-Golgi/TGN does not decrease in mutants defective in GPI-anchored protein sorting. This may suggest that traffic from trans-Golgi/TGN gets stuck due to impaired protein transport at the levels of post-Golgi in the mutants (Fig. 4). It is believed that the plasma membrane is organized into domains of different protein and lipid composition, and GPI-anchored proteins are enriched in liquid-ordered (Lo) domains distinct from non-GPI-anchored proteins [26,27]. Given this model, it is conceivable that the trans-Golgi/TGN-derived transport vesicles containing missorted GPI-anchored proteins may not be able to take the direct route to the Lo domain of the plasma membrane, resulting in delayed transport and no reduction in the number of trans-Golgi/TGN. Although how different types of cargoes are distributed and behave remains elusive [28], if GPI-anchored and non-GPI-anchored proteins are equally distributed in the Golgi cisternae and do not mix through the maturation process, the above hypothesis was confirmed by our simple mathematical model (Fig. S1A,B). Specifically, the deficiency of ERES for GPI-anchored proteins leads to a reduction in the number of cis- and medial-Golgi. However, if this is accompanied by an incomplete formation of the Ld and Lo domains, resulting in reduced transport from the trans-Golgi to the plasma membrane, then the number of trans-Golgi/TGN does not decrease. The delayed anterograde transport at the post-Golgi level may affect

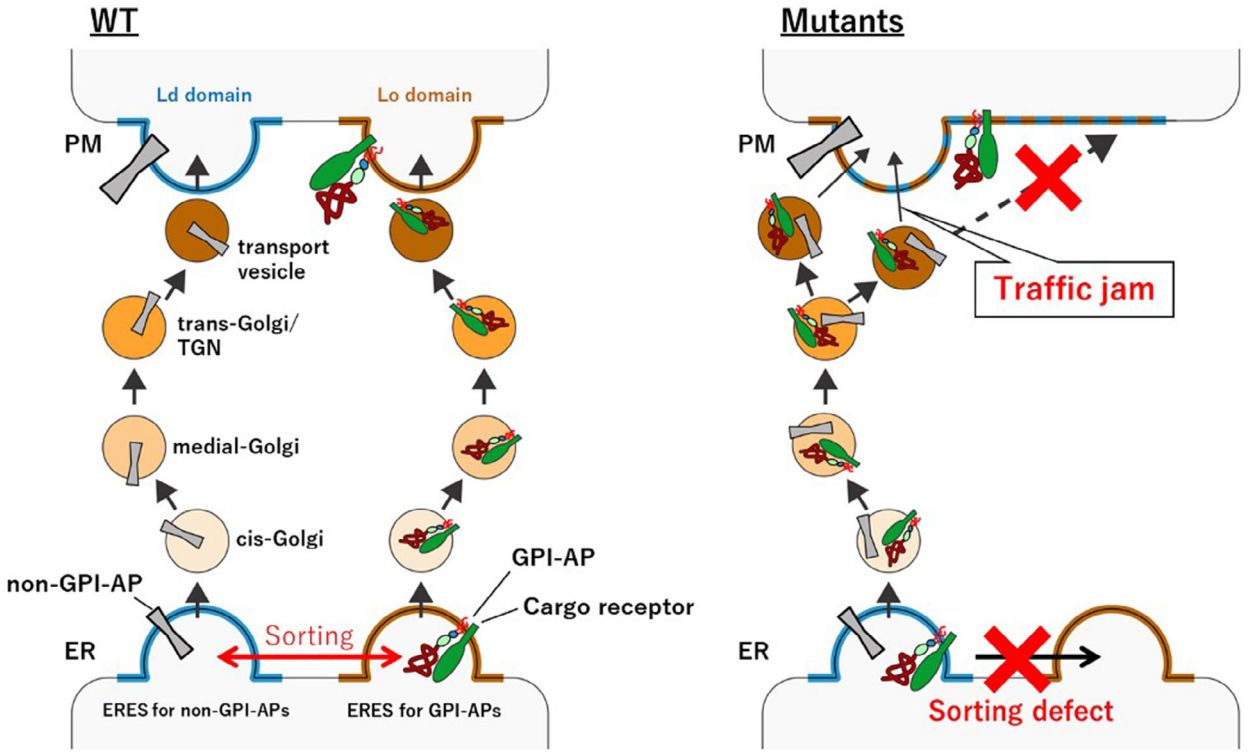

Fig. 4. A model for Golgi biogenesis dominated by cargo sorting in the ER. In wild-type cells, GPI-anchored proteins (GPI-APs) and non-GPI-APs are sorted in the ER and incorporated into different COPII vesicles. They are transported to the cis-Golgi compartments, which mature separately, and fuse with their corresponding domains of the PM to ensure efficient transport. On the other hand, in the mutants defective in cargo sorting in the ER, GPI-APs, and non-GPI-APs are incorporated into the same COPII vesicles and transported together to the TGN. As definite domains corresponding to those cargoes no longer exist in the PM, the PM sites where the transport vesicles from the TGN fuse may be limited, leading to a traffic jam at the post-trans-Golgi/TGN.

retrograde direction through COPI-coated vesicles, which is linked to the maintenance of Golgi cisternae. Thus, protein sorting in the ER plays a critical role in maintaining Golgi biogenesis. This study supports our previously proposed model [22] that proteins sorted in the ER are transported to their respective domains of the plasma membrane via at least two distinct lines, with maturation of the Golgi, to ensure their efficient transport.

Acknowledgements

We thank Dr Howard Riezman and Dr Akihiko Nakano for providing yeast strains and plasmids, respectively. This work was supported by Japan Society for the Promotion of Science (JSPS), Grants-in-Aid for Scientific Research (KAKENHI) (19H02922 to KF), grant PID2020-119505GB-I00/AEI/10.13039/501100011033 to MM, and grants co-financed by the Junta de Andalucía and the ERDF PY20_01240 and US-1380893 to MM.

Author contributions

SS, PS, KH, and HN performed the experiments and analyzed the data. HM performed modeling and simulation analysis. MM and KF conceived and designed the experiments. PS, HM, MM, and KF contributed to the writing of the manuscript.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/1873-3468.14830.

Data accessibility

The data that support the findings of this study are available in Figs 1–4 and supporting information. Further information can be requested from the corresponding authors (mmuniz@us.es, kfunato@hiroshima-u.ac.jp).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Modeling of traffic jam.

Table S1. Strains of *Saccharomyces cerevisiae* used for this study.