Retro-2 alters Golgi structure

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Retro-2 directly interacts with an ER exit site protein, Sec16A, inhibiting ER exit of a Golgi tSNARE, Syntaxin5, which results in rapid re-distribution of Syntaxin5 to the ER. Recently, it was shown that SARS-CoV-2 infection disrupts the Golgi apparatus within 6–12 h, while its replication was effectively inhibited by Retro-2 in cultured human lung cells. Yet, exactly how Retro-2 may influence ultrastructure of the Golgi apparatus have not been thoroughly investigated. In this study, we characterized the effect of Retro-2 treatment on ultrastructure of the Golgi apparatus using electron microscopy and EM tomography. Our initial results on protein secretion showed that Retro-2 treatment does not significantly influence secretion of either small or large cargos. Ultrastructural study of the Golgi, however, revealed rapid accumulation of COPI-like vesicular profiles in the perinuclear area and a partial disassembly of the Golgi stack under electron microscope within 3–5 h, suggesting altered Golgi organization in these cells. Retro-2 treatment in cells depleted of GRASP65/55, the two well-known Golgi structural proteins, induced complete and rapid disassembly of the Golgi into individual cisternae. Taken together, these results suggest that Retro-2 profoundly alters Golgi structure to a much greater extent than previously anticipated.

The Golgi apparatus plays important roles in post-translational modifications of newly synthesized proteins and is also considered as a major sorting center at the cross-road of the secretory and the endocytic pathways. Its stacked architecture, a hallmark feature of the Golgi apparatus, is crucial for its post-translational role and is known to be mediated by a group of Golgin tethers, including GM130 and Golgin45, and two Golgi Reassembly and Stacking proteins (GRASPs; GRASP65/55), and the Golgi undergoes dynamic disassembly and reassembly during mitotic cell division. GRASPs-mediated Golgi stacking by their PDZ domain interaction in trans had been widely accepted in the field. However, two recent studies conclusively showed that GRASPs are not required for Golgi stacking in vivo, but necessary for Golgi ribbon formation. On the other hand, Golgins, such as GM130, seem to be essential for SNARE-mediated membrane fusion of mitotic Golgi membranes during post-mitotic Golgi reassembly. GM130 had been shown to directly interact with two Rab-GTPases, Rab1 and Rab33b as well as Golgi tSNARE Syntaxin5. While it is still largely unknown by what mechanism these Golgins contribute to Golgi stack assembly or maintenance, recent studies suggest that many of these Golgins may phase-separate to form liquid-like condensates, potentially contributing to Golgi stack assembly/orientation. Overall, exact mechanism of cisternal stacking/adhesion still remains elusive after many years of research.

We had previously shown that the two Syntaxin5-interacting Golgins, GM130 and Golgin45, could substitute for GRASPs to such an extent that their exogenous over-expression could create morphologically and functionally normal Golgi stacks in GRASP65/55-depleted mammalian cells, suggesting that the two Golgins and GRASPs may play extensively complementary roles in Golgi structure maintenance. Under steady state condition, the more enigmatic Golgin, Golgin45, forms a multimeric protein complex with Acyl-CoA Binding-Domain-3 (ACBD3), GRASP55, Rab2-GTP and TBC1D22, a Rab-GTPase activating protein, which seems to assist in domain organization and regulate Golgi structural proteins. In addition, Golgin45 was also shown to recruit Tankyrase1 (TNKS1), a regulator of Wnt-signaling and telomere maintenance, to the Golgi and is subjected to TNKS1-dependent poly (ADP-Ribosyl)ation and subsequent proteasomal degradation, which greatly influences anterograde cargo secretion and protein N-glycosylation. Strikingly, inter-cisternal membrane fusion frequently occurs in cells simultaneously depleted of GM130 and Golgin45 or in cells transfected with a Golgin45 mutant (D171A) that abrogates the interaction between Golgin45 and Syntaxin5, suggesting that Golgin-Syntaxin5 interaction may contribute to structural integrity of the Golgi stack during interphase. In that study and a previous study by Wang's group, RNAi-mediated depletion of Golgin45 and GRASP55 were shown to result in statistically significant reduction in the number of cisternae per stack. Overall, these studies point to a possibility that Golgin45 and GRASP55 may not be required

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Syntaxin5-binding Golgin tethers. in Golgi cisternal adhesion and maintenance of Golgi structure during interphase by interacting with various possibilities that a certain percentage of these vesicles may be COPII vesicles, arriving from the ER.

Retro-2 treatment results in accumulation of COPI-like vesicles at the Golgi. Results and discussion

Retro-2 treatment results in accumulation of COPI-like vesicles at the Golgi.

COPI-like vesicular profiles accumulate in the peri-Golgi region of HeLa cells treated with Retro-2. As accumulation of these vesicular profiles occurred very rapidly within a few hours in peri-Golgi area, we reasoned that these vesicles could have been derived from the Golgi itself, but unable to fuse back with the Golgi, due to lack of the essential Golgi SNARE, Syntaxin5 by Retro-2 treatment. Alternatively, a portion of these vesicles could be COPII vesicles from the ER, but unable to fuse with the Golgi. To help understand the nature of these vesicular profiles, we measured diameter of a large number of these accumulated vesicles and found that they display an average diameter of 57 ± 7.4 nm with a median diameter of 55 nm (n = 213) (Fig. 2A). This measured diameter closely matched the observed size of uncoated COPI-vesicles, which had been shown to be ranging from ~ 60 nm in in vivo studies to 45 ± 6 nm in an in vitro study28–30, suggesting that COPI-vesicles may account for a significant fraction of these accumulated vesicular profiles, although we cannot rule out the possibility that a certain percentage of these vesicles may be COPII vesicles, arriving from the ER.

Anterograde secretion of cargo proteins remains unaffected by Retro-2 treatment. In order to study how Retro-2 treatment and resulting vesicle accumulation may influence basic functions of the Golgi apparatus, we tested cargo secretion using ss-HRP and collagen IV23. The results showed that secretion of both small cargo, such as ss-HRP and large cargo, collagen IV, are largely unaffected in the presence of Retro-2 (Fig. 2B–D). These results are consistent with a prevalent view in the field that COPII vesicles are mainly responsible for retrograde transport of Golgi resident glycosyltransferases, whereas cisternal maturation or rim progression...
Figure 1. Acute displacement of Syntaxin5 by Retro-2 results in significant turnover of Golgi membranes to vesicular profiles, accumulated in peri-Golgi area. (A) Retro-2 treatment re-distributes Syntaxin5 from the Golgi within ~5 h. Confocal micrographs showing that Syntaxin5 is largely absent at the Golgi in HeLa cells treated with 25 μM Retro2 for 5 h. Golgi markers, GT-GFP and GM130, did not show any noticeable change in Retro2-treated cells. (scale bar 10 μm). (B,C) 3D-SIM images of the Golgi, stained with anti-GM130 (cis-Golgi) and anti-Golgin97 (trans-Golgi) or anti-Giantin (Golgi rim) and anti-Golgin97 antibodies in cells treated with either DMSO (control) or 25 μM Retro-2 for 5 h. Note that Golgi ribbon structure still localized to perinuclear region in both DMSO and Retro-2 treated cells (scale bar 2 μm). (D,E) Representative EM photos showing accumulation of vesicular profiles in Retro-2-treated cells. We observed striking amount of vesicular profiles in Retro-2-treated cells, but failed to notice any significant Golgi disassembly or cisternal dilation, as often observed in GRASP or Golgin knockdown experiments. M mitochondria, N nucleus, G Golgi apparatus (scale bar 1 μm).
may account for anterograde secretion of cargo proteins. Taken together, these results indicate that Retro-2 significantly alters Golgi structure to a greater extent than previously anticipated.

**Figure 2.** Retro-2 treatment induces COPI-like vesicles accumulation in the peri-Golgi region and does not affect anterograde secretion of cargo proteins. (A) Size distribution analysis of Retro-2-induced vesicular profiles suggest that a large fraction of these accumulated vesicles are likely to be uncoated COPI vesicles. For diameter distribution of the vesicles, we measured maximum diameter from membrane to membrane. Distribution median value is 55 and mean value is 57 nm (n = 213) (scale bar 200 nm). (B, C) Retro-2 treatment does not affect secretion of soluble secretory cargo, ss-HRP, in HeLa or COS7 cells. Cells were transfected with ss-HRP overnight, then the cells were changed with fresh medium and treated with DMSO or Retro-2 for 5 h. The activity of secreted HRP was estimated measured by TMB ELISA, as described in the methods. Relative secreted ss-HRP (mean ± SD) was shown and experiments were repeated for three times. Statistical analysis was performed using two-tailed, paired t-test (mean ± SD, n.s. not significant). (D) Retro-2 treatment does not affect secretion of large cargo, collagen IV, in COS7 cells. COS7 cells pre-treated with DMSO or Retro-2 for 2 h before folding block (40 ºC, 3 h) without ascorbate and transport pulse condition, was later induced by shifting cells to 32 ºC in the presence of 100 μg/ml ascorbate and 50 μg/ml Cycloheximide for 5 h. Conditioned media were harvested and assessed by collagen IV ELISA Kit, as described in the methods. Relative secreted collagen IV (mean ± SD) was shown and experiments were repeated for three times. Statistical analysis was performed using two-tailed, paired t-test (mean ± SD, n.s. not significant).

Retro-2 treatment alters Golgi morphology in GRASP65/55-double depleted cells, as examined by structured illumination microscopy. We then considered the possibility that Retro-2 may be exploited to investigate whether Syntaxin5 is required for Golgi structure maintenance in interphase cells. We had previously reported that the two Syntaxin5-binding Golgi stacking proteins, GM130 and Golgin45, can entirely substitute for GRASP65/55 (when overexpressed) to maintain functionally and morphologically normal Golgi stacks in interphase cells. In the previous study, we consistently observed that Golgi cisternae appeared to be significantly dilated, but not unstacked, in cells depleted of GRASP65/55. Furthermore, we also demonstrated that Golgin45-Syntaxin5 interaction contributes to structural integrity of the Golgi stack in interphase cells.

Therefore, we postulated that acute displacement of Syntaxin5 via Retro-2 treatment could be used as a type of 'knock-side' strategy to investigate the hypothesis that interaction between Syntaxin5 and the two Syntaxin5-binding Golgin tethers may contribute a cisternal adhesion mechanism to restore morphologically and functionally normal Golgi stacking in GRASPs-depleted cells.
Since significant Golgi unstacking occurs only during mitotic Golgi disassembly34–37 or in cells treated with Golgi-disturbing agents, such as Brefeldin A38, this experimental approach offers a unique opportunity to test whether Golgin-Syntaxin5 interaction may assist or support Golgi stack maintenance during interphase.

Thus, HeLa cells were treated with GRASP65/55 siRNAs (Fig. 3A) or GM130/Golgin45 siRNAs (Fig. 3B) for 48 h, followed by Retro-2 treatment for 5 h at 37 °C (Fig. 3C for knockdown efficiency). These cells were then fixed and stained with anti-Giantin and anti-Golgin97 antibodies to examine any change in overall Golgi organization by super-resolution SIM microscopy. Prior to Retro-2 treatment, we observed that Giantin staining always showed distinct peripheral staining pattern with hallow space. However, upon Retro-2 treatment, this distinct feature was largely lost in GRASP65/55-double depleted cells (Fig. 3A; lower panel; as indicated by line analysis at the insets), but not in control or GM130/Golgin45-double depleted cells (Fig. 3B,C for knockdown efficiency).

Taken together, these results suggested that Retro-2 treatment induce greater disruption of Golgi organization in GRASP-depleted cells than in GM130/Golgin45-depleted cells. These changes were quite subtle and difficult to quantify by co-localization analysis, which then prompted us to use EM for more detailed structural analysis.

**Retro-2 induces rapid and complete Golgi unstacking in GRASP65/55-depleted cells within 5 h.** In order to further study this structural alteration at the ultrastructural level, the cells were then processed for EM study. Strikingly, upon examination by EM, we found that the Golgi was completely unstacked by Retro-2 treatment in GRASP65/55 double-depleted cells, but not in control or in GM130/Golgin45 double-depleted cells (Fig. 4A–E). While Golgi cisternae were still well stacked, they were highly dilated in both GRASP-depleted and Golgin-depleted HeLa cells, which is consistent with our previous observation35. These results suggested that Syntaxin5 may contribute to Golgi stack maintenance in GRASP65/55-depleted HeLa cells during interphase. Due to highly heterogeneous morphologies of the unstacked Golgi, it was difficult to quantitatively summarize these results, but we found that over 90% of the cells showed similar cisternal membrane de-adhesion in GRASP65/55-depleted cells upon Retro-2 treatment within 5 h. This was not due to disruption of the secretory pathway through the Golgi. Although GRASP double KD and Golgin double KD led to greatly increased ss-HRP secretion, consistent with previous work by ourselves and Wang’s group21,25, there were no statistically significant changes in the ss-HRP secretion for GRASP double KD or GM130/Golgin45 double KD cells, treated with DMSO or Retro-2 (Fig. 4F).

**Retro-2-induced Golgi unstacking in GRASP65/55-depleted cells is a time-dependent process.** To more thoroughly characterize this rapid Golgi unstacking, we performed a time-course study using Retro-2 and EM, and found that the alteration of Golgi structure by Retro-2 is a time-dependent process. During the first hour of Retro-2 treatment, we observed negligible changes in cisternal adhesion (Fig. 5A; left panel). However, there was noticeable cisternal membrane de-adhesion at 3 h of Retro-2 treatment (Fig. 5A; middle panel), which eventually led to almost complete Golgi unstacking at 5 h (Fig. 5A; right panel). Unlike Golgi disassembly during mitosis, Golgi cisternal membranes did not appear to undergo complete vesiculation in Retro-2-treated cells in this time frame and remained as relatively large membranous structures (see Golgi cisternal membranes, indicated by black arrowheads). Most of these membranous structures appeared to be invaginated with hallow space, although a few of them looked like large tubular structures.

In order to further characterize these novel membranous structures, we performed EM tomography of the Golgi in cells depleted of GRASP65/55 and treated with Retro-2 for 5 h. The resulting EM tomographs revealed that the unstacked Golgi membranes were cup-shaped, invaginated membrane structures, typically ranging in 100–250 nm diameter, as shown in EM tomograph tilt series (Fig. 5B; also see the movie#S1 and colored rendition of a cup-shaped membrane structure below).

Although it was difficult to further characterize these unstacked Golgi cisternae in the present study, the unstacked Golgi cisternae in our experiments appears to be strikingly similar to rapidly disrupted Golgi membranes in SARS-CoV-2-infected cells27, raising an interesting possibility that SARS-CoV-2 may have an ability to either directly or indirectly disrupt GRASP-mediated cisternal adhesion and alter the intracellular trafficking of certain SNARE proteins for efficient viral replication, which requires future study. In support of this hypothesis, SARS-CoV-2 spike protein has already been shown to directly bind and hijack COPI and COPII for progeny biogenesis38–40.

Taken together, our new results indicate that Syntaxin5 may assist Golgin-mediated Golgi structure maintenance during interphase. These results do not unequivocally prove that GM130/Golgin45-Syntaxin5 interaction provides the fundamental mechanism for cisternal adhesion during post-mitotic Golgi reassembly. However, these results do suggest that this unique Golgin-SNARE interaction may contribute to Golgi structure maintenance during interphase by inhibiting intercisternal fusion among adjacent Golgi cisternae and by simultaneously providing additional support for cisternal adhesion.

Thus, we propose that direct interaction between Syntaxin5 and Golgin45/GM130 (or any other Syntaxin5-binding Golgin tethers) may assist Golgi structure maintenance during interphase, as illustrated in the model (Fig. 6). For a mechanistic point of view, this would be quite distinct from GRASP65/55-dependent cisternal adhesion, where GRASP65/55 contribute to cisternal stacking via their PDZ-domain-mediated homo-oligomerization in trans between two apposed cisternae9,10,34,41,42. Instead, Golgin-SNARE interaction may provide a weak reversible binding, facilitating dynamic membrane adhesion/de-adhesion for regulation of cargo transport through the Golgi and basic Golgi structure maintenance. It would be especially intriguing to investigate in the future how this proposed role may intertwine with recently reported Syntaxin 5 phosphorylation under proteostatic stress39,44.
Figure 3. Structured Illumination microscopy data suggest a significant morphological/structural alteration of the Golgi in GRASP65/55-double depleted cells upon Retro-2 treatment. (A) 3D-SIM images of the Golgi stained with anti-Giantin and anti-Golgin97 antibodies in GRASP65/55-depleted cells (top panel) and in GRASP65/55-depleted cells, treated with Retro-2 for 5 h (bottom panel). (B) 3D-SIM images of the Golgi stained with anti-Giantin and anti-Golgin97 antibodies in GM130/Golgin45-depleted cells (top panel) and in GM130/Golgin45-depleted cells, treated with Retro-2 for 5 h (bottom panel). (scale bar 2 μm). (C) Immunoblot analysis of knockdown efficiency of indicated siRNAs used in 3D-SIM experiments.
Overall, these new results implicate Syntaxin5 in Golgi structure maintenance and regulation during interphase, which may have been overlooked thus far. As Syntaxin5 is localized throughout the Golgi stacks, we suggest that the Golgi SNARE may be optimally positioned to perform such a structural role.

**Methods**

**Reagents and antibodies.** All common reagents were purchased from Sigma-Aldrich, unless otherwise mentioned. The following antibodies were used: anti-Giantin (ab174655, Abcam), anti-Golgin97 (A21270, Thermo), anti-GM130 (610822, BD bioscience), anti-GRASP65 (MA5-25148, Thermo), anti-GRASP55 (10598-1-AP, Proteintech), anti-Golgin45 (PA530714, Thermo), anti-Syntaxin 5 (110053, Synaptic Systems), anti-GAPDH (KC-5G5, Kangchen Bio-tech). Retro-2 was obtained from Sigma-Aldrich. Anti-Rabbit Alexa Fluor
Figure 5. Retro-2-induced Golgi unstacking in GRASP65/55-depleted HeLa cells occurs in a time-dependent manner. (A) Representative EM micrographs showing that Golgi unstacking by Retro-2 treatment in GRASP65/55-depleted cells is a time-dependent process. These images show morphological and structural changes of the Golgi in GRASP65/55-depleted HeLa cells after Retro-2 treatment for 1, 3, 5 h, respectively. Black arrow heads indicate Golgi cisternal membranes during Retro-2-induced Golgi unstacking (scale bar 500 nm). (B) Sequential slice views from the tomogram showing more detailed structures of the ‘cup’-shaped, unstacked cisternal membranes. Below are the magnified views and segmentation of rendition from one of the unstacked cisternae (light blue; yellow indicates vesicular profiles in peri-Golgi area). (scale bar 200 nm). See movie#S1 for more information.
488 (A21441), Alexa Fluor 568 (A10037), Alexa Fluor 647 (A21236) for immunofluorescence were obtained from ThermoFisher. All siRNA oligos were purchased from Shanghai GenePharma, China and the target sequences were as following: human Golgin45 (GGG AAC AGT TTC GTC AAG A), human GRASP55 (GGC ATT GGA TAT GGT TAT T), human GM130 (GGA CAA TGC TGC TAC TCT ACA ACC A), human GRASP65 (CCT GAA GGC ACT ACT GAA AGC CAA T). The sequence of the non-targeting control siRNA was UUC UCC GAA CGU GUC ACG U.

Cell culture and treatments. HeLa (ATCC, CCL-2) and COS7 ( Stem Cell Bank, Chinese Academy of Sciences) cells were grown in DMEM supplemented with 10% FBS (Thermo) at 37 °C. HeLa cells were authenticated by STR profiling. The authentication of COS7 is provided by Stem Cell Bank, Chinese Academy of Sciences. All cell lines were routinely tested for the mycoplasma contamination and were negative. Transfection of DNA constructs and siRNAs was performed using Lipofectamine 2000 and RNAiMAX (ThermoFisher), respectively, according to the manufacturer’s instructions.

Immunofluorescence staining. Cells grown on glass coverslips (72,230–01, Electron Microscopy Sciences) were grown in DMEM supplemented with 10% FBS (Thermo) at 37 °C. HeLa cells were authenticated by STR profiling. The authentication of COS7 is provided by Stem Cell Bank, Chinese Academy of Sciences. All cell lines were routinely tested for the mycoplasma contamination and were negative. Transfection of DNA constructs and siRNAs was performed using Lipofectamine 2000 and RNAiMAX (ThermoFisher), respectively, according to the manufacturer’s instructions.

Immunoblotting. For immunoblotting, proteins were separated by SDS-PAGE (Genscript) and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked with 3% bovine serum albumin (BSA).
and then probed with specific primary antibodies and then with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). The bands were visualized with chemiluminescence (Clarity Western ECL Substrate, Bio-Rad) and imaged by a ChemiDoc Touch imaging system (Bio-Rad). Representative blots are shown from several experiments.

Sample preparation and image acquisition for Electron microscopy/tomography. The cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 1 h. They were then fixed in 0.1 M sodium cacodylate buffer, scraped and pelleted in 2% agar. Samples were trimmed and post-fixed in 1% osmium tetroxide for 1 h, en bloc stained in 2% uranyl acetate in maleate buffer pH 5.2 for a further hour, rinsed then dehydrated in an ethanol series and infiltrated with resin (Embed812, Electron Microscopy Science) and cured overnight at 60 °C. Hardened blocks were cut using a Leica UC7 Ultracutermotor, 60 nm sections were collected onto formvar/carbon coated nickel grids, 10 nm gold particles added on both sides of the grids (Utrecht UMC). A tomography tilt series was acquired using SerialEM software on an FEI Tecnai TF20 FEG TEM at 200 kV. Tomograms were viewed FEI Tecnai Biotwin TEM at 80 kV. Images were taken using Morada CCD and iTEM (Olympus) software typically at 26,000 × magnification. For electron tomography, 250 nm sections were collected on formvar/carbon copper grids, 10 nm gold particles added on both sides of the grids (Utrecht UMC). A tomography tilt series was acquired using SerialEM software on an FEI Tecnai TF20 FEG TEM at 200 kV. Tomograms were reconstructed using IMOD software (University of Colorado, Boulder, CO).

ss-HRP and collagen IV secretion assays. After overnight transfection with ss-HRP plasmid, the cells were changed with fresh medium and treated with DMSO or Retro-2 for 5 h. Extracellular media were harvested and HRP activity was measured using 1-Step Ultra TMB-ELISA (ThermoFisher), according to the manufacturer’s instructions. For collagen IV secretion, COS7 cells pre-treated with DMSO or Retro-2 for 2 h before folding block (40 °C, 3 h) without ascorbate and transport pulse condition, was later induced by shifting cells to 32 °C in the presence of 100 mg/mL ascorbate and 50 µg/mL Cycloheximide for 5 h. Extracellular media were harvested and assessed by collagen IV ELISA Kit (FineTest, EH2867), according to the manufacturer’s protocols.

Image processing and data presentation. Line intensity of 3D-SIM images were analyzed by Fiji software. Results are displayed as mean of results from each experiment or dataset, as indicated in figure legends. Analyses were performed with GraphPad Prism 9.0 software.

Data availability
The datasets used and/or analyzed during the current study is available from the corresponding author on reasonable request.

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Author contributions

X.Y., L.Z., B.G., C.T., Y.Q and I.L. performed confocal and other cell biological experiments. M.G., X.L. and I.L. carried out EM experiments. X.Y. performed size analysis of vesicular profiles, induced upon Retro-2 treatment. The authors thank Ms. Mijjeong Kim for colored rendition of unstacked Golgi cisternae from EM tomograph. X.Y. and I.L. wrote the manuscript. Financial support of this study was provided by ShanghaiTech University. The authors declare no conflict of interest.

Competing interests

The authors declare no competing interests.

Additional information

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