

Quantitative intra-Golgi transport and organization data suggest the stable compartment nature of the Golgi

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This **important** study offers **convincing** evidence that intra-Golgi transport slows from cis to trans and varies between cargos even within the same cisternae, supporting a more stable compartment model. Using nocodazole-induced ministacks, the authors show cargo-specific transport kinetics with distinct velocities and residence times. These findings refine the cisternal progression model and prompt further investigation into alternative mechanisms, such as rapid partitioning or rim progression. This study will be of interest to cell biologists studying membrane trafficking, Golgi organization, and protein secretion, as well as researchers investigating the mechanisms of organelle dynamics and the molecular basis of intracellular transport.

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Abstract

How the intra-Golgi secretory transport works remains a mystery. The cisternal progression and the stable compartment models have been proposed and are under debate. Classic cisternal progression model posits that both the intra-Golgi transport and Golgi exit of secretory cargos should occur at a constant velocity dictated by the cisternal progression; furthermore, COPI-mediated intra-Golgi retrograde transport is essential for maintaining the Golgi organization. Leveraging our recently developed Golgi imaging tools in nocodazole-induced Golgi ministacks, we found that the intra-Golgi transport velocity of a secretory cargo decreases during their transition from the *cis* to the *trans*-side of the Golgi, and different cargos exhibit distinct velocities even within the same cisternae. We observed a vast variation in the Golgi residence times of different cargos. Remarkably, truncation of the luminal domain causes the Golgi residence time of Tac — a standard transmembrane secretory cargo without intra-Golgi recycling signals — to extend from 16 min to a notable 3.4 h. Additionally, when COPI-mediated intra-Golgi retrograde transport was inhibited by brefeldin A, we found that nocodazole-induced Golgi can remain stacked for over 30 - 60 minutes. Therefore, our findings challenge the classical cisternal progression model and suggest the stable compartment nature of the Golgi.

Introduction

The Golgi complex in mammalian cells plays a crucial role in membrane trafficking and post-translational modification of proteins and lipids (cargos). Centrally positioned around the microtubule organization center (Glick and Luini, 2011 [↗](#); Klumperman, 2011 [↗](#)), it comprises laterally connected Golgi stacks, each containing 4 – 7 tightly adjacent membrane sacs known as cisternae. Conventionally, a Golgi stack is recognized to have three regions: the *cis*, medial, and *trans*-Golgi. The *trans*-Golgi network (TGN) assembles outside the *trans*-side of a Golgi stack and is mainly composed of tubular and vesicular membranes (De Matteis and Luini, 2008 [↗](#)). In the secretory pathway, newly synthesized cargos exit the ER at the ER exit site (ERES), pass the ER and Golgi intermediate compartment (ERGIC), and reach the Golgi. Cargos subsequently transit from the *cis*-side through the medial and eventually reach the *trans*-side of the Golgi, where they exit the Golgi and target the plasma membrane (Bergmann and Singer, 1983 [↗](#); Castle et al., 1972 [↗](#)).

Despite decades of research, the Golgi remains one of the most enigmatic organelles, particularly regarding the mechanism of intra-Golgi transport of secretory cargos (Emr et al., 2009 [↗](#); Glick and Luini, 2011 [↗](#)). Two primary intra-Golgi transport models have been proposed and are currently the subject of debate. In the classic cisternal progression or maturation model, secretory cargos passively reside within Golgi cisternae, which progress or mature from the *cis* to the *trans*-Golgi cisternae to facilitate the forward or anterograde transport of cargos. Simultaneously, post-translational modification enzymes such as glycosyltransferases move in reverse or retrograde transport, altering cisternal properties to become the next cisternae of the Golgi stack. Under this perspective, the Golgi functions at a dynamic equilibrium of anterograde and retrograde intra-Golgi transport. The model predicts that all secretory cargos should have the same and constant intra-Golgi transport and Golgi exit velocity. It can provide a plausible explanation for the intra-Golgi transition of oversized secretory cargos, such as procollagen I (Bonfanti et al., 1998 [↗](#); Mironov et al., 2001 [↗](#)). Moreover, direct observations of cisternal maturation have been made in budding yeast *Saccharomyces cerevisiae* (Kurokawa et al., 2019 [↗](#); Losev et al., 2006 [↗](#); Matsuura-Tokita et al., 2006 [↗](#)), although similar observation has not been reported in mammalian cells. However, the budding yeast Golgi differs significantly from the mammalian one in the cisternal organization – it scatters throughout the cytoplasm as unstacked compartments. This substantial difference casts doubt on the general applicability of the budding yeast Golgi observation to higher eukaryotes.

In contrast, the stable compartment model posits that Golgi cisternae are stable entities. During intra-Golgi transport, carriers actively move secretory cargos from one cisterna or compartment to the next, from the *cis* to the *trans*-side, while post-translational modification enzymes remain stationary. At the *trans*-side of the Golgi, cargos are sorted into carriers bound for the plasma membrane. As a result, different cargos can exhibit distinct intra-Golgi transport and Golgi exit velocities under this model. To account for the intra-Golgi transport of oversized secretory cargos, such as procollagen I, a modified version of the stable compartment model, the rim progression model, has been proposed (Lavie et al., 2013 [↗](#); Pfeffer, 2010 [↗](#); Volchuk et al., 2000 [↗](#)). In this model, each Golgi cisterna seems to have a stable interior domain and a dynamic rim domain; the rim domain of Golgi cisternae undergoes constant en bloc fission and fusion, carrying small or large cargos to the next cisternae for intra-Golgi cargo transport. The rim partitioning of large secretory cargos is supported with previous EM studies of procollagen I (Bonfanti et al., 1998 [↗](#)), FM4 aggregates (Lavie et al., 2013 [↗](#)), and algal protein aggregates (Engel et al., 2015 [↗](#)). Furthermore, we have recently demonstrated in fluorescence microscopy that during their intra-Golgi transition, small cargos, such as CD59, E-cadherin, and VSVG, localize to the cisternal interior, coinciding with Golgi glycosyltransferases, while large cargos, such as FM4 aggregates and collagenX, position themselves at the cisternal rim, where trafficking machinery components localize (Tie et al., 2018 [↗](#)).

Once secretory cargos transit the Golgi stack, they reach the *trans*-side of the Golgi and are packed into carriers destined for the plasma membrane. The classic cisternal progression model posits that secretory cargos linearly depart the *trans*-Golgi. Contrary to this, cargo exit kinetics have been shown to adhere to a first-order exponential relationship rather than a linear one (Hirschberg et al., 1998 [DOI](#); Patterson et al., 2008 [DOI](#); Sun et al., 2020 [DOI](#)). In an attempt to reconcile these observations, Patterson *et al.* introduced the rapid partitioning model, which stands distinct from the classic cisternal progression and stable compartment models (Patterson et al., 2008 [DOI](#)). This model suggests that cargos rapidly diffuse throughout the Golgi stack, segregating into multiple post-translational processing and export domains, where cargos are packed into carriers bound for the plasma membrane. Nonetheless, synchronized traffic waves have been observed through various techniques, including electron microscopy (EM) (Trucco et al., 2004 [DOI](#)) and advanced light microscopy methods we developed, such as GLIM and side-averaging (Tie et al., 2016 [DOI](#); Tie et al., 2022 [DOI](#)). These findings suggest that the rapid partitioning model might not accurately represent the true nature of the intra-Golgi transport.

The ongoing debate among these models underscores the complexity of the Golgi and reflects the insufficiency of the existing experimental data, especially the intra-Golgi transport kinetics data. Two semi-quantitative approaches exist for studying the intra-Golgi transport kinetics of ER-synchronized secretory cargos. The first is the biochemical approach, where intra-Golgi transport kinetics can be indirectly deduced by subtracting ER-to-Golgi and Golgi-to-plasma membrane transport from the overall secretion (ER-to-plasma membrane transport) during the chase (Ernst et al., 2018 [DOI](#)). However, this indirect approach provides only an averaged intra-Golgi transport kinetics, making it incapable of measuring the instantaneous intra-Golgi transport velocity at a sub-Golgi region. The second approach involves EM imaging of immuno-gold labeled secretory cargos at Golgi cisternae (Beznoussenko et al., 2014 [DOI](#); Trucco et al., 2004 [DOI](#)). The distribution of gold particles among individual Golgi cisternae during the chase directly indicates intra-Golgi transport kinetics. Nevertheless, this approach demands specialized techniques and equipment and considerable manual work in EM imaging and subsequent image examination, limiting its widespread use in other labs.

The limitations in current approaches call for novel methods, especially ones based on fluorescence microscopy, to resolve the intra-Golgi transport spatially and kinetically. However, optically resolving the intra-Golgi secretory transport in mammalian cells is challenging due to the thinness (200 – 400 nm) and random orientation of Golgi stacks. To overcome this, we leverage the uniform and rotationally symmetrical arrangement of nocodazole-induced Golgi ministacks (hereafter referred to as Golgi ministacks). Extensive studies have provided strong evidence that ministacks obtained under prolonged nocodazole treatment (≥ 3 h), the condition employed in our studies, largely represent the native Golgi stack (Cole et al., 1996 [DOI](#); Fourriere et al., 2016 [DOI](#); Rogalski et al., 1984 [DOI](#); Schueder et al., 2024 [DOI](#); Tie et al., 2018 [DOI](#); Trucco et al., 2004 [DOI](#); Van De Moortele et al., 1993 [DOI](#)). We developed a numerical Golgi localization tool called Golgi localization by imaging centers of mass (GLIM) that can precisely pinpoint a Golgi protein's cisternal localization with nanometer accuracy in Golgi ministacks (Tie et al., 2017 [DOI](#); Tie et al., 2016 [DOI](#)). Alongside this, we propose using the metric of Golgi residence time to quantify a Golgi protein's retention in the Golgi (Sun et al., 2021 [DOI](#); Sun et al., 2020 [DOI](#)).

Here, we utilized past and newly acquired GLIM and Golgi residence time data to quantitatively analyze intra-Golgi transport and Golgi exit kinetics. Our data revealed that neither intra-Golgi transport nor Golgi exit exhibits a constant velocity. We discovered that when the luminal domain of Tac — a conventional transmembrane secretory cargo lacking intra-Golgi recycling signals — is truncated, its Golgi residence time increases from 16 min to a substantial 3.4 h. Through GLIM, we examined the cisternal organization of Golgi ministacks under brefeldin A (BFA) treatment, which halts retrograde intra-Golgi transport. Remarkably, under this condition, we found that

nocodazole-induced Golgi ministacks can remain stacked for 30 - 60 min. Therefore, our findings underscore the stable compartment nature of Golgi cisternae. They challenge the classical cisternal progression model and favor the stable compartment model.

Results and discussion

The intra-Golgi transport is not a motion with constant velocity

The classic cisternal progression model postulates that the intra-Golgi transport velocity of a secretory cargo should remain constant. However, this prediction has not been directly tested due to the inherent challenge of measuring the intra-Golgi transport velocity. The advent of GLIM has allowed us to address this issue. In GLIM, briefly, HeLa cells expressing the RUSH secretory cargo (RUSH reporter) (Boncompain et al., 2012) and GalT-mCherry, a *trans*-Golgi marker containing amino acids 1-81 of B4GALT1, were initially treated with nocodazole. Subsequently, cells were chased in biotin with nocodazole and cycloheximide (a protein synthesis inhibitor) for various lengths of time (t) before immunofluorescence labeling for endogenous GM130, a *cis*-Golgi marker. Additionally, apart from SBP-GFP, a soluble secretory protein, and SBP-GFP-CD59 (Tie et al., 2016), a glycosylphosphatidylinositol-anchored protein, all RUSH reporters in this study are transmembrane proteins, including TNF α -SBP-GFP, TfR-SBP-GFP (Chen et al., 2017), SBP-GFP-CD8a-furin-WT (Tie et al., 2016), SBP-GFP-CD8a-furin-YA, SBP-GFP-CD8a-furin-AC (Tie et al., 2016), SBP-GFP-CD8a-furin-Y+AC (Tie et al., 2016), SBP-GFP-Tac (Sun et al., 2020), SBP-GFP-Tac-TC (Sun et al., 2020), and SBP-GFP-E-cadherin (Boncompain et al., 2012). Except for TNF α -SBP-GFP, which uses Ii-streptavidin as the ER hook, all RUSH reporters employ signal sequence fused streptavidin-KDEL as their ER hook (Fig. 1).

We calculated centers of mass as the positions of GM130, RUSH reporter, and GalT-mCherry within each analyzable Golgi ministack. The RUSH reporter's Golgi localization quotient, or LQ , is calculated by dividing its distance from GM130 by GalT-mCherry's distance from GM130 (Supplementary Fig. 1 A-G). The LQ is a linear numerical metric to indicate a cargo's axial localization within the Golgi, with a nanometer range of precision (Tie et al., 2017; Tie et al., 2016). We previously linearly defined regions of Golgi: ERES/ERGIC ($LQ < -0.25$), *cis* ($-0.25 \leq LQ < 0.25$), medial ($0.25 \leq LQ < 0.75$), *trans*-Golgi ($0.75 \leq LQ < 1.25$), and TGN ($LQ \geq 1.25$).

To analyze the intra-Golgi transport kinetics of secretory cargos, we measured the LQ s of RUSH reporters after various durations of biotin administration (chase). Most kinetic data was previously reported in HeLa cells (Tie et al., 2017; Tie et al., 2016) and re-analyzed here. Additionally, we generated new data and replicated specific measurements. GLIM involves laborious manual image analysis. To increase image analysis efficiency, we developed a software tool, Auto-GLIM, to automatically analyze ministack images and calculate LQ s by using a deep learning algorithm (the manuscript will be published elsewhere). We found that Auto-GLIM can produce LQ s similar to the conventional manual analysis method (Supplementary Fig. 1 H-M) but requires much less user interaction, therefore increasing our image analysis efficiency. We employed the Auto-GLIM tool to analyze the intra-Golgi transport of RUSH reporters, SBP-GFP-CD59 and SBP-GFP-Tac-TC, in HEK293T cells.

Figure 2, Supplementary Figure 2, and Supplementary File 1 illustrate that our LQ vs. t plots are highly reproducible. As previously reported, all LQ vs. t plots fit the following first-order exponential function (Equation 1) well with an adjusted R^2 (adj. R^2) ≥ 0.85 (Fig. 2A-J and Supplementary Fig. 2A-T, left panels).

$$LQ = y_0 - Ae^{\left(-\frac{\ln 2}{t_{\text{intra}}}t\right)} \quad (\text{Equation 1})$$

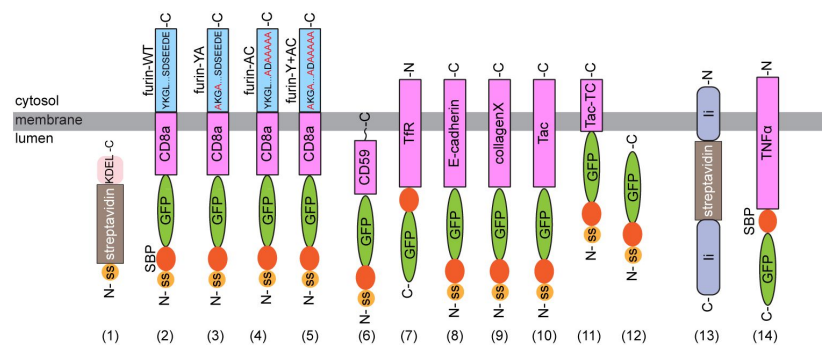


Figure 1.

A schematic diagram showing the domain organizations of ER hooks and RUSH reporters used in this study.

(1) streptavidin-KDEL (ER hook), (2) SBP-GFP-CD8a-furin-WT, (3) SBP-GFP-CD8a-furin-YA, (4) SBP-GFP-CD8a-furin-AC, (5) SBP-GFP-CD8a-furin-Y+AC, (6) SBP-GFP-CD59, (7) TfR-SBP-GFP, (8) SBP-GFP-E-cadherin, (9) SBP-GFP-collagenX, (10) SBP-GFP-Tac, (11) SBP-GFP-Tac-TC, (12) SBP-GFP, (13) Ii-streptavidin (ER hook), (14) TNF α -SBP-GFP. ss, signal sequence. SBP, streptavidin binding peptide. (1) is the ER hook for RUSH reporters (2-12), while (13) is the ER Hook for the RUSH reporter (14). Key cytosolic amino acid motifs are indicated for furin cytosolic tail RUSH reporters. YA is YKGL, a tyrosine-based motif, to AKGL mutation, while AC is SDSEEDL, an acidic cluster sequence, to ADAAAAA mutation (Tie et al., 2016). Y + AC indicates mutations in both sites (Tie et al., 2016).

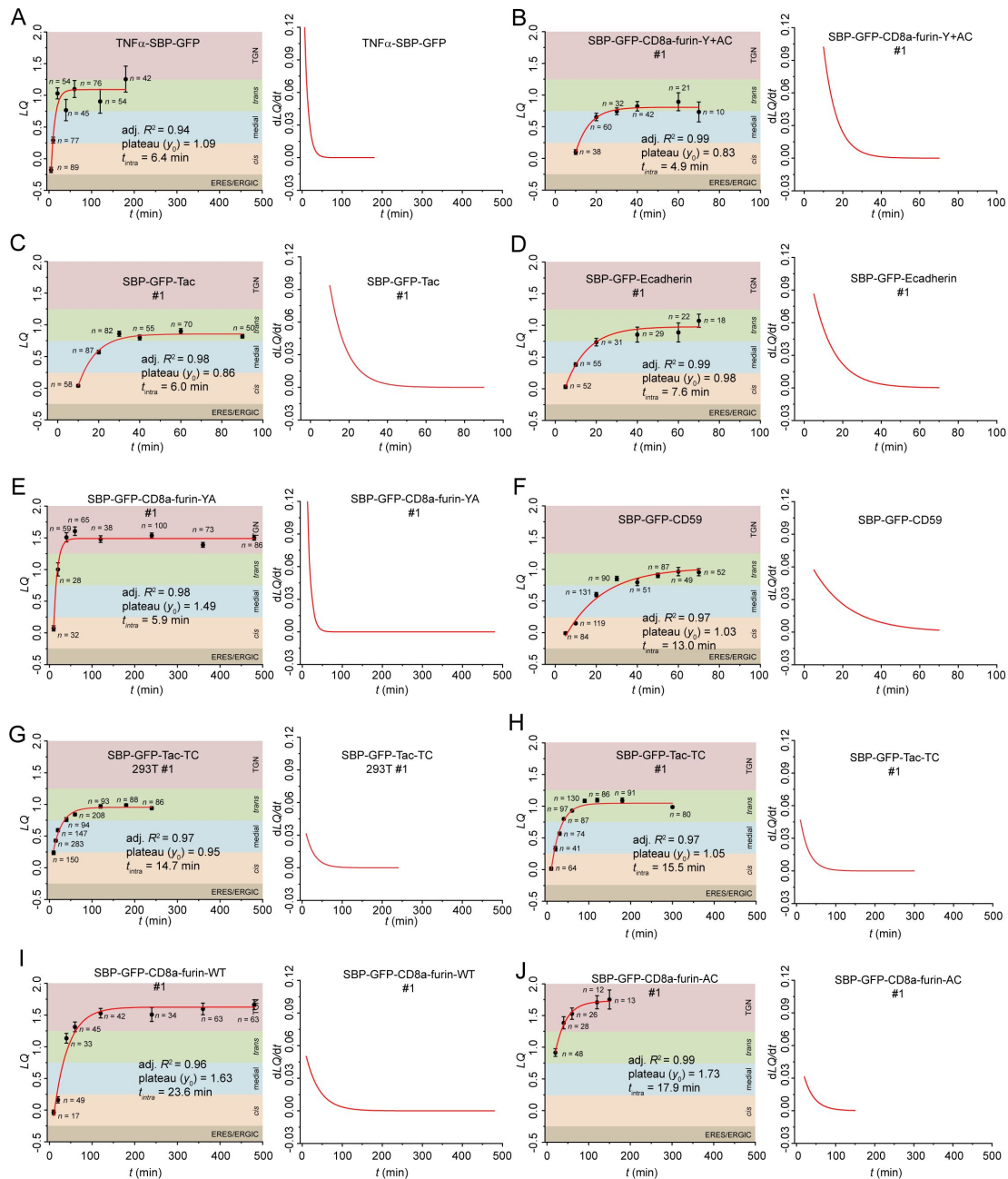


Figure 2.

The intra-Golgi transport kinetics for RUSH Reporters in Golgi ministacks.

HeLa or 293T cells transiently co-expressing individual RUSH reporter and GalT-mCherry were incubated with nocodazole for 3 h. This was followed by a chase with nocodazole, cycloheximide, and biotin for variable time durations (t) before fixation and GM130 immunostaining. Except for TNF α -SBP-GFP, which uses Ii-streptavidin as the ER hook, all RUSH reporters employ signal sequence fused streptavidin-KDEL as their ER hook. Using GLIM, LQ s for the RUSH reporters were then calculated and plotted. Each panel has an LQ vs. t plot on the left, with n (the number of analyzable Golgi ministacks), adj. R^2 , y_0 , and t_{intra} indicated. On the right side of each panel, the dLQ/dt vs. t plot represents the first-order derivative of its corresponding LQ vs. t plot on the left. ERES/ERGIC ($LQ < -0.25$), *cis* ($-0.25 \leq LQ < 0.25$), medial ($0.25 \leq LQ < 0.75$), *trans*-Golgi ($0.75 \leq LQ < 1.25$), and TGN ($1.25 \leq LQ$) zones were color-shaded. The LQ vs. t plot in panel G was acquired in 293T cells and analyzed by the Auto-GLIM tool, while the rest are from our previous reports (see **Table 1**). Panels are arranged according to their RUSH reporters' t_{intra} means (see **Table 1**). See Supplementary File 1 for the raw data.

Table 1.

Intra-Golgi transport kinetics of secretory RUSH reporters in Golgi ministacks.

See the legend of **Figure 2** for details. #1-4 indicate independent replicates. All data were acquired from HeLa cells except for those labeled with "293T", which were acquired using 293T cells. Except for TNF α -SBP-GFP, which uses Ii-streptavidin as the ER hook, all RUSH reporters employ signal sequence fused streptavidin-KDEL as their ER hook. Superscripts 1 and 2 indicate data were re-analyzed from previous publications, (Tie et al., 2016) and (Sun et al., 2020), respectively. WT, wild type. t_{intra} (intra-Golgi transport time), y_0 , and adj. R^2 were calculated by fitting measured LQ vs. time kinetics data to Equation 1. dLQ/dt (at LQ = 0.40) was calculated by Equation 3, and converted to nm/min by multiplying 274 nm per LQ unit. SD, standard deviation.

RUSH reporter	t_{intra} (min) Mean $\pm$ SD	t_{intra} (min)	y_0	Adj. R^2	dLQ/dt (at LQ = 0.40)	dLQ/dt (at LQ = 0.40) (nm/min)
SBP-GFP-CD59 #1 (293T)	5 $\pm$ 4	0.4	0.66	0.94	0.244	66.7
SBP-GFP-CD59 #2 (293T)		7.3	0.63	0.92	0.022	6.0
SBP-GFP-CD59 #3 (293T)		6.8	0.79	0.85	0.040	10.9
TNF α -SBP-GFP ¹	6.4	6.4	1.09	0.94	0.075	20.5
TfR-SBP-GFP	7	7.0	1.16	0.99	0.075	20.6
SBP-GFP-CD8a-furin-Y+AC #1 ¹	7 $\pm$ 1	4.9	0.83	0.99	0.061	16.7
SBP-GFP-CD8a-furin-Y+AC #2 ¹		7.1	1.00	0.99	0.059	16.0
SBP-GFP-CD8a-furin-Y+AC #3 ¹		7.7	1.04	0.92	0.058	15.8
SBP-GFP-Tac #1 ²	7 $\pm$ 2	6.0	0.86	0.98	0.053	14.6
SBP-GFP-Tac #2		8.2	0.91	0.98	0.043	11.8
SBP-GFP-E-cadherin #1 ¹	8 $\pm$ 1	7.6	0.98	0.99	0.053	14.5
SBP-GFP-E-cadherin #2 ¹		8.7	1.15	0.96	0.060	16.5
SBP-GFP-E-cadherin #3 ¹		6.5	1.11	0.98	0.075	20.6
SBP-GFP-collagenX #1	8 $\pm$ 1	9.0	1.00	0.94	0.046	12.7
SBP-GFP-collagenX #2		9.5	0.84	0.97	0.032	8.8
SBP-GFP-collagenX #3		6.9	0.91	0.97	0.051	14.0
SBP-GFP	10.6	10.6	0.74	0.98	0.022	6.1
SBP-GFP-CD8a-furin-YA #1 ¹	11 $\pm$ 5	5.9	1.49	0.98	0.13	35.0
SBP-GFP-CD8a-furin-YA #2 ¹		11.1	1.51	0.93	0.069	19.0
SBP-GFP-CD8a-furin-YA #3 ¹		16.3	1.48	0.95	0.046	12.6
SBP-GFP-CD59 ¹	13.0	13.0	1.03	0.97	0.034	9.2
SBP-GFP-Tac-TC #1 (293T)	14.2 $\pm$ 0.6	14.7	0.95	0.97	0.026	7.1
SBP-GFP-Tac-TC #2 (293T)		14.3	1.00	0.98	0.029	8.0
SBP-GFP-Tac-TC #3 (293T)		13.6	0.98	0.89	0.030	8.1
SBP-GFP-Tac-TC #1 ²	17 $\pm$ 3	15.5	1.05	0.97	0.029	8.0
SBP-GFP-Tac-TC #2		19.1	1.16	0.94	0.028	7.6
SBP-GFP-CD8a-furin-WT #1 ¹	18 $\pm$ 5	23.6	1.63	0.96	0.036	9.9
SBP-GFP-CD8a-furin-WT #2 ¹		18.7	1.58	0.97	0.044	12.0
SBP-GFP-CD8a-furin-WT #3 ¹		13.1	1.55	0.97	0.061	16.7
SBP-GFP-CD8a-furin-AC #1 ¹	21 $\pm$ 5	17.9	1.73	0.99	0.052	14.1
SBP-GFP-CD8a-furin-AC #2 ¹		26.9	1.50	1.00	0.028	7.8
SBP-GFP-CD8a-furin-AC #3 ¹		23.2	1.58	0.96	0.035	9.7
SBP-GFP-CD8a-furin-AC #4 ¹		16.8	1.62	0.99	0.050	13.8

In Equation 1 [\[1\]](#), t represents chase time in minutes (biotin treatment starts at $t = 0$); $\ln 2$ is the natural logarithm of 2; A is a constant; y_0 represents the LQ of the Golgi exit site; t_{intra} is the time that the cargo reaches half of the transport range and is hereafter referred to as the intra-Golgi transport time. **Table 1** [\[1\]](#) lists t_{intra} , y_0 , and adj. R^2 of each data set. We define the instantaneous intra-Golgi transport velocity as the derivative of LQ with respect to time, dLQ/dt , which measures the axial transport velocity of the center of mass of the synchronized cargo wave. It should also follow the first-order exponential function (**Equation 2** [\[1\]](#)).

$$\frac{dLQ}{dt} = \frac{A \ln 2}{t_{\text{intra}}} e^{\left(-\frac{\ln 2}{t_{\text{intra}}} t\right)} \quad (\text{Equation 2})$$

In this context, the intra-Golgi transport velocity is the highest when the cargo enters the secretory pathway ($t = 0$). However, it is crucial to approach this extrapolation cautiously due to the lack of experimental data at $t \leq 5$ min, when a RUSH reporter's high ER background and low Golgi signal make it challenging to select analyzable Golgi ministacks. It is evident that the dLQ/dt of all our RUSH reporters slows to zero as they transit across the Golgi stack to reach $LQ = y_0$ at the *trans*-Golgi or TGN (**Fig. 2A-J** [\[1\]](#) and **Supplementary Fig. 2A-T** [\[1\]](#), right panels). At the *trans*-Golgi, we propose that RUSH reporters that do not target the TGN exit Golgi ministacks in carriers en route to the plasma membrane (Tie et al., 2018 [\[1\]](#); Tie et al., 2016 [\[1\]](#); Tie et al., 2022 [\[1\]](#)). Hence, the intra-Golgi transport velocity of a secretory cargo does not remain constant, contradicting the prediction of the classic cisternal progression model.

The intra-Golgi transport kinetics of collagenX, a cisternal rim partitioned secretory cargo, resemble those of conventional cargos

CollagenX has been known to assemble into oligomers (Kwan et al., 1991 [\[1\]](#)). We previously reported that the collagenX RUSH reporter, SBP-GFP-collagenX, forms large aggregates containing ~ 190 copies (Tie et al., 2018 [\[1\]](#)). With an estimated mean size of ~ 40 nm, these aggregates are much smaller than FM4 aggregates and procollagen I (> 300 nm) (Bonfanti et al., 1998 [\[1\]](#); Volchuk et al., 2000 [\[1\]](#)) and, therefore, are not excluded from conventional transport vesicles, which typically have a size of 50 – 100 nm. However, our previous findings showed that while conventional secretory cargos partition to the cisternal interior during intra-Golgi transport, large cargos such as FM4 aggregates and collagenX preferentially localize to the cisternal rim (Tie et al., 2018 [\[1\]](#)), highlighting distinct intra-Golgi transport behavior for different cargo sizes. Hence, we asked if collagenX follows the same intra-Golgi transport kinetics as conventional secretory cargos.

Using the RUSH assay, our LQ vs. t data demonstrated that the intra-Golgi transport of SBP-GFP-collagenX followed a first-order exponential function, with a t_{intra} of 8 ± 1 (mean $\pm$ SD, $n = 3$) (**Fig. 3 A-C** [\[1\]](#); **Table 1** [\[1\]](#)). SBP-GFP-collagenX exited at the *trans*-Golgi, with a y_0 of 0.92 ± 0.08 (mean $\pm$ SD, $n = 3$). Additionally, the instantaneous intra-Golgi transport velocity (dLQ/dt) of SBP-GFP-collagenX also decreases in accordance with a first-order exponential function (**Fig. 3 A-C** [\[1\]](#); **Table 1** [\[1\]](#)).

Using side averaging, we visualized the synchronized traffic wave of SBP-GFP-collagenX as it transitioned across the ministack (**Fig. 3 D and E** [\[1\]](#); **Supplementary Fig. 3** [\[1\]](#)). When passing through the medial and *trans*-Golgi regions at $t = 10$ and 20 min after biotin chase, SBP-GFP-collagenX appeared as double puncta, supporting its cisternal rim localization (**Fig. 3 D** [\[1\]](#)). 40 min after biotin chase, numerous SBP-GFP-collagenX-positive carriers were observed surrounding the *trans*-Golgi, indicating a process of Golgi exiting (**Fig. 3 D** [\[1\]](#)). In summary, our findings demonstrated that cisternal rim partitioned large size secretory cargos might follow intra-Golgi transport kinetics similar to those of cisternal interior partitioned conventional secretory cargos.

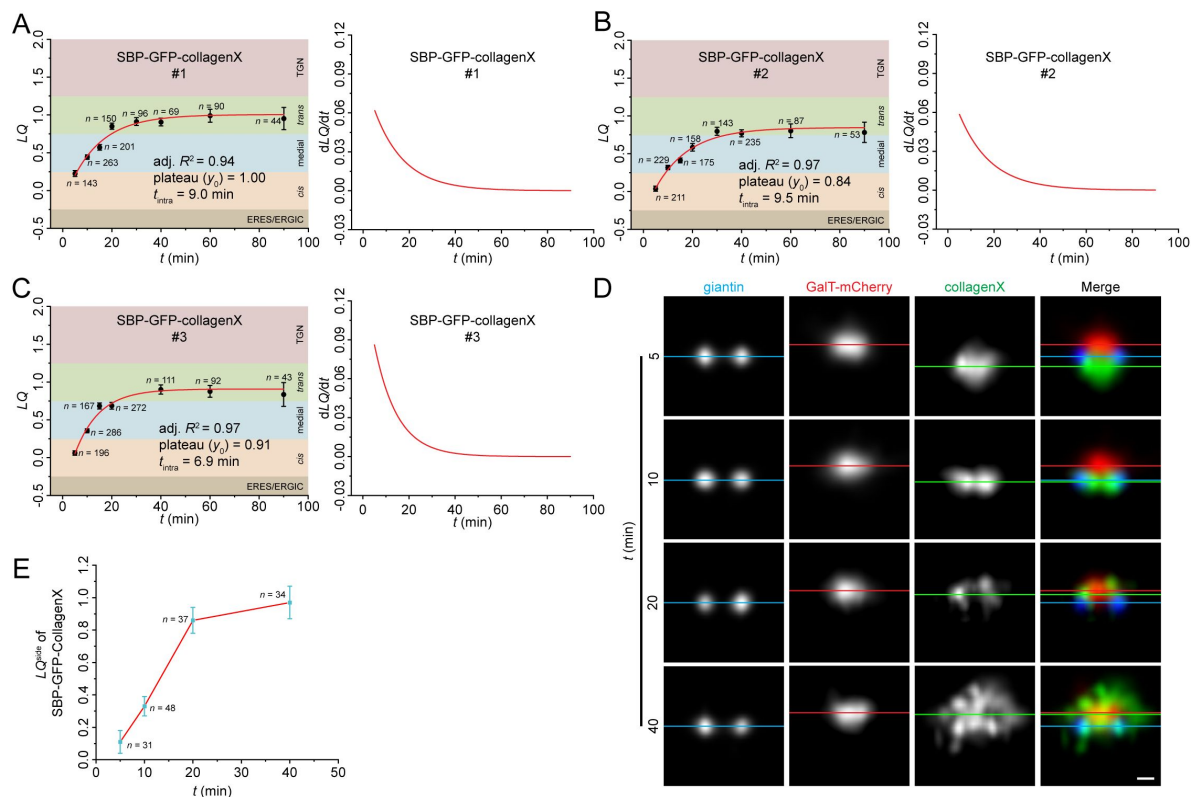


Figure 3.

The intra-Golgi transport of SBP-GFP-collagenX quantified by GLIM and visualized by side averaging.

(A-C) GLIM. HeLa cells transiently co-expressing the RUSH reporter, SBP-GFP-collagenX, and GalT-mCherry were imaged during biotin chase (t) and subjected to GLIM. See [Figure 2](#) legend for details. (D) Side averaging. HeLa cells transiently co-expressing the RUSH reporter, SBP-GFP-collagenX, and GalT-mCherry were incubated with nocodazole for 3 h. This was followed by a chase with nocodazole, cycloheximide, and biotin for variable time durations (t) before fixation and giantin immunostaining. Images were acquired using Airyscan microscopy and subjected to side averaging guided by giantin double puncta. Blue, red, and green horizontal lines represent the center of mass positions of giantin, GalT-mCherry, and SBP-GFP-collagenX, respectively. Scale bar, 200 nm. (E) The LQ^{side} vs. biotin chase time (t) plot shows the transition of SBP-GFP-collagenX from the *cis* to the *trans*-region of the Golgi ministack. LQ^{side} is an approximate metric corresponding to LQ (see Materials and Methods). n , the number of ministacks quantified. Error bar, SEM.

Distinct intra-Golgi transport velocities for different cargos at the same cisternae

From [Equations 1](#) and [2](#), we derive the following relationship ([Equation 3](#)):

$$\frac{dLQ}{dt} = \frac{\ln 2}{t_{\text{intra}}} (y_0 - LQ) \quad (\text{Equation 3})$$

In our previous work, through side-averaging, we determined that one LQ unit corresponds to 274 nm ([Tie et al., 2022](#)). Hence, we scaled [Equation 3](#) by 274 nm to derive the instantaneous intra-Golgi transport velocity in nm/min. When plotting the instantaneous intra-Golgi transport velocity (nm/min) against the LQ for selected RUSH reporters, as seen in [Figure 4A](#), we observed that different secretory cargos exhibit varied transport velocities even within the same cisternae or at the same LQ values. At $LQ = 0.40$, corresponding to the medial-Golgi region, our RUSH reporters' instantaneous intra-Golgi transport velocities are calculated in [Table 1](#) according to [Equation 3](#). For instance, instantaneous intra-Golgi transport velocities of SBP-GFP, SBP-GFP-Tac-TC, SBP-GFP-CD59, SBP-GFP-Tac, and TNF α -SBP-GFP are 6.1, 8.0, 9.2, 14.6, and 20.5 nm/min, respectively ([Table 1](#)). These findings highlighted that different secretory cargos possess distinct intra-Golgi transport velocities within the same Golgi cisternae, challenging the prediction made by the classic cisternal progression model.

Golgi residence times vary significantly among different secretory cargos

Once cargos transit through the Golgi stack, the classic cisternal progression model predicts that cargos depart the Golgi in a linear kinetics. However, studies have demonstrated that cargos exit the Golgi by following the first-order exponential kinetics ([Hirschberg et al., 1998](#); [Patterson et al., 2008](#); [Sun et al., 2020](#)). While we could apply a hypothetical rate-limiting step to the classic cisternal progression and stable compartment models to rationalize the exponential kinetics of cargo exit ([Luini, 2011](#)), the classic cisternal progression model encounters more significant challenges. Firstly, the first-order exponential Golgi exit implies that clearing a synchronized wave of secretory cargo from the *trans*-cisternae would take an indefinite time, which is inconsistent with the transient nature of the *trans*-cisternae as described by the classic cisternal progression model. Secondly, since cargos are considered passive in the classic cisternal progression model, the exit kinetics, as measured by the Golgi residence times, should be the same across all secretory cargos.

The Golgi residence time is a cargo's duration at the *trans*-Golgi cisternae before exit and is a metric for Golgi retention ([Sun et al., 2021](#); [Sun et al., 2020](#)). The first step to determine this metric involves synchronizing a transmembrane protein at the Golgi. For Golgi transmembrane resident proteins like glycosyltransferases and secretory transmembrane proteins with a substantial Golgi localization at the steady state, such as GFP-Tac-TC, synchronization is not required. However, for secretory transmembrane cargos lacking a significant Golgi pool at the steady state, such as TfR-GFP, GFP-Tac, and RUSH reporters like TNF α -SBP-GFP, synchronization is achieved through a 20°C temperature block. Subsequently, live imaging at 37 °C is performed to capture the Golgi fluorescence intensity decay of the protein in the presence of cycloheximide. The Golgi residence time is calculated as the half-time ($t_{1/2}$) by fitting the intensity decay to a first-order exponential function.

Our extensive measurement demonstrated that Golgi residence times of secretory cargos display a wide range of values ([Table 2](#)). For example, TNF α -SBP-GFP has a Golgi residence time of 6.0 ± 0.4 min (mean $\pm$ SEM, $n = 73$), one of the shortest, while SBP-GFP-CD59 has a Golgi residence time

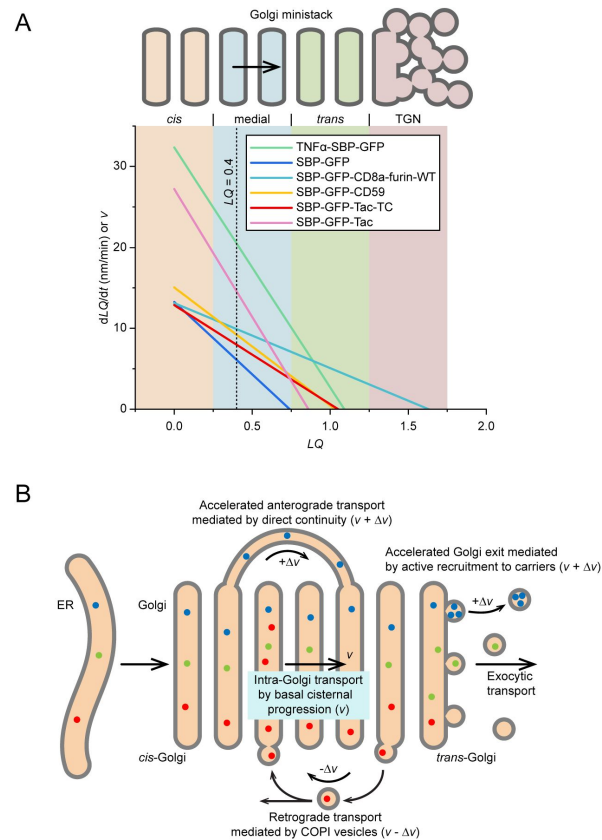


Figure 4.

Different secretory cargos exhibit distinct intra-Golgi transport velocities within the same cisternae of Golgi ministacks.

(A) Plots of dLQ/dt vs. LQ for selected RUSH reporters reveal diverse intra-Golgi transport velocities at the same cisternae. These plots were constructed based on [Equation 3](#), using parameters from [Table 1](#). Different regions within the Golgi — *cis* ($-0.25 \leq LQ < 0.25$), medial ($0.25 \leq LQ < 0.75$), *trans*-Golgi ($0.75 \leq LQ < 1.25$), and TGN ($1.25 \leq LQ$) — are color-shaded for easier identification. To convert dLQ/dt to nm/min, we multiplied dLQ/dt by 274 nm per LQ unit. A dotted vertical line marks the LQ value of 0.40. (B) Modifying the classic cisternal progression model to explain the distinct intra-Golgi transport velocities at the same cisternae. According to this model, Golgi cisternae progress at a constant velocity (v). Direct-continuity-based anterograde transport could accelerate the intra-Golgi transport velocity ($v + \Delta v$), while the COPI-mediated retrograde transport could reduce it ($v - \Delta v$).

of 16 ± 2 min (mean $\pm$ SEM, $n = 29$) (**Supplementary Fig. 4A**). Hence, our data suggest that different secretory cargos reside at the *trans*-Golgi cisternae for varying durations, contradicting predictions from the classic cisternal progression model.

Cargos exhibiting prolonged Golgi residence times suggest the *trans*-Golgi interior might be a stable domain

The classic cisternal progression model could be modified to explain the diverse intra-Golgi transport kinetics. For example, accelerated anterograde or retrograde transport might be introduced on top of the basal cisternal progression to account for the wide range of intra-Golgi transport velocities and Golgi residence times we observed (**Fig. 4B**). Accelerated anterograde transport mechanisms might include continuity-based direct diffusion across cisternae via heterologous cisternal connections (Beznoussenko et al., 2014; Marsh et al., 2004; Trucco et al., 2004). Hence, secretory cargo with such a mechanism would have a faster intra-Golgi transport velocity. This mechanism might explain the rapid and diverse intra-Golgi transport velocity of secretory cargos such as VSVG, insulin, albumin, and alpha1-antitrypsin (Beznoussenko et al., 2014; Marsh et al., 2004; Trucco et al., 2004). Similarly, active recruitment to exocytic carriers budding at the *trans*-Golgi possibly might shorten the Golgi residence time. On the other hand, COPI-coated carriers might facilitate retrograde intra-Golgi transport to counter the cisternal progression, accounting for the slow intra-Golgi transport velocity and prolonged Golgi residence time of cargos. Indeed, COPI has been known to maintain certain glycosyltransferases' Golgi retention by direct or indirect interactions (Ali et al., 2012; Eckert et al., 2014; Liu et al., 2018; Pereira et al., 2014; Rizzo et al., 2021; Schmitz et al., 2008; Tu et al., 2008). However, such a retrograde mechanism requires the interaction between secretory cargo and the COPI coat.

Earlier, we identified truncation mutants of two typical secretory cargos, GFP-Tac (Sun et al., 2020) and GFP-CD8a, which reside in the Golgi nearly as stably as Golgi glycosyltransferases. GFP-Tac and GFP-CD8a are type I transmembrane proteins comprising from their N- to C-termini a signal sequence, GFP, luminal domain, transmembrane domain (TMD), and cytosolic tail (**Fig. 5A**). Previously, we and others demonstrated that fully glycosylated luminal domain can function as a Golgi exit signal (Gut et al., 1998; Sun et al., 2020). Their Golgi residence time was documented at 16 ± 2 min (mean $\pm$ SEM, $n = 45$) and 7.8 ± 0.7 min (mean $\pm$ SEM, $n = 26$), respectively (**Table 2**). However, after truncating the luminal domain, the Golgi residence time for the resultant chimeras, GFP-Tac-TC and GFP-CD8a-TC, extended significantly to 3.4 ± 0.3 h (mean $\pm$ SEM, $n = 22$) and 66 ± 7 min (mean $\pm$ SEM, $n = 19$) (**Table 2**) (**Supplementary Fig. 4B**), respectively. Consequentially, their long Golgi residence times ensure a significant steady-state Golgi pool.

The following evidence suggests that GFP-Tac-TC and GFP-CD8a-TC behave like bona fide Golgi resident transmembrane proteins. Firstly, their Golgi residence times, 3.4 h and 66 min, are comparable to that of a typical Golgi glycosyltransferase, such as ST6GAL1 (5.3 ± 0.6 h, mean $\pm$ SEM, $n = 21$) (Sun et al., 2021). Second, their *LQ*s, 0.94 ± 0.02 (mean $\pm$ SEM, $n = 118$) and 0.86 ± 0.05 (mean $\pm$ SEM, $n = 76$), combined with their cisternal interior localization (**Fig. 5B**) (Sun et al., 2020), indicate that they primarily reside within the interior of the *trans*-cisternae, similar to many Golgi glycosyltransferases (Tie et al., 2018). Additionally, GFP-Tac-TC follows GalT-mCherry to localize to the ER under the BFA treatment reversibly (Sun et al., 2020).

According to the modified cisternal progression model above, GFP-Tac-Tc could have an active retrieval mechanism mediated by an unidentified signal in its cytosolic tail or TMD to counter the cisternal progression. Considering that GFP-Tac-TC's *LQ* (0.94) is close to its Golgi exit site, y_0 (0.95 – 1.16) (**Table 1**; **Fig. 2G,H** and **Supplementary Fig. 2M-O**), measured by its RUSH version, there are two possible retrieval pathways, acting at either post-Golgi or intra-Golgi stages. To test if

Transmembrane protein	Golgi residence time ($t_{1/2}$)	n	SEM
TNF α -SBP-GFP ¹	6.0 min	73	0.4 min
GFP-CD8a ¹	7.8 min	26	0.7 min
TfR-GFP ¹	10 min	12	1 min
GFP-Tac ¹	16 min	45	2 min
SBP-GFP-CD59	16 min	29	2 min
GFP-Tac (5A) ¹	47 min	57	3 min
GFP-CD8a-TC	66 min	19	7 min
GFP-Tac-TC ¹	3.4 h	22	0.3 h
MGAT2-GFP ²	4.9 h	26	0.5 h
ST6GAL1-GFP ¹	5.3 h	21	0.6 h

Table 2.

Golgi residence times of transmembrane secretory cargos and Golgi glycosyltransferases in native Golgi (without nocodazole).

Superscripts 1 and 2 indicate data were from previous publications, (Sun et al., 2020 [DOI](#)) and (Sun et al., 2021 [DOI](#)), respectively. The RUSH reporter, TNF α -SBP-GFP, employs signal sequence fused streptavidin-KDEL as the ER hook. n , the number of quantified cells; SEM, standard error of the mean.

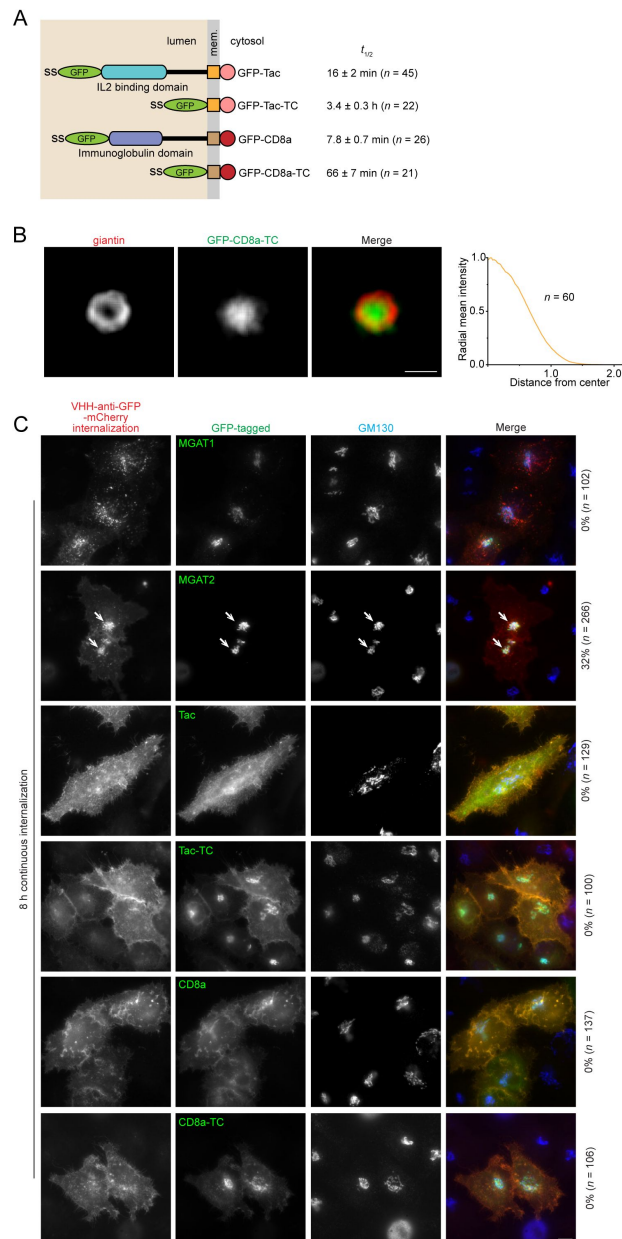


Figure 5.

GFP-Tac-TC and GFP-CD8a-TC might not have a Golgi retrieval mechanism once exiting the Golgi.

(A) Domain organization of GFP-tagged Tac, Tac-TC, CD8a, and CD8a-TC. ss, signal sequence. Mem., membrane. $t_{1/2}$ values are from [Table 2](#) and shown as mean $\pm$ SEM. n , the number of cells analyzed. (B) GFP-CD8a-TC localizes to the Golgi cisternal interior at the steady state. HeLa cells transiently expressing GFP-CD8a-TC were treated with nocodazole to induce the formation of ministacks before fixation and immunostaining for the endogenous giantin. En face averaging images of giantin and GFP-CD8a-TC are shown on the left. Scale bar, 500 nm. The radial mean intensity profile of en face averaged GFP-CD8a-TC is shown on the right. The x-axis represents the distance from the center of fluorescence mass (normalized to the giantin radius), and the y-axis represents the radial mean intensity (normalized). n , the number of averaged ministacks. (C) The plasma membrane-localized GFP-Tac-TC and GFP-CD8a-TC are not retrieved to the Golgi. HeLa cells transiently expressing indicated GFP-tagged protein were incubated continuously with VHH-anti-GFP-mCherry for 8 h before fixation and immunostaining for the endogenous Golgi marker, GM130. Images were acquired under a wide-field microscope. The internalized VHH-anti-GFP-mCherry has negative Golgi localization for all GFP-tagged proteins except for MGAT2 (arrows), a positive control. The percentage of cells showing the Golgi localization of VHH-anti-GFP-mCherry is labeled on the right. n , the number of cells analyzed. Scale bar, 10 μ m.

GFP-Tac-Tc possesses a Golgi retrieval pathway after its Golgi exit, we incubated HeLa cells transiently expressing MGAT1-GFP (negative control) (Sun et al., 2021 [DOI](#)), MGAT2-GFP (positive control) (Sun et al., 2021 [DOI](#)), GFP-Tac, or GFP-Tac-TC with a recombinant mCherry-fused anti-GFP nanobody (VHH-anti-GFP-mCherry) continuously for 8 h (**Fig. 5C** [DOI](#)). By binding to the cell surface-exposed GFP, VHH-anti-GFP-mCherry serves as a sensitive probe to track the endocytic trafficking itinerary of the above GFP-fused transmembrane proteins.

We found that VHH-anti-GFP-mCherry localized to the Golgi in a significant fraction of MAGT2-GFP-expressing cells (32%, $n = 266$) but not in MGAT1-GFP cells (0%, $n = 102$) after 8 h continuous internalization (**Fig. 5C** [DOI](#)). This result is consistent with our previous report that MGAT2, but not MAGT1, has a Golgi retrieval mechanism (Sun et al., 2021 [DOI](#)), although its molecular mechanism is still unknown. In contrast, VHH-anti-GFP-mCherry did not localize to the Golgi in cells expressing other constructs, including GFP-Tac-TC, suggesting that GFP-Tac-TC might not possess a post-Golgi retrieval mechanism targeting the Golgi (**Fig. 5C** [DOI](#)). Next, we reason that given GFP-Tac's short Golgi residence time (16 min), its cytosolic tail and TMD might not facilitate any active intra-Golgi retrograde transport mechanism, such as COPI coat binding, to recycle GFP-Tac-TC from Golgi exiting. Since GFP-Tac and GFP-Tac-TC share identical TMD and cytosolic tail sequences, if such a retrograde mechanism existed, GFP-Tac would have a similar Golgi residence time to GFP-Tac-TC. The same observation and reasoning also apply to GFP-CD8a-TC (**Fig. 5C** [DOI](#)).

Therefore, we argue that GFP-Tac-TC and GFP-CD8a-TC might not have retrieval signals to facilitate their Golgi residence, although proving a protein does not possess a transport signal is challenging. Since the cisternal interior is continuous with the cisternal rim both in membrane and lumen, our findings suggest that the cisternal interior at the *trans*-Golgi might be a stable domain. In summary, our data implies that retention within the *trans*-side stable domain, rather than continuous retrieval to counter the cisternal progression (treadmilling), could be the primary mechanism for the long Golgi residence times of GFP-Tac-TC and GFP-CD8a-TC.

The Golgi maintains its stacked organization after 30 min BFA treatment

COPI functions in the retrograde direction at the ER-Golgi interface and within the Golgi (Glick and Luini, 2011 [DOI](#); Popoff et al., 2011 [DOI](#); Rabouille and Klumperman, 2005 [DOI](#)). According to the classic cisternal progression model, COPI-mediated retrograde intra-Golgi transport recycles resident transmembrane proteins, such as glycosyltransferases and transport machinery components. This model predicts that upon the compromise of COPI, intra-Golgi recycling would stop, and the Golgi stack would continuously lose its materials and eventually disappear, depending on the cisternal progression rate. In addition to its retrograde role, COPI has also been documented to function in the anterograde ER-to-Golgi transport (Monetta et al., 2007 [DOI](#); Weigel et al., 2021 [DOI](#)).

To test the role of COPI in the Golgi organization, we employed BFA, a small molecule fungal metabolite that rapidly dissociates COPI and clathrin from the Golgi, inhibits the ER-to-Golgi trafficking, and causes the fusion of the Golgi with the ER in 10 - 20 min (Klausner et al., 1992 [DOI](#)). It does so by rapidly inactivating class I ARFs, which recruit COPI and clathrin coats to the Golgi membrane (Donaldson et al., 1992 [DOI](#); Helms and Rothman, 1992 [DOI](#)). In contrast to the native Golgi, nocodazole-induced Golgi ministacks have been documented to be more resistant to BFA, and, hence, the disappearance of Golgi occurs at a much later time (> 30 min) (Lippincott-Schwartz et al., 1990 [DOI](#)). However, it is unclear if the nocodazole-induced Golgi maintains a similar stacked organization under the BFA treatment.

To address this, we studied the LQs of several Golgi markers following 30 – 60 min of BFA treatment (**Fig. 6A-E** [DOI](#) and **Supplementary Fig. 5A,B** [DOI](#)). The fast dissociation of Arf1-GFP from Golgi ministacks confirmed the effectiveness of BFA (**Supplementary Fig. 5A** [DOI](#)). We also observed that extended BFA treatment considerably reduced the number of intact Golgi ministacks. We

measured the LQ s of six transmembrane Golgi markers, GFP-golgin-84, GS15, ST6GAL1-GFP, TGN46, CD8a-furin, and CD8a-CI-M6PR (**Fig. 6A-F** and **Supplementary Fig. 5B**). GS15's LQ was monitored for 60 min, with the rest for 30 min. Strikingly, we observed that Golgi puncta are still stacked as all measured LQ s exhibited a relative arrangement similar to those of the pre-BFA treatment ($t = 0$ min) (**Fig. 6F**). For example, LQ s of ST6GAL1 and GS15 remained largely unchanged after 30 min BFA treatment. Although the LQ of golgin-84 increased from 0.2 to 0.4, it still remained between the LQ s of GM130 and GS15. The stacked organization of BFA-treated ministacks was further confirmed by the side-averaging images of GM130, Giantin, GalT-mCherry, and CD8a-furin (**Fig. 6G,H**).

However, BFA treatment altered the physical dimensions of ministacks. We defined the distance between two Golgi markers as the distance between their Gaussian peak centers in the axial line intensity profile (see the Materials and Methods). We observed the distance from GM130 to GalT-mCherry, $d_{(GM130-GalT-mCherry)}$, decreased from 300 ± 10 nm (mean $\pm$ SEM, $n = 34$) to 190 ± 30 nm (mean $\pm$ SEM, $n = 13$) after 30 min of BFA treatment, indicating axial shrinkage of ministacks (**Fig. 6G**). Furthermore, LQ s of all three TGN markers, TGN46, CD8a-furin, and CD8a-CI-M6PR, decreased and approached that of the *trans*-Golgi, suggesting a collapse of the TGN (**Fig. 6F**), possibly due to the dissociation of clathrin and its adaptor proteins. This collapse of the TGN was further supported by the side-averaging images, in which CD8a-furin gradually approached the *trans*-Golgi labeled by GalT-mCherry over time (**Fig. 6H**). We found the distance from GalT-mCherry to CD8a-furin, $d_{(GalT-mCherry-CD8a-furin)}$, decreased from 200 ± 20 nm (mean $\pm$ SEM, $n = 30$) to 100 ± 30 nm (mean $\pm$ SEM, $n = 20$) after 30 min of BFA treatment.

Despite dramatic changes in the TGN and shrinkage of the axial length of the Golgi ministack, our data demonstrate that the Golgi maintains its stacked organization for at least 30 – 60 min, even in the absence of COPI-mediated intra-Golgi retrograde transport. Our findings suggest that the Golgi might not be a dynamic equilibrium between the cisternal progression and retrograde trafficking and argue for a by-default stable nature of the Golgi stack. It is worth noting that the cellular effect of BFA is complex and pleiotropic. For example, in addition to Arfs, it can inhibit lipid metabolic enzymes (De Matteis et al., 1994). So, the disassociation of COPI might not be the sole factor responsible for our observations.

Our data supports the rim progression model, a modified version of the stable compartment model

Previously, we discovered that Golgi glycosyltransferases tend to localize to the cisternal interior, while trafficking machinery components localize primarily to the cisternal rim (Tie et al., 2018). Combined with our current observations of differential intra-Golgi transport velocities among distinct secretory cargos (**Fig. 2-4** and **Supplementary Fig. 2**) and the stable *trans*-Golgi interior localization of GFP-Tac-TC (Sun et al., 2020) and GFP-CD8a-TC (**Fig. 5B** and **Table 2**), these findings support the modified version of the stable compartment model – the rim progression model. This model explains the retention of Golgi glycosyltransferases in the cisternal interior, given that many do not recycle via COPI-coated vesicles (Liu et al., 2018) or possess a post-Golgi retrieval pathway (Sun et al., 2021). It also readily accounts for the diverse intra-Golgi transport velocities of secretory cargos by suggesting that each cargo type may have a different retention time within the stable cisternal interior domain.

The Golgi glycosyltransferases at the cisternal interior might assemble a dense protein matrix based on fluorescence microscopy (Tie et al., 2018) and EM data (Engel et al., 2015). It is tempting to speculate that the enzyme matrix could provide the molecular basis for the stable cisternal interior domain and functionally mirror a gel-filtration chromatography matrix with a defined porosity. Hence, large secretory cargos, such as FM4 aggregates and collagenX, are excluded from the interior, where the enzyme matrix localizes, while small secretory cargos can enter and become kinetically trapped there. The progressive reduction in intra-Golgi transport of

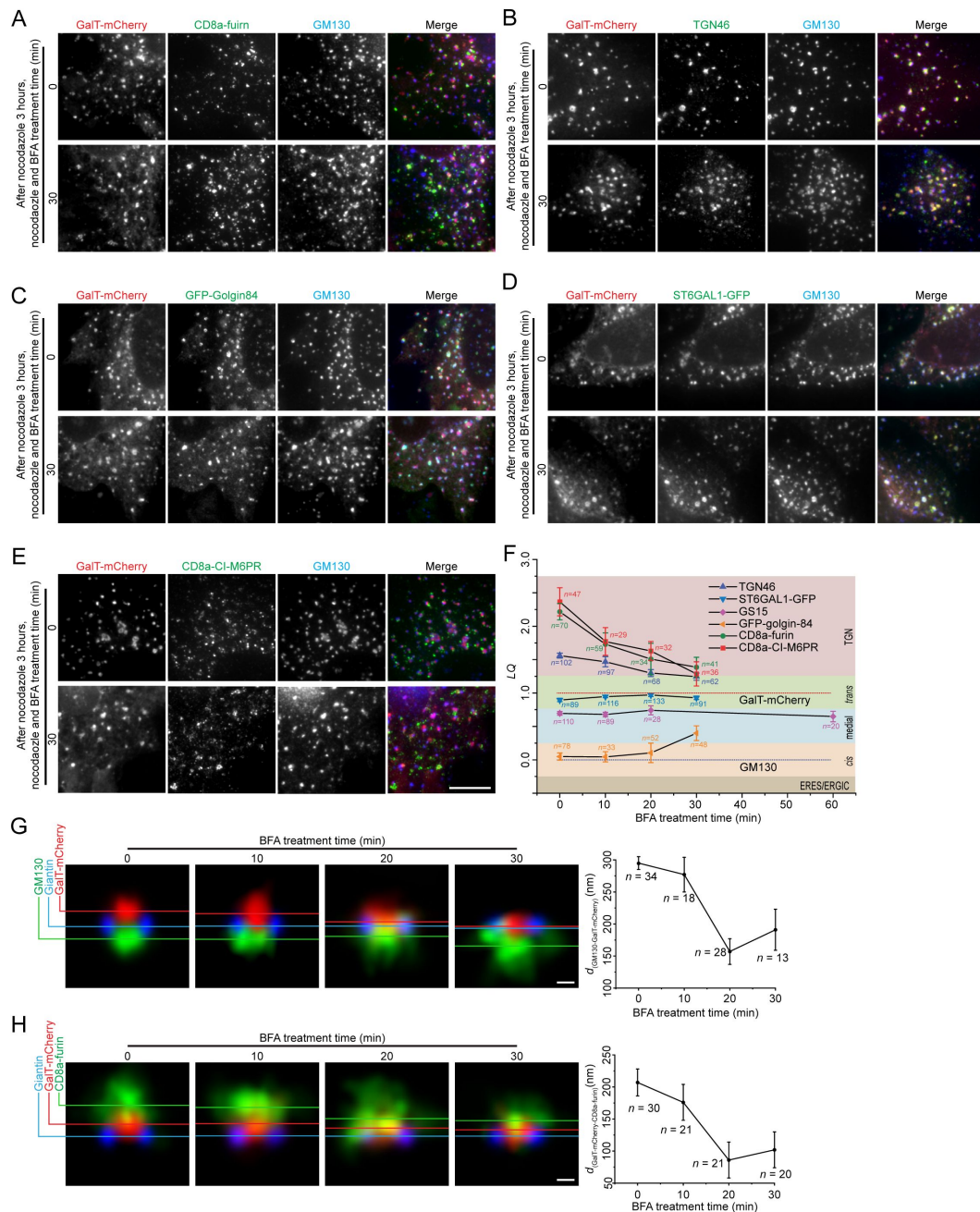


Figure 6.

The organization of Golgi ministacks under the BFA treatment.

(A-E) HeLa cells transiently co-expressing GalT-mCherry and indicated GFP or CD8a-tagged Golgi proteins were incubated with nocodazole for 3 h. This was followed by additional treatment with nocodazole and 5 μM BFA for variable durations before fixation. Endogenous TGN46 was immunostained. Images were acquired by wide-field microscopy. Representative images at 0 and 30 min are shown. Scale bar, 10 μm . (F) LQs of different Golgi proteins were determined using GLIM. The dotted lines represent LQs for GM130 ($LQ = 0.00$) and GalT-mCherry ($LQ = 1.00$). n , the number of analyzed Golgi ministacks. (G-H) HeLa cells transiently expressing GalT-mCherry alone (G) or together with CD8a-furin (H) were incubated with nocodazole for 3 h. This was followed by additional treatment with nocodazole and 5 μM BFA for variable durations before immunostaining endogenous giantin or GM130. After Airyscan imaging, images were subjected to side averaging. Horizontal color lines indicate centers of mass of corresponding side-averaged Golgi proteins. $d_{\text{GM130-GalT-mCherry}}$ and $d_{\text{GalT-mCherry-CD8a-furin}}$ in nm are plotted against the BFA treatment time in the right panels. n , the number of ministacks quantified. Error bar, SEM. Scale bar, 200 nm.

secretory cargo might result from the enzyme matrix's retention at the *trans*-Golgi. As the secretory cargos progress along the Golgi stack from the *cis* to the *trans*-side, more and more cargos become temporarily retained in the *trans*-Golgi region, gradually reducing their overall intra-Golgi transport velocity. If the release or Golgi exit of these cargos from the enzyme matrix follows a constant probability per unit time, i.e., a first-order kinetics process, the rate of cargo exiting from the Golgi should follow the first-order exponential function. Since the mechanism underlying intra-Golgi transport kinetics reflects fundamental molecular and cellular processes of the Golgi, further experimental data are essential to rigorously test this hypothesis.

Limitations of the study

We introduced new quantitative data on the intra-Golgi transport dynamics. However, our study has limitations. First, our approach relied on the overexpression of fluorescence protein-tagged cargos. The synchronized release of a large amount of cargo could significantly saturate and skew the intra-Golgi transport. Second, we utilized nocodazole-induced ministacks instead of the native Golgi to analyze the intra-Golgi transport, which could raise concerns about the impact of depolymerizing microtubules on the intra-Golgi transport and Golgi organization. Third, with the exception of furin and its mutants, all RUSH reporters used in this study are constitutive secretory cargos. As a result, the intra-Golgi transport dynamics observed here might not reflect those of regulated secretion, which involves the synchronized release of a large quantity of cargo in response to a specific signal.

Our findings suggest that the Golgi cisternal interior might be a stable domain, therefore supporting a modified version of the stable compartment model, the rim progression model. However, we do not think our data alone can resolve the two models, which have been the subject of debate for several decades. Further refinement of the classic cisternal progression model might also account for our data. We anticipate further development using our approach will provide more systematic data to test and refine future intra-Golgi transport models. Moreover, we hope that our study will stimulate further research into this longstanding and intriguing question.

Materials and methods

DNA plasmids, antibodies, and small molecules

To clone RUSH reporter, SBP-GFP, two PCRs were performed using li-Strep_{ss}-SBP-EGFP-Ecadherin (a gift plasmid from F. Perez) (Boncompain et al., 2012 [DOI](#)) and pEGFP-C1 (Clontech) as templates and the following primer pairs (GAT GCA CCC GGG AGG CGC GCC ATG and CTC CTC GCC CTT GCT CAC ACC TGC AGG TGG TTC ACG) and (CGT GAA CCA CCT GCA GGT GTG AGC AAG GGC GAG GAG and GAT GCA TCT AGA TTA CTT GTA CAG CTC GTC CAT), respectively. The mixture of the two purified PCR fragments was used as the template for the third round of PCR amplification using the first and the fourth primer listed above. The resulting PCR fragment was digested by XmaI and XbaI and ligated to XmaI and XbaI digested li-Strep_{ss}-SBP-EGFP-Ecadherin DNA plasmid. To clone GFP-CD8a-TC, the coding sequence of the TMD and cytosolic tail of CD8a was amplified by PCR using GFP-CD8a (Sun et al., 2020 [DOI](#)) as a template and the following primers, CAG TGC CTC GAG GAC TTC GCC TGT GAT ATC TA and GAC CGT GAA TTC TTA GAC GTA TCT CGC CGA AAG GCT G. The resulting PCR fragment was digested by EcoRI and XhoI and ligated to EcoRI and XhoI digested GFP-CD8a DNA plasmid.

ST6GAL1-GFP (ST-GFP) (Sun et al., 2020 [DOI](#)), CD8a-furin (Mahajan et al., 2013 [DOI](#)), CD8a-CI-M6PR (Mahajan et al., 2013 [DOI](#)) were previously described. RUSH TfR-SBP-GFP was a gift plasmid from J. Bonifacino (Chen et al., 2017 [DOI](#)). GFP-golgin-84 (Diao et al., 2003 [DOI](#)) was a gift plasmid from M. Lowe. Arf1-GFP (Wessels et al., 2006 [DOI](#)) was a gift plasmid from F. van Kuppeveld.

Mouse monoclonal antibody anti-CD8a (OKT8) was from the hybridoma culture supernatant. Mouse monoclonal antibodies against GM130 (#610822) and GS15 (#610960) were purchased from BD Bioscience. Rabbit polyclonal antibody against TGN46 was from Abcam (#ab50595). Alexa Fluor 488, 594, and 647-conjugated goat anti-mouse or anti-rabbit secondary antibodies were purchased from Thermo Fisher Scientific.

Small molecules

Nocodazole (#487928; working concentration: 33 μ M), BFA (working concentration: 5 μ M), and cycloheximide (working concentration: 10 μ g/ml) were purchased from Merck, Epicenter Technologies, and Sigma Aldrich, respectively.

Cell culture and transfection

HeLa and 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to make it complete DMEM. For cell transfection, Lipofectamine 2000 (Thermo Fisher Scientific) was used per the manufacturer's instructions. For BFA treatment, cells were treated with complete DMEM containing 5 μ M BFA for the specified duration. For nocodazole treatment, cells were treated with complete DMEM containing 33 μ M nocodazole for 3 h to induce the formation of Golgi ministacks.

Immunofluorescence

Cells for immunofluorescence were grown on No. 1.5 Φ 12 mm glass coverslips. They were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS). Following a PBS wash to remove residual paraformaldehyde, any remaining paraformaldehyde within the cells was neutralized with 100 mM NH_4Cl . The cells were then processed for immunofluorescence labeling by first incubating with mouse or rabbit primary antibodies, followed by Alexa Fluor 488, 594, and/or 647 conjugated goat anti-mouse or anti-rabbit secondary antibodies. Both primary and secondary antibodies were diluted in PBS containing 5% fetal bovine serum, 2% bovine serum albumin, and 0.1% saponin (Sigma-Aldrich). The labeled cells were mounted in the Mowiol mounting medium, composed of 12% Mowiol 4-88 (EMD Millipore), 30% glycerol, and 100 mM Tris pH 8.5. After the mounting medium had dried, the coverslips were sealed with nail polish and stored at -20°C .

Acquiring LQs

To analyze LQs of intra-Golgi transport RUSH reporters, HeLa cells transiently co-expressing individual GFP-tagged RUSH reporter and GalT-mCherry were cultured in complete DMEM supplemented with 16 nM His-tagged streptavidin (in-house purified using Addgene #20860, a gift plasmid from A. Ting) (Howarth et al., 2006 [DOI](#)). Following a 3 h nocodazole treatment, cells were chased with 50 μ M biotin, 10 μ g/ml cycloheximide, and 33 μ M nocodazole for various durations before fixation.

In another set of experiments to study LQs of Golgi markers under the BFA treatment, nocodazole-treated HeLa cells transiently expressing GalT-mCherry were further incubated with 5 μ M BFA and 33 μ M nocodazole for various durations before fixation. For these experiments, the Golgi markers were either co-expressed with GalT-mCherry as a GFP-tagged construct or detected as an endogenous protein by immunostaining.

The methodology for acquiring LQs through GLIM has been described in our previous studies (Tie et al., 2017 [DOI](#); Tie et al., 2016 [DOI](#)). Briefly, cells were further immuno-labeled to visualize endogenous GM130. Ministacks exhibiting fluorescence signals of GM130, transfected GalT-mCherry, and the testing protein were imaged using a wide-field microscope. Ministacks were manually selected for analysis, and fluorescence centers for GM130, GalT-mCherry, and the testing protein were acquired using Fiji (<https://imagej.net/software/fiji/> [DOI](#)). After chromatic aberration

correction, coordinates of centers were used to calculate LQs , defined as the ratio of axial distances from GM130 to the testing protein and from GM130 to GalT-mCherry. Intra-Golgi transport kinetic LQ data were fitted to the first-order exponential function using OriginPro 2020.

In addition to the manual method mentioned above, we employed a newly developed software tool, Auto-GLIM (<https://github.com/Chokyotager/AutoGLIM>), to automatically analyze our ministack images. To this end, three-color images were acquired as described above. Three z-sections centered around the plane of interest were averaged (average z-projection) for beads and ministacks images. Automated background subtraction was performed using a deep learning segmentation model to first extract the cell contours, followed by a dual annealing optimization algorithm to perform background subtraction to extract the highest number of valid ROIs according to criteria of GLIM (Tie et al., 2016). Final LQs were further subjected to analysis in OriginPro 2020.

Acquisition of Golgi residence times

The methodology follows protocols previously described (Sun et al., 2021; Sun et al., 2020). Nocodazole was not used in these experiments. Briefly, HeLa cells on a Φ 35mm glass-bottom Petri dish were transiently transfected to co-express a GFP-tagged reporter and GalT-mCherry. For RUSH reporters, cells were treated with 50 μ M biotin at 20 °C for 2 h to accumulate the reporter at the Golgi. Live imaging was performed in a CO₂-independent medium (Thermo Fisher Scientific) with 10% FBS, 4 mM glutamine, and 10 μ g/ml cycloheximide, using a wide-field microscope until the cellular GFP fluorescence at the Golgi nearly vanished. The resulting time-lapse images were segmented based on GalT-mCherry using Fiji. Total GFP fluorescence within the Golgi was quantified and fitted to the first-order exponential function $y = y_0 + A1\exp(-x/t_1)$ in OriginPro 2020. Golgi residence time, $t_{1/2}$, was calculated as $0.693 \cdot t_1$. We only included time-lapse data with adj. $R^2 \geq 0.80$ and acquisition length $\geq 1.33 \cdot t_{1/2}$.

VHH-anti-GFP-mCherry internalization assay

6 $\times$ His-tagged VHH-anti-GFP-mCherry was purified as previously described (Sun et al., 2021; Sun et al., 2020). In the internalization assay, HeLa cells transiently expressing GFP-tagged reporters were continuously incubated with 5 μ g/ml VHH-anti-GFP-mCherry at 37 °C for 8 h. After washing, cells were fixed and imaged.

Microscopy for GLIM and Golgi residence time

Golgi residence times and most LQs were measured using a wide-field microscope based on Olympus IX83. The microscope featured a 100 $\times$ oil objective lens (NA 1.40), a motorized stage for sample positioning, and automated filter cubes to accommodate different fluorescence channels. Dichroic mirrors and filters were optimized for GFP/Alexa fluor 488, mCherry/Alexa Fluor 594, and Alexa Fluor 647. Imaging was captured with an sCMOS (scientific complementary metal oxide semiconductor) camera (Neo) by Andor. A 200W metal halide light source (Lumen Pro 200) by Prior Scientific provided illumination. Operational control and data collection were facilitated through Metamorph software by Molecular Devices. The image pixel size is 65 nm. The range of exposure time is 400 – 5000 ms for each channel.

GS15 LQs during the BFA treatment time course were measured using a spinning disk confocal microscope system comprising Olympus IX81 equipped with a 100 $\times$ oil objective lens (NA 1.45), a piezo z stage, Yokogawa CSU-X1 spinning head, 50 mW solid state lasers (488, 561 and 640 nm) (Sapphire; Coherent Inc., Santa Clara, CA, United States), an electron multiplying charge-coupled device (Evolve; Photometrics, Tucson, AZ, United States), and filters optimized for GFP/Alexa fluor 488, mCherry/Alexa Fluor 594, and Alexa Fluor 647. The system was controlled by Metamorph software (Molecular Devices). The image pixel size is 89 nm. The range of exposure time is 200 – 500 ms for each channel.

Airyscan microscopy

The Airyscan microscopy was performed using a Zeiss LSM710 confocal microscope, equipped with alpha Plan-Apochromat 100× NA 1.46 objective and the Airyscan module (Carl Zeiss). The system operation was controlled by Zen software (Carl Zeiss). Three lines of laser lights were used, 488, 561, and 640 nm. The emission band was selected to optimize the capture of the emission light while minimizing channel crosstalk. For side averaging, images were acquired under 63× objective (NA 1.40), zoomed in 3.5× to achieve 45 nm pixel size using the SR mode. The pixel dwelling time is 1.16 μs. The raw images were processed by Airyscan Zen software.

Side averaging and en face averaging

En face and side averaging were performed as described previously using Airyscan images (Tie et al., 2018; Tie et al., 2022). Ministacks with en face views were identified by giantin rings. They were subsequently normalized, expanded, and aligned with the center of the image, followed by averaging using Fiji. The radial mean intensity profile was acquired by Fiji macros (Tie et al., 2018). Ministacks with side views were identified by giantin double puncta and subjected to rotation, expansion, and normalization before averaging in Fiji (Tie et al., 2022). In a side average image, we define the axial position of a Golgi protein i as the y component of its center of mass coordinate, y_i . In **Figure 3E**, LQ^{side} of SBP-GFP-collagenX is calculated as below.

$$LQ^{\text{side}} = \frac{y_{\text{SBP-GFP-collagenX}} - y_{\text{GM130}}}{y_{\text{GalT-mCherry}} - y_{\text{GM130}}}$$

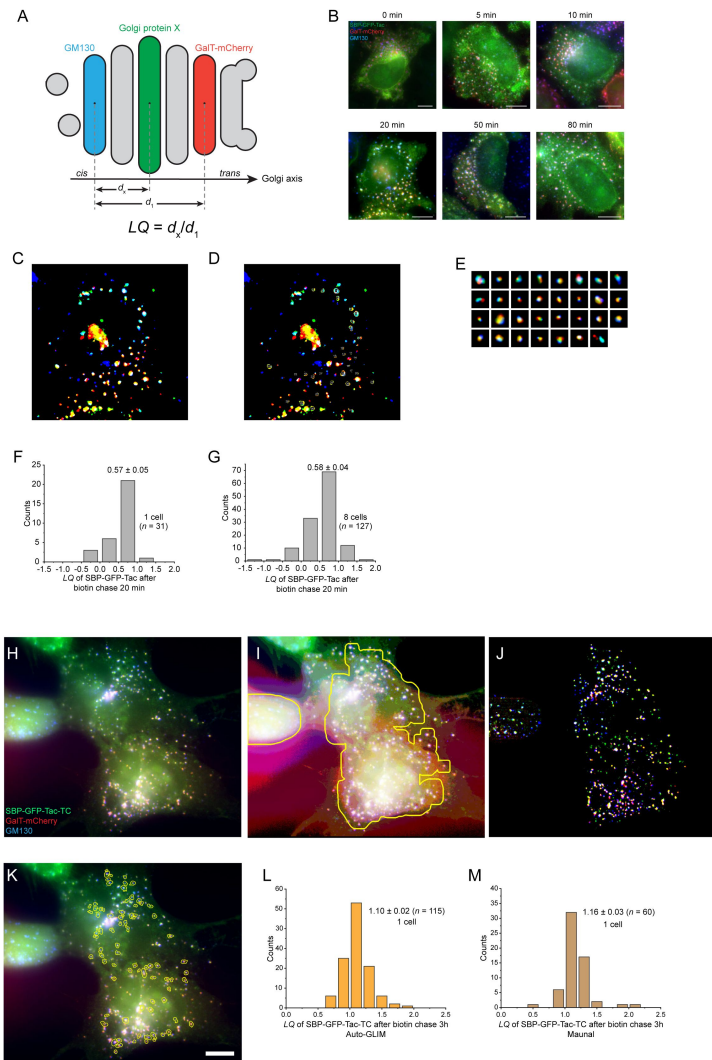
$y_{\text{SBP-GFP-collagenX}}$, y_{GM130} , and $y_{\text{GalT-mCherry}}$ are axial positions of SBP-GFP-collagenX, GM130, and GalT-mCherry in their side average images, respectively. y_{GM130} and $y_{\text{GalT-mCherry}}$ were measured previously (Tie et al., 2022).

In **Figure 6G**, the distance from GM130 to GalT-mCherry, $d_{(\text{GM130-GalT-mCherry})}$, was measured from individual ministacks with side views. The axial line intensity profile of GM130 or GalT-mCherry was subjected to Gaussian fitting in OriginPro2020 (Analysis>Fitting>Non-Linear Curve Fit). The calculated X_c represents the axial position of each protein. $d_{(\text{GM130-GalT-mCherry})}$ of each Golgi ministack is then calculated as

$$d_{\text{GM130-GalT-mCherry}} = X_{c_{\text{GalT-mCherry}}} - X_{c_{\text{GM130}}}$$

and subjected to statistical analysis. $d_{(\text{GalT-mCherry-CD8a-furin})}$ in **Figure 6H** was calculated similarly.

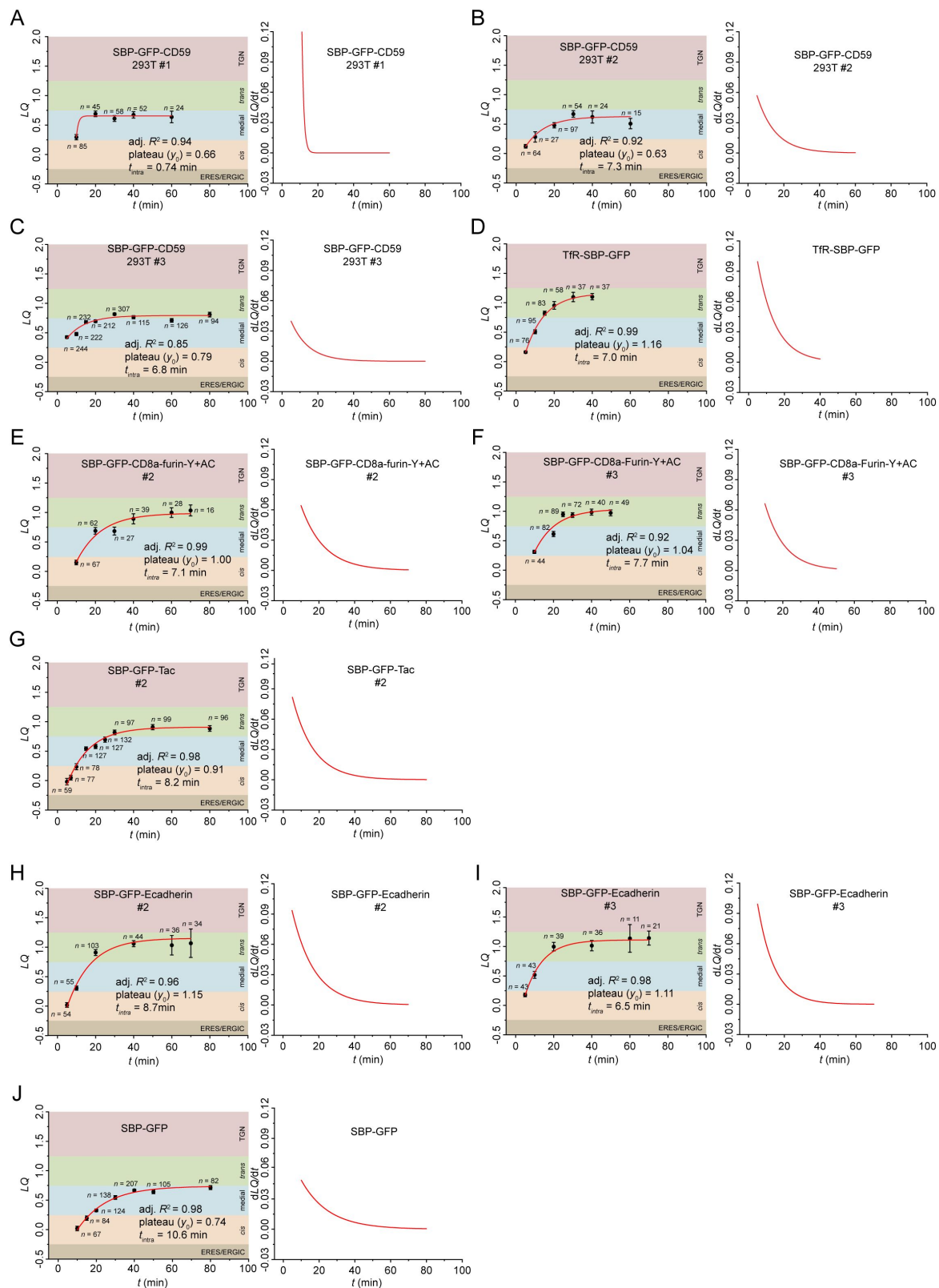
Supplementary information



Supplementary Figure 1.

GLIM and the Auto-GLIM tool.

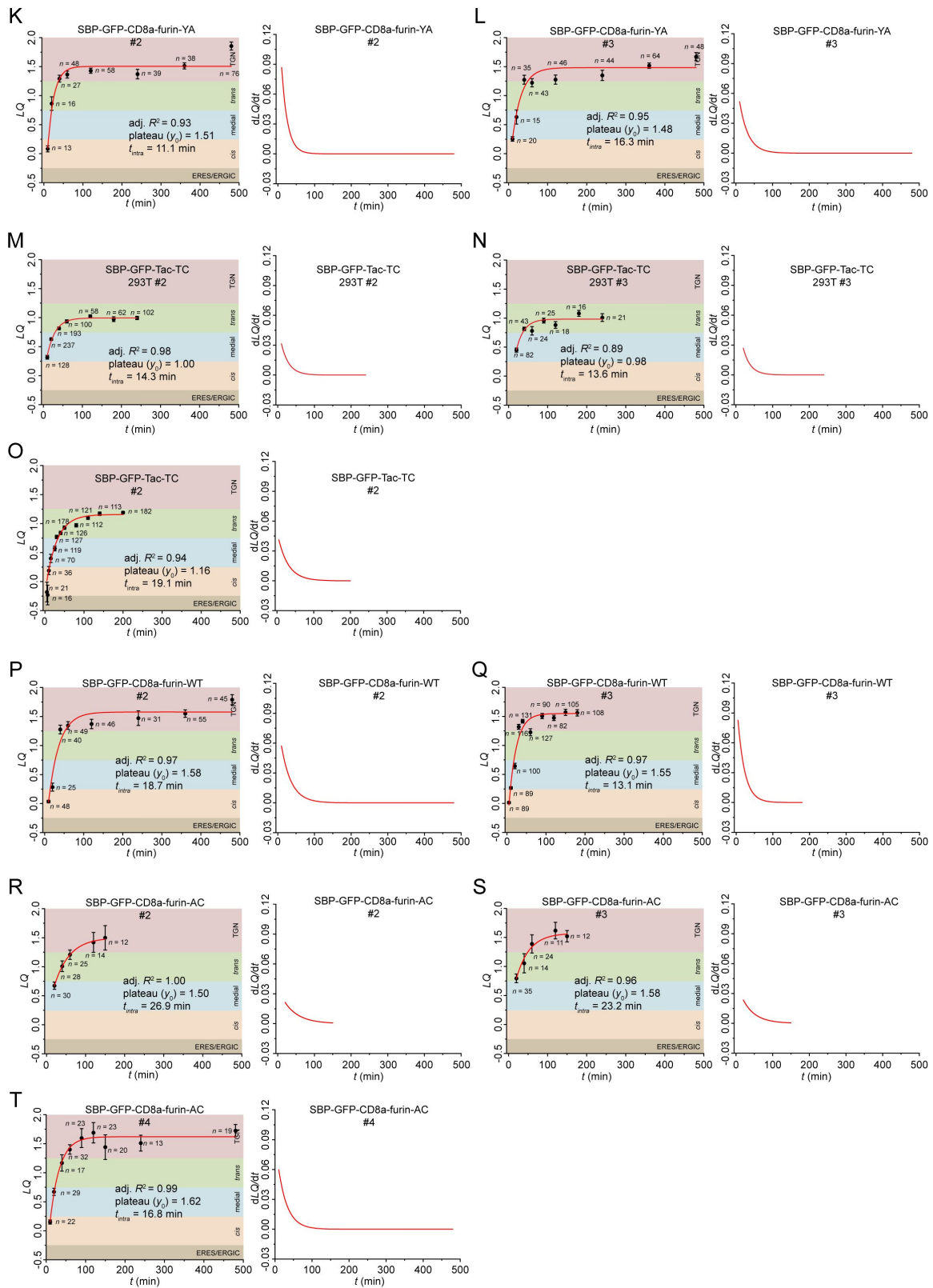
(A) A diagram that illustrates the principle behind GLIM. In the Golgi ministack, three proteins, GM130 (endogenous, *cis*-Golgi marker), GalT-mCherry (transfected, *trans*-Golgi marker), and protein X (transfected or endogenous, Golgi protein of interest) are fluorescently labeled. The centers of fluorescence mass (centers) of the three proteins can be calculated. The Golgi axis is defined as the vector from the center of GM130 to that of GalT-mCherry. The distance from the center of GM130 to that of GalT-mCherry is denoted as d_1 , while the distance from the center of GM130 to that of protein X is d_x . The LQ is then calculated as d_x/d_1 . (B-E) An example that illustrates the study of intra-Golgi transport kinetics using the RUSH reporter (SBP-GFP-Tac) and GLIM. HeLa cells co-expressing SBP-GFP-Tac and GalT-mCherry were treated with nocodazole for 3 h, followed by a biotin chase for various durations. Cells were then immunostained for endogenous GM130 and imaged using wide-field microscopy (B). Scale bar, 10 μ m. The image acquired after 20 min biotin chase was selected to demonstrate the image processing for GLIM. The background-subtracted image is displayed in binary intensity to enhance the visualization of ministacks with varying intensities (C). Analyzable ministacks (outlined by yellow contours) were manually selected after intensity thresholding (D). A gallery of 31 analyzable ministacks from (D) and the corresponding LQ histogram ($n = 1$ cell) are shown in (E) and (F), respectively. The LQ histogram of $n = 8$ cells is shown in (G). (H-M) Assessing the Auto-GLIM tool. HeLa cells co-expressing GalT-mCherry and SBP-GFP-Tac-TC were treated with nocodazole for 3 h, fixed, and immunostained for endogenous GM130. The image in (H) was acquired by wide-field microscopy. It is subjected to the Auto-GLIM tool with a frame of 72×72 pixels to detect the cell contour (marked by the yellow line) (I), conduct background-subtraction (J), and select analyzable Golgi ministacks (marked by yellow lines) (K). Scale bar, 10 μ m. The resulting histogram of LQ s is shown in (L). We also acquired LQ s using the conventional manual method (M), demonstrating strong agreement between the two approaches.



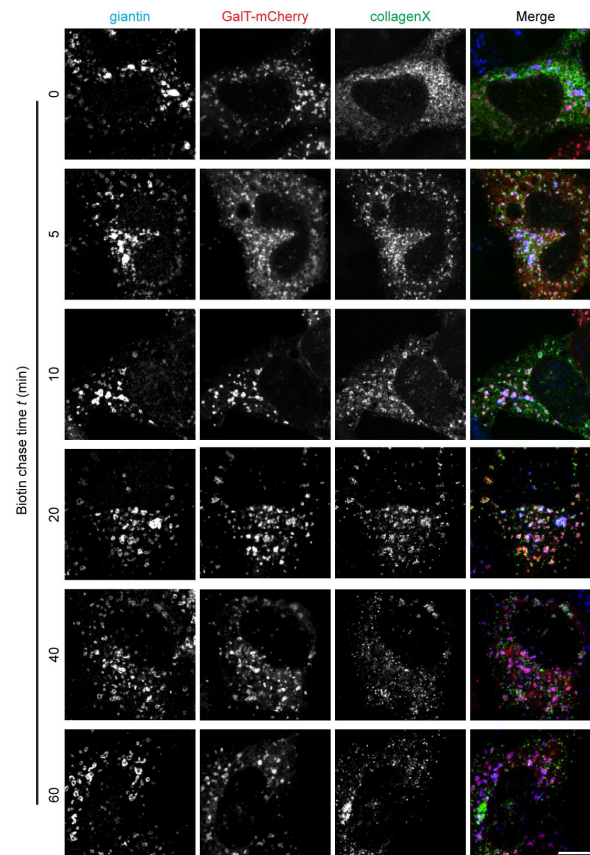
Supplementary Figure 2.

Additional intra-Golgi transport kinetics of RUSH reporters.

LQ vs. t plots (in A-D, G, J, and M-O) are generated in this study while the rest are from our previous reports (see [Table 1](#)). See the legend of [Figure 2](#) for details.



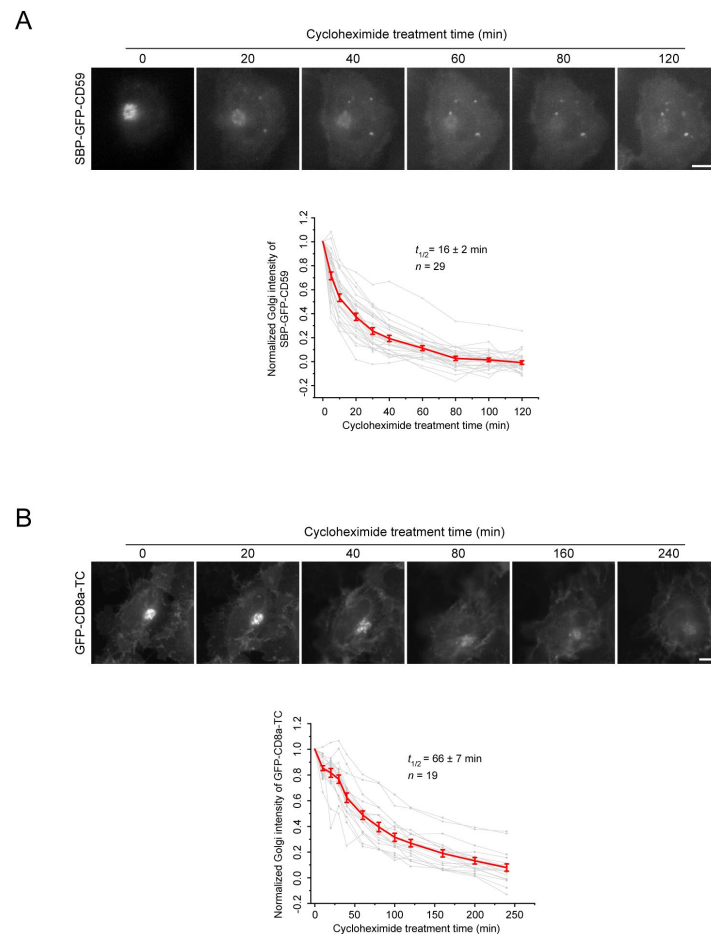
Supplementary Figure 2. (continued)



Supplementary Figure 3.

Example images used for side averaging in Figure 3D [↗](#).

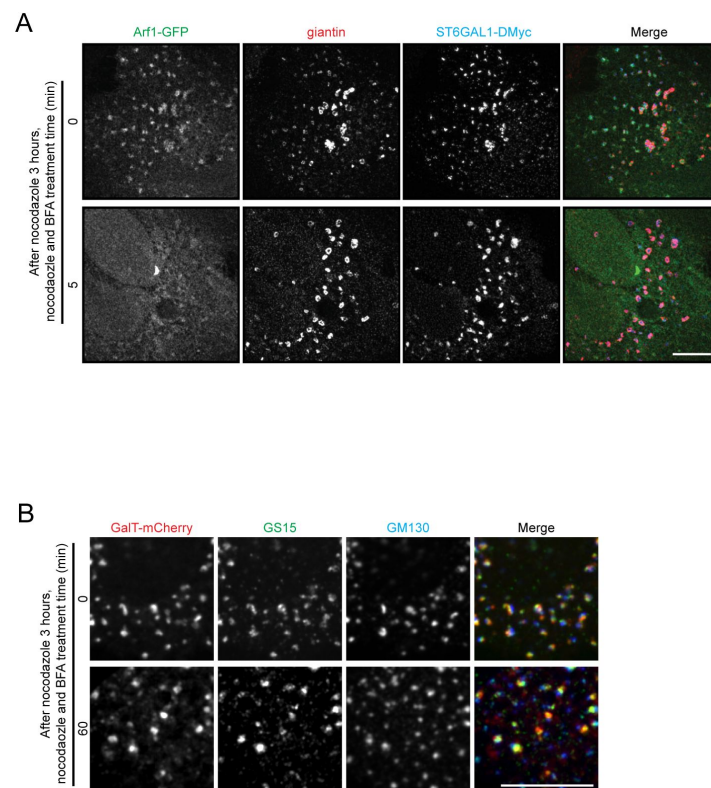
See the legend of Figure 3D [↗](#) for details. Scale bar, 10 μm .



Supplementary Figure 4.

Acquiring Golgi residence times of SBP-GFP-CD59 and GFP-CD8a-TC.

(A) HeLa cells were transiently transfected to co-express RUSH reporter, SBP-GFP-CD59, and GalT-mCherry. Cells were incubated with biotin and cycloheximide at 20 °C to accumulate the RUSH reporter in the Golgi before live-cell imaging under a wide-field microscope at 37 °C. Only SBP-GFP-CD59 images are shown. (B) HeLa cells transiently co-expressing GFP-CD8a-TC and GalT-mCherry were treated with cycloheximide and imaged live under a wide-field microscope. Only GFP-CD8a-TC images are shown. In both (A) and (B), the total fluorescence intensity within the Golgi was quantified, normalized, and plotted against the time. Each intensity series was fitted to the first-order exponential function to calculate $t_{1/2}$. Grey and red indicate individual and averaged time series, respectively. $t_{1/2}$ is expressed as mean $\pm$ SEM. n , the number of quantified cells. Scale bar, 10 μm .



Supplementary Figure 5.

The effect of BFA treatment on Golgi markers.

(A) Arf1 quickly dissociates from the Golgi under the treatment of nocodazole and BFA. HeLa cells transiently expressing Arf1-GFP and ST6GAL1-DMyc were treated with nocodazole for 3 h. This was followed by additional treatment with nocodazole and 5 μ M BFA for 5 min before fixation and immunostaining of Myc-tag and endogenous giantin. Images were acquired by Airyscan microscopy. (B) HeLa cells transiently expressing GalT-mCherry were treated with nocodazole for 3 h. This was followed by additional treatment with nocodazole and 5 μ M BFA for variable durations before fixation and immunostaining endogenous GS15 and GM130. Images were acquired by spinning disk confocal microscopy. Representative images at 0 and 60 min are shown. Scale bar, 10 μ m.

Fig. 2

A. SBP-TNF α -GFP

Chase time (min)	<i>n</i>	LQ	SEM	Cell number
5	89	-0.18	0.04	12
10	77	0.29	0.05	15
20	54	1.03	0.09	15
40	45	0.77	0.17	30
60	76	1.10	0.13	38
120	54	0.90	0.19	29
180	42	1.25	0.21	27

B. SBP-GFP-CD8 α -furin-Y+AC #1

10	38	0.10	0.04	9
20	60	0.65	0.06	9
30	32	0.74	0.05	7
40	42	0.82	0.07	14
60	21	0.89	0.14	8
70	10	0.73	0.16	9

C. SBP-GFP-Tac

10	58	0.04	0.02	4
20	87	0.57	0.03	3
30	82	0.86	0.04	6
40	55	0.80	0.04	2
60	70	0.90	0.04	3
90	50	0.82	0.03	2

D. SBP-GFP-Ecadherin #1

5	52	0.03087	0.02753	11
10	55	0.38	0.03	15
20	31	0.74	0.06	14
40	29	0.85	0.12	11
60	22	0.89	0.15	11
70	18	1.07	0.10	11

E. SBP-GFP-CD8 α -furin-YA #2

10	13	0.08	0.05	11
20	16	0.86	0.12	10
40	27	1.29	0.06	16
60	48	1.36	0.06	11
120	58	1.43	0.04	6
240	39	1.37	0.08	11
360	38	1.51	0.05	10
480	76	1.85	0.07	5

F. SBP-GFP-CD59

5	84	-0.01	0.02	12
10	119	0.15	0.02	19
20	131	0.60	0.03	16
30	90	0.85	0.04	18
40	51	0.80	0.05	10
50	87	0.90	0.04	15
60	49	0.96	0.07	15
70	52	0.95	0.06	12

10	150	0.24	0.03	14
15	283	0.43	0.02	14

G. SBP-GFP-Tac-TC
293T

20	147	0.59	0.03	11
40	94	0.76	0.03	8
60	208	0.84	0.02	13
120	93	0.97	0.03	10
180	88	0.99	0.02	9
240	86	0.94	0.02	10

H. SBP-GFP-Tac-TC

10	64	0.02	0.03	2
20	41	0.33	0.04	5
30	74	0.57	0.03	3
40	87	0.80	0.02	5
60	97	0.93	0.02	6
90	130	1.08	0.03	5
120	86	1.10	0.03	2
180	91	1.09	0.04	4
300	80	0.99	0.02	2

I. SBP-GFP-CD8a-furin-WT #1

10	17	-0.03	0.04	14
20	49	0.16	0.05	16
40	33	1.13	0.08	14
60	45	1.31	0.08	10
120	42	1.53	0.07	10
240	34	1.51	0.11	17
360	63	1.60	0.09	18
480	63	1.66	0.08	16

J. SBP-GFP-CD8a-furin-AC #1

20	48	0.92	0.06	11
40	28	1.38	0.10	23
60	26	1.53	0.09	21
120	12	1.71	0.10	21
150	13	1.75	0.15	11

Fig. 3

A. SBP-GFP-Collagen X
#1

Chase time (min)	<i>n</i>	<i>LQ</i>	SEM	Cell number
5	143	0.23	0.04	11
10	263	0.44	0.03	17
15	201	0.57	0.04	11
20	150	0.85	0.04	12
30	96	0.91	0.05	9
40	69	0.91	0.05	10
60	90	0.99	0.08	13
90	44	0.95	0.15	11

B. SBP-GFP-Collagen X
#2

5	211	0.04	0.03	11
10	229	0.32	0.03	11
15	175	0.41	0.03	8
20	158	0.59	0.05	9
30	143	0.80	0.05	15
40	235	0.77	0.05	15
60	87	0.80	0.09	16
90	53	0.78	0.13	12

C. SBP-GFP-Collagen X
#3

5	196	0.06	0.03	8
10	286	0.36	0.03	15
15	167	0.68	0.04	9
20	272	0.69	0.04	14
40	111	0.90	0.06	13
60	92	0.88	0.08	13
90	43	0.84	0.16	13

E. SBP-GFP-Collagen X
side-averaging

Chase time (min)	<i>n</i>	<i>LQ</i> ^{side}	SEM	Cell number
5	31	0.11	0.07	10
10	48	0.33	0.06	10
20	37	0.86	0.08	8
40	34	0.97	0.10	8

Fig. 6

TGN46	BFA treatment time(min)	<i>n</i>	<i>LQ</i>	SEM	Cell number
	0	102	1.56	0.04	5
	10	97	1.47	0.08	18
	20	68	1.30	0.05	13
ST6GAL1-moxGFP	30	62	1.25	0.05	19
	0	89	0.90	0.03	8
	10	116	0.95	0.02	7
	20	133	0.97	0.02	17
GS15	30	91	0.93	0.03	12
	0	110	0.70	0.03	9
	10	89	0.68	0.03	10
	20	28	0.74	0.07	11
GFP-Golgin-84	60	20	0.65	0.08	12
	0	78	0.05	0.05	8
	10	33	0.04	0.08	15
	20	52	0.10	0.15	14
CD8a-furin	30	48	0.40	0.11	13
	0	70	2.22	0.12	9
	10	59	1.73	0.17	18
	20	34	1.51	0.24	16
CD8a-CI-M6PR	30	41	1.39	0.15	19
	0	47	2.37	0.21	15
	10	29	1.77	0.21	19
	20	32	1.63	0.14	12
$d_{(GM130-GalTmCherry)}$ side-averaging	30	36	1.29	0.18	19
	BFA treatment time(min)	<i>n</i>	<i>d</i>	SEM	Cell number
	0	34	300	10	9
	10	18	280	27	11
$d_{(GalTmCherry-CD8afurin)}$ side-averaging	20	28	160	20	15
	30	13	190	32	7
	BFA treatment time(min)	<i>n</i>	<i>d</i>	SEM	Cell number
	0	30	210	21	7
	10	21	180	28	10
	20	21	90	28	9
	30	20	100	28	10

Fig. S2

A. SBP-GFP-CD59 293T#1	Chase time (min)	<i>n</i>	<i>LQ</i>	SEM	Cell number
	10	85	0.29	0.04	10
	20	45	0.69	0.05	7
	30	58	0.61	0.05	5
	40	52	0.67	0.05	7
	60	24	0.64	0.10	5
B. SBP-GFP-CD59 293T#2	5	64	0.12	0.03	15
	10	27	0.28	0.09	8
	20	97	0.48	0.04	16
	30	54	0.67	0.06	10
	40	24	0.62	0.10	11
	60	15	0.51	0.09	12
C. SBP-GFP-CD59 293T#3	5	244	0.43	0.02	13
	10	222	0.48	0.03	12
	15	232	0.68	0.02	14
	20	212	0.70	0.03	15
	30	307	0.82	0.02	16
	40	115	0.76	0.03	8
	60	126	0.71	0.03	16
	80	94	0.81	0.04	9
D. TfR-SBP-GFP	5	76	0.16	0.02	10
	10	95	0.51	0.03	8
	15	83	0.82	0.03	11
	20	58	0.95	0.06	10
	30	37	1.10	0.08	10
	40	37	1.10	0.05	13
E. SBP-GFP-CD8a-furin-Y+AC #2	10	67	0.15	0.04	13
	20	62	0.69	0.06	11
	30	27	0.68	0.07	13
	40	39	0.89	0.09	14
	60	28	1.00	0.08	15
	70	16	1.04	0.09	13
F. SBP-GFP-CD8a-furin-Y+AC #3	10	44	0.31	0.03	9
	20	82	0.62	0.04	12
	25	89	0.95	0.04	11
	30	72	0.94	0.04	15
	40	40	0.99	0.05	14
	50	49	0.97	0.05	14
G. SBP-GFP-Tac #2	5	59	-0.02	0.06	12
	7	77	0.05	0.04	12
	10	78	0.23	0.05	13
	15	127	0.54	0.03	15
	20	127	0.58	0.04	13
	25	132	0.70	0.04	14

H. SBP-GFP-Ecadherin #2

30	97	0.82	0.04	10
50	99	0.91	0.04	13
80	96	0.89	0.05	12

5	54	0.02	0.04	12
10	55	0.31	0.04	16
20	103	0.91	0.05	15
40	44	1.06	0.05	13
60	36	1.03	0.17	13
70	34	1.07	0.24	13

I. SBP-GFP-Ecadherin #3

5	43	0.18	0.03	10
10	43	0.52	0.06	12
20	39	1.00	0.07	8
40	36	1.01	0.09	16
60	11	1.14	0.23	9
70	21	1.14	0.12	13

J. SBP-GFP

10	67	0.02	0.04	6
15	84	0.19	0.04	9
20	124	0.33	0.02	6
30	138	0.55	0.03	10
40	207	0.67	0.02	10
50	105	0.64	0.03	8
80	82	0.71	0.03	9

K. SBP-GFP-CD8a-furin-YA #2

10	32	0.07	0.04	5
20	28	1.00	0.10	6
40	59	1.51	0.07	12
60	65	1.61	0.07	11
120	38	1.48	0.05	6
240	100	1.54	0.04	11
360	73	1.39	0.04	11
480	86	1.50	0.04	5

L. SBP-GFP-CD8a-furin-YA #3

10	20	0.25	0.04	15
20	15	0.63	0.12	14
40	35	1.27	0.08	13
60	43	1.22	0.07	10
120	46	1.28	0.08	6
240	44	1.35	0.09	16
360	64	1.52	0.05	8
480	48	1.67	0.07	6

M. SBP-GFP-Tac-TC
293T#2

10	128	0.32	0.03	15
20	237	0.63	0.02	13
40	193	0.82	0.02	13
60	100	0.94	0.03	7
120	58	1.02	0.03	10
180	62	0.97	0.04	15
240	102	0.99	0.03	13

N. SBP-GFP-Tac-TC
293T#3

20	83	0.45	0.04	24
40	43	0.81	0.03	15
60	24	0.78	0.07	8
90	25	0.96	0.04	7
120	18	0.88	0.06	8
180	16	1.08	0.05	4
240	21	1.01	0.07	11

O. SBP-GFP-Tac-TC #2

5	21	-0.18	0.17	11
7	16	-0.23	0.17	9
10	36	0.19	0.07	12
15	70	0.40	0.07	9
25	119	0.69	0.09	11
30	127	0.77	0.03	10
40	126	0.84	0.03	10
50	178	0.93	0.03	11
80	112	0.99	0.02	9
110	121	1.11	0.03	9
140	113	1.18	0.03	12
200	182	1.19	0.02	11

P. SBP-GFP-CD8a-furin-WT #2

10	48	0.04	0.02	8
20	25	0.28	0.07	9
40	40	1.28	0.08	10
60	49	1.34	0.07	15
120	46	1.37	0.08	13
240	31	1.47	0.13	11
360	55	1.55	0.06	14
480	45	1.79	0.09	10

Q. SBP-GFP-CD8a-furin-WT #3

5	89	0.02	0.02	9
10	89	0.27	0.02	10
20	100	0.64	0.05	8
30	116	1.32	0.04	18
40	131	1.42	0.03	9
60	127	1.23	0.06	17
90	90	1.51	0.05	9
120	82	1.47	0.05	17
150	105	1.57	0.05	13
180	108	1.56	0.05	13

R. SBP-GFP-CD8a-furin-AC #2

20	30	0.67	0.06	9
40	28	1.01	0.09	8
60	25	1.21	0.08	7
120	14	1.42	0.17	9
150	12	1.50	0.21	7

S. SBP-GFP-CD8a-furin-AC #3

20	35	0.80	0.07	10
40	14	1.06	0.17	10
60	24	1.39	0.16	15
120	11	1.62	0.14	8
150	12	1.52	0.10	11

T. SBP-GFP-CD8a-furin-AC #4

10	22	0.15	0.04	12
20	29	0.67	0.06	15
40	17	1.17	0.14	18
60	32	1.40	0.08	19
90	23	1.60	0.16	17
120	23	1.69	0.18	17
150	20	1.44	0.21	11
240	13	1.51	0.14	10
480	19	1.73	0.11	8

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Additional files

Supplementary file 1. LQ data employed in plots presented in Figure 2, Figure 6, and Supplementary Figure 2. *n*, the number of quantified cells. SEM, standard error of the mean. [↗](#)

References

- Ali M.F., Chachadi V.B., Petrosyan A., Cheng P.W. (2012) **Golgi phosphoprotein 3 determines cell binding properties under dynamic flow by controlling Golgi localization of core 2 N-acetylglucosaminyltransferase 1** *J Biol Chem* **287**:39564–39577 [Google Scholar](#)
- Bergmann J.E., Singer S.J. (1983) **Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells** *J Cell Biol* **97**:1777–1787 [Google Scholar](#)
- Bezoussenko G.V., Parashuraman S., Rizzo R., Polishchuk R., Martella O., Di Giandomenico D., Fusella A., Spaar A., Sallese M., Capestrano M.G., Pavelka M., Vos M.R., Rikers Y.G., Helms V., Mironov A.A., Luini A. (2014) **Transport of soluble proteins through the Golgi occurs by diffusion via continuities across cisternae** *eLife* **3** [Google Scholar](#)
- Boncompain G., Divoux S., Gareil N., de Forges H., Lescure A., Latreche L., Mercanti V., Jollivet F., Raposo G., Perez F. (2012) **Synchronization of secretory protein traffic in populations of cells** *Nat Methods* **9**:493–498 [Google Scholar](#)
- Bonfanti L., Mironov A.A., Martinez-Menarguez J.A., Martella O., Fusella A., Baldassarre M., Buccione R., Geuze H.J., Mironov A.A., Luini A. (1998) **Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation** *Cell* **95**:993–1003 [Google Scholar](#)
- Castle J.D., Jamieson J.D., Palade G.E. (1972) **Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit** *J Cell Biol* **53**:290–311 [Google Scholar](#)
- Chen Y., Gershlick D.C., Park S.Y., Bonifacino J.S. (2017) **Segregation in the Golgi complex precedes export of endolysosomal proteins in distinct transport carriers** *J Cell Biol* **216**:4141–4151 [Google Scholar](#)
- Cole N.B., Sciaky N., Marotta A., Song J., Lippincott-Schwartz J. (1996) **Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites** *Mol Biol Cell* **7**:631–650 [Google Scholar](#)
- De Matteis M.A., Di Girolamo M., Colanzi A., Pallas M., Di Tullio G., McDonald L.J., Moss J., Santini G., Bannykh S., Corda D., et al. (1994) **Stimulation of endogenous ADP-ribosylation by brefeldin A** *Proc Natl Acad Sci U S A* **91**:1114–1118 [Google Scholar](#)
- De Matteis M.A., Luini A. (2008) **Exiting the Golgi complex** *Nat Rev Mol Cell Biol* **9**:273–284 [Google Scholar](#)
- Diao A., Rahman D., Pappin D.J., Lucocq J., Lowe M. (2003) **The coiled-coil membrane protein golgin-84 is a novel rab effector required for Golgi ribbon formation** *J Cell Biol* **160**:201–212 [Google Scholar](#)

- Donaldson J.G., Finazzi D., Klausner R.D. (1992) **Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein** *Nature* **360**:350–352 [Google Scholar](#)
- Eckert E.S., Reckmann I., Hellwig A., Rohling S., El-Battari A., Wieland F.T., Popoff V. (2014) **Golgi phosphoprotein 3 triggers signal-mediated incorporation of glycosyltransferases into coatamer-coated (COPI) vesicles** *J Biol Chem* **289**:31319–31329 [Google Scholar](#)
- Emr S., Glick B.S., Linstedt A.D., Lippincott-Schwartz J., Luini A., Malhotra V., Marsh B.J., Nakano A., Pfeffer S.R., Rabouille C., Rothman J.E., Warren G., Wieland F.T. (2009) **Journeys through the Golgi--taking stock in a new era** *J Cell Biol* **187**:449–453 [Google Scholar](#)
- Engel B.D., Schaffer M., Albert S., Asano S., Plitzko J.M., Baumeister W. (2015) **In situ structural analysis of Golgi intracisternal protein arrays** *Proc Natl Acad Sci U S A* **112**:11264–11269 [Google Scholar](#)
- Ernst A.M., Syed S.A., Zaki O., Bottanelli F., Zheng H., Hacke M., Xi Z., Rivera-Molina F., Graham M., Rebane A.A., Bjorkholm P., Baddeley D., Toomre D., Pincet F., Rothman J.E. (2018) **S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi** *Dev Cell* **47**:479–493 [Google Scholar](#)
- Fourriere L., Divoux S., Roceri M., Perez F., Boncompain G. (2016) **Microtubule-independent secretion requires functional maturation of Golgi elements** *J Cell Sci* **129**:3238–3250 [Google Scholar](#)
- Glick B.S., Luini A. (2011) **Models for Golgi traffic: a critical assessment** *Cold Spring Harb Perspect Biol* **3**:a005215 [Google Scholar](#)
- Gut A., Kappeler F., Hyka N., Balda M.S., Hauri H.P., Matter K. (1998) **Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins** *EMBO J* **17**:1919–1929 [Google Scholar](#)
- Helms J.B., Rothman J.E. (1992) **Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF** *Nature* **360**:352–354 [Google Scholar](#)
- Hirschberg K., Miller C.M., Ellenberg J., Presley J.F., Siggia E.D., Phair R.D., Lippincott-Schwartz J. (1998) **Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells** *J Cell Biol* **143**:1485–1503 [Google Scholar](#)
- Howarth M., Chinnapen D.J., Gerrow K., Dorrestein P.C., Grandy M.R., Kelleher N.L., El-Husseini A., Ting A.Y. (2006) **A monovalent streptavidin with a single femtomolar biotin binding site** *Nat Methods* **3**:267–273 [Google Scholar](#)
- Klausner R.D., Donaldson J.G., Lippincott-Schwartz J. (1992) **Brefeldin A: insights into the control of membrane traffic and organelle structure** *J Cell Biol* **116**:1071–1080 [Google Scholar](#)
- Klumperman J (2011) **Architecture of the mammalian Golgi** *Cold Spring Harb Perspect Biol* **3** [Google Scholar](#)
- Kurokawa K., Osakada H., Kojidani T., Waga M., Suda Y., Asakawa H., Haraguchi T., Nakano A. (2019) **Visualization of secretory cargo transport within the Golgi apparatus** *J Cell Biol* **218**:1602–1618 [Google Scholar](#)

- Kwan A.P., Cummings C.E., Chapman J.A., Grant M.E. (1991) **Macromolecular organization of chicken type X collagen in vitro** *J Cell Biol* **114**:597–604 [Google Scholar](#)
- Lavieu G., Zheng H., Rothman J.E. (2013) **Stapled Golgi cisternae remain in place as cargo passes through the stack** *eLife* **2**:e00558 <https://doi.org/10.7554/eLife.00558> | [Google Scholar](#)
- Lippincott-Schwartz J., Donaldson J.G., Schweizer A., Berger E.G., Hauri H.P., Yuan L.C., Klausner R.D. (1990) **Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway** *Cell* **60**:821–836 [Google Scholar](#)
- Liu L., Doray B., Kornfeld S. (2018) **Recycling of Golgi glycosyltransferases requires direct binding to coatomer** *Proc Natl Acad Sci U S A* **115**:8984–8989 [Google Scholar](#)
- Losev E., Reinke C.A., Jellen J., Strongin D.E., Bevis B.J., Glick B.S. (2006) **Golgi maturation visualized in living yeast** *Nature* **441**:1002–1006 [Google Scholar](#)
- Luini A (2011) **A brief history of the cisternal progression-maturation model** *Cell Logist* **1**:6–11 [Google Scholar](#)
- Mahajan D., Boh B.K., Zhou Y., Chen L., Cornvik T.C., Hong W., Lu L. (2013) **Mammalian Mon2/Ysl2 regulates endosome-to-Golgi trafficking but possesses no guanine nucleotide exchange activity toward Arl1 GTPase** *Sci Rep* **3**:3362 [Google Scholar](#)
- Marsh B.J., Volkmann N., McIntosh J.R., Howell K.E. (2004) **Direct continuities between cisternae at different levels of the Golgi complex in glucose-stimulated mouse islet beta cells** *Proc Natl Acad Sci U S A* **101**:5565–5570 [Google Scholar](#)
- Matsuura-Tokita K., Takeuchi M., Ichihara A., Mikuriya K., Nakano A. (2006) **Live imaging of yeast Golgi cisternal maturation** *Nature* **441**:1007–1010 [Google Scholar](#)
- Mironov AA, Beznoussenko GV, Nicoziani P, Martella O, Trucco A, Kweon HS, Di Giandomenico D, Polishchuk RS, Fusella A, Lupetti P, Berger EG, Geerts WJ, Koster AJ, Burger KN, Luini A (2001) **Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae** *J Cell Biol* **155**:1225–1238 [Google Scholar](#)
- Monetta P., Slavin I., Romero N., Alvarez C. (2007) **Rab1b interacts with GBF1 and modulates both ARF1 dynamics and COPI association** *Mol Biol Cell* **18**:2400–2410 [Google Scholar](#)
- Patterson G.H., Hirschberg K., Polishchuk R.S., Gerlich D., Phair R.D., Lippincott-Schwartz J. (2008) **Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system** *Cell* **133**:1055–1067 [Google Scholar](#)
- Pereira N.A., Pu H.X., Goh H., Song Z. (2014) **Golgi phosphoprotein 3 mediates the Golgi localization and function of protein O-linked mannose beta-1,2-N-acetylglucosaminyltransferase 1** *J Biol Chem* **289**:14762–14770 [Google Scholar](#)
- Pfeffer S.R (2010) **How the Golgi works: a cisternal progenitor model** *Proc Natl Acad Sci U S A* **107**:19614–19618 [Google Scholar](#)
- Popoff V., Adolf F., Brugger B., Wieland F. (2011) **COPI budding within the Golgi stack** *Cold Spring Harb Perspect Biol* **3**:a005231 [Google Scholar](#)

Rabouille C., Klumperman J. (2005) **Opinion: The maturing role of COPI vesicles in intra-Golgi transport** *Nat Rev Mol Cell Biol* **6**:812–817 [Google Scholar](#)

Rizzo R., Russo D., Kurokawa K., Sahu P., Lombardi B., Supino D., Zhukovsky M.A., Vocat A., Pothukuchi P., Kunnathully V., Capolupo L., Boncompain G., Vitagliano C., Zito Marino F., Aquino G., Montariello D., Henklein P., Mandrich L., Botti G., Clausen H., Mandel U., Yamaji T., Hanada K., Budillon A., Perez F., Parashuraman S., Hannun Y.A., Nakano A., Corda D., D'Angelo G., Luini A. (2021) **Golgi maturation-dependent glycoenzyme recycling controls glycosphingolipid biosynthesis and cell growth via GOLPH3** *EMBO J* **40**:e107238 [Google Scholar](#)

Rogalski A.A., Bergmann J.E., Singer S.J. (1984) **Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane** *J Cell Biol* **99**:1101–1109 [Google Scholar](#)

Schmitz K.R., Liu J., Li S., Setty T.G., Wood C.S., Burd C.G., Ferguson K.M. (2008) **Golgi localization of glycosyltransferases requires a Vps74p oligomer** *Dev Cell* **14**:523–534 [Google Scholar](#)

Schueder F., Rivera-Molina F., Su M., Marin Z., Kidd P., Rothman J.E., Toomre D., Bewersdorf J. (2024) **Unraveling cellular complexity with transient adapters in highly multiplexed super-resolution imaging** *Cell* **187**:1769–1784 [Google Scholar](#)

Sun X., Mahajan D., Chen B., Song Z., Lu L. (2021) **A quantitative study of the Golgi retention of glycosyltransferases** *J Cell Sci* **134** [Google Scholar](#)

Sun X., Tie H.C., Chen B., Lu L. (2020) **Glycans function as a Golgi export signal to promote the constitutive exocytic trafficking** *J Biol Chem* **295**:14750–14762 [Google Scholar](#)

Tie H.C., Chen B., Sun X., Cheng L., Lu L. (2017) **Quantitative Localization of a Golgi Protein by Imaging Its Center of Fluorescence Mass** *J Vis Exp* [Google Scholar](#)

Tie H.C., Ludwig A., Sandin S., Lu L. (2018) **The spatial separation of processing and transport functions to the interior and periphery of the Golgi stack** *eLife* **7** [Google Scholar](#)

Tie H.C., Mahajan D., Chen B., Cheng L., VanDongen A.M., Lu L. (2016) **A novel imaging method for quantitative Golgi localization reveals differential intra-Golgi trafficking of secretory cargoes** *Mol Biol Cell* **27**:848–861 [Google Scholar](#)

Tie H.C., Mahajan D., Lu L. (2022) **Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks** *J Cell Biol* **221** [Google Scholar](#)

Trucco A., Polishchuk R.S., Martella O., Di Pentima A., Fusella A., Di Giandomenico D., San Pietro E., Beznoussenko G.V., Polishchuk E.V., Baldassarre M., Buccione R., Geerts W.J., Koster A.J., Burger K.N., Mironov A.A., Luini A. (2004) **Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments** *Nat Cell Biol* **6**:1071–1081 [Google Scholar](#)

Tu L., Tai W.C., Chen L., Banfield D.K. (2008) **Signal-mediated dynamic retention of glycosyltransferases in the Golgi** *Science* **321**:404–407 [Google Scholar](#)

Van De Moortele S., Picart R., Tixier-Vida A., Tougard C. (1993) **Nocodazole and taxol affect subcellular compartments but not secretory activity of GH3B6 prolactin cells** *Eur J Cell Biol* **60**:217–227 [Google Scholar](#)

Volchuk A., Amherdt M., Ravazzola M., Brugger B., Rivera V.M., Clackson T., Perrelet A., Sollner T.H., Rothman J.E., Orci L. (2000) **Megavesicles implicated in the rapid transport of intracisternal aggregates across the Golgi stack** *Cell* **102**:335–348 [Google Scholar](#)

Weigel A.V., Chang C.L., Shtengel G., Xu C.S., Hoffman D.P., Freeman M., Iyer N., Aaron J., Khuon S., Bogovic J., Qiu W., Hess H.F., Lippincott-Schwartz J. (2021) **ER-to-Golgi protein delivery through an interwoven, tubular network extending from ER** *Cell* **184**:2412–2429 [Google Scholar](#)

Wessels E., Duijsings D., Niu TK, Neumann S, Oorschot VM, de Lange F, Lanke KH, Klumperman J, Henke A, Jackson CL, Melchers WJ, van Kuppeveld FJ (2006) **A viral protein that blocks Arf1-mediated COP-I assembly by inhibiting the guanine nucleotide exchange factor GBF1** *Dev Cell* **11**:191–201 [Google Scholar](#)

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Reviewer #1 (Public review):**Summary:**

In the manuscript by Tie et.al., the authors couple the methodology which they have developed to measure LQ (localization quotient) of proteins within the Golgi apparatus along with RUSH based cargo release to quantify the speed of different cargos traveling through Golgi stacks in nocodazole induced Golgi ministacks to differentiate between cisternal progression vs stable compartment model of the Golgi apparatus. The debate between cisternal progression model and stable compartment model has been intense and going on for decades and important to understand the basic way of function/organization of the Golgi apparatus. As per the stable compartment model, cisterna are stable structures, and cargo moves along the Golgi apparatus in vesicular carriers. While as per cisternal progression model, Golgi cisterna themselves mature acquiring new identity from the cis face to the trans face and act as transport carriers themselves. In this work, authors provide a missing part regarding intra-Golgi speed for transport of different cargoes as well as the speed of TGN exit and based on the differences in the transport velocities for different cargoes tested favor a stable compartment model. The argument which authors make is that if there is cisternal progression, all the cargoes should have a similar intra-Golgi transport speed which is essentially the rate at which the Golgi cisterna mature. Furthermore, using a combination of BFA and Nocodazole treatments authors show that the compartments remain stable in cells for at least 30-60 minutes after BFA treatment.

Strengths:

The method to accurately measure localization of a protein within the Golgi stack is rigorously tested in the previous publications from the same authors and in combination with pulse chase approaches has been used to quantify transport velocities of cargoes through the Golgi. This is a novel aspect in this paper and differences in intra-Golgi velocities for different cargoes tested makes a case for a stable compartment model.

Weaknesses:

None noted in the revised version of the manuscript.

<https://doi.org/10.7554/eLife.98582.2.sa3>

Reviewer #2 (Public review):**Summary:**

This manuscript describes the use of quantitative imaging approaches, that have been a key element of the labs work over the past years, to address one of the major unresolved discussions in trafficking: intra-Golgi transport. The approach used has been clearly described in the labs previous papers, and is thus clearly described. The authors clearly address the weaknesses in this manuscript, and do not overstate the conclusions drawn from the data. The only weakness not addressed is the concept of blocking COPI transport with BFA, which is a strong inhibitor and causes general disruption of the system. This is an interesting element of the paper, which I think could be improved upon by using more specific COPI inhibitors instead, although I understand that this is not necessarily straightforward.

I commend the authors on their clear and precise presentation of this body of work, incorporating mathematical modelling with a fundamental question in cell biology. In all, I think that this is a very robust body of work, that provides a sound conclusion in support of the stable compartment model for the Golgi.

General points:

The manuscript contains a lot of background in its results sections, and the authors may wish to consider rebalancing the text: The section beginning at Line 175 is about 90% background and 10% data. Could some data currently in supplementary be included here to redress this balance, or this part combined with another?

Minor points:

Equation 2: A should be in front of the $\ln 2$. It's already resolved in equation 3, so likely only needs changing in the text

Line 152: Why is there a lack of experimental data? High ER background and low golgi signal make it difficult to select ministacks: would be good to see examples of these images. Is 0 a relevant timepoint as cargo is still at the ER? Instead would a timepoint $<5'$ be better demonstrate initial arrival in fast cargo, and 0' discarded?

Table 1 Line 474: 1-3 independent replicates: is there a better way of incorporating this into the table to make it more streamlined? It would be useful to see each cargo as a mean with error. Is there a more demonstrative way to present the table, for example (but does not have to be) fastest cargo first (Tindra) as in Table 2?

Line 264 / Fig 3B: It's unclear to me why the VHH-anti-GFP-mCherry internalisation approach was used, when the cells were expressing GFP, that could be used for imaging. Also, this introduces a question over trafficking of the VHH itself, to access the same compartments as the GFP-proteins are localised. It would be useful to describe the choice of this approach briefly in the text.

446 Typo "internalization"

Post-Revision

I thank the authors for their work revising the paper in light of our comments. I am satisfied with their response, and I have no other comments.

<https://doi.org/10.7554/eLife.98582.2.sa2>

Reviewer #3 (Public review):

The manuscript by Tie et al. provides a quantitative assessment of intra-Golgi transport of diverse cargos. Quantitative approaches using fluorescence microscopy of RUSH synchronized cargos, namely GLIM and measurement of Golgi residence time, previously developed by the author's team (publications from 2016 to 2022), are being used here.

Most of the results have been already published by the same team in 2016, 2017, 2020 and 2021. In this manuscript, the authors have put together measurement of intra-Golgi transport kinetics and Golgi residence time of many cargos. The quantitative results are supported by a large number of Golgi mini-stacks/cells analyzed. They are discussed with regard to the intra-Golgi transport models being debated in the field, namely the cisternal maturation/progression model and the stable compartments model.

The authors show that different cargos have distinct intra-Golgi transport kinetics and that the Golgi residence time of glycosyltransferases is high. From this and experiment using brefeldinA, the authors suggest that the rim progression model, adapted from the stable compartments model, fits with their experimental data.

Strengths:

The major strength of this manuscript is to put together many quantitative results that the authors previously obtained and to discuss them to advance our understanding of the intra-Golgi transport mechanisms.

The analysis by fluorescence microscopy of intra-Golgi transport is tough and this is a tour de force of the authors even though their approach shows limitations, which are clearly stated. Their work is remarkable in regards of the numbers of Golgi markers and secretory cargos which have been analyzed.

Weaknesses:

Most of the data provided here were already published and thus accessible for the community. The tubular connections between cisternae and the diffusion/biochemical properties of cargos are not taken into account to interpret the results. Indeed, tubular connections and biochemical properties of the cargos may affect their transit through the Golgi and the kinetics with which they reach the TGN for Golgi exit.

The use of nocodazole might affect cellular homeostasis but this is clearly stated by the authors and is acceptable as we need to perturb the system to conduct this analysis.

The manual selection of the Golgi mini-stack being analyzed (where the cargo and the Golgi reference markers are clearly detectable) might introduce a bias in the analysis.

<https://doi.org/10.7554/eLife.98582.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:**Reviewer #1 (Public Review):***Summary:*

In the manuscript by Tie et al., the authors couple the methodology which they have developed to measure LQ (localization quotient) of proteins within the Golgi apparatus along with RUSH based cargo release to quantify the speed of different cargos traveling

through Golgi stacks in nocodazole induced Golgi ministacks to differentiate between cisternal progression vs stable compartment model of the Golgi apparatus. The debate between cisternal progression model and stable compartment model has been intense and going on for decades and important to understand the basic way of function/organization of the Golgi apparatus. As per the stable compartment model, cisterna are stable structures and cargo moves along the Golgi apparatus in vesicular carriers. While as per cisternal progression model, Golgi cisterna themselves mature acquiring new identity from the cis face to the trans face and act as transport carriers themselves. In this work, authors provide a missing part regarding intra-Golgi speed for transport of different cargoes as well as the speed of TGN exit and based on the differences in the transport velocities for different cargoes tested favor a stable compartment model. The argument which authors make is that if there is cisternal progression, all the cargoes should have a similar intra-Golgi transport speed which is essentially the rate at which the Golgi cisterna mature. Furthermore, using a combination of BFA and Nocodazole treatments authors show that the compartments remain stable in cells for at least 30-60 minutes after BFA treatment.

Strengths:

The method to accurately measure localization of a protein within the Golgi stack is rigorously tested in the previous publications from the same authors and in combination with pulse chase approaches has been used to quantify transport velocities of cargoes through the Golgi. This is a novel aspect in this paper and differences in intra-Golgi velocities for different cargoes tested makes a case for a stable compartment model.

Weaknesses:

Experiments are only tested in one cell line (HeLa cells) and predominantly derived from experimental paradigm using RUSH assays where a secretory cargo is released in a wave (not the most physiological condition) and therefore additional approaches would make a more compelling case for the model.

We have added datasets from 293T cells in the revamped manuscript.

Reviewer #2 (Public Review):

Summary:

This manuscript describes the use of quantitative imaging approaches, which have been a key element of the labs work over the past years, to address one of the major unresolved discussions in trafficking: intra-Golgi transport. The approach used has been clearly described in the labs previous papers, and is thus clearly described. The authors clearly address the weaknesses in this manuscript and do not overstate the conclusions drawn from the data. The only weakness not addressed is the concept of blocking COPI transport with BFA, which is a strong inhibitor and causes general disruption of the system. This is an interesting element of the paper, which I think could be improved upon by using more specific COPI inhibitors instead, although I understand that this is not necessarily straightforward.

I commend the authors on their clear and precise presentation of this body of work, incorporating mathematical modelling with a fundamental question in cell biology. In all, I think that this is a very robust body of work, that provides a sound conclusion in support of the stable compartment model for the Golgi.

General points:

The manuscript contains a lot of background in its results sections, and the authors may wish to consider rebalancing the text: The section beginning at Line 175 is about 90% background and 10% data. Could some data currently in supplementary be included here to redress this balance, or this part combined with another?

In the revamped manuscript, we have moved the background information on rapid partitioning and rim progression models to the Introduction.

Reviewer #3 (Public Review):

The manuscript by Tie et al. provides a quantitative assessment of intra-Golgi transport of diverse cargos. Quantitative approaches using fluorescence microscopy of RUSH synchronized cargos, namely GLIM and measurement of Golgi residence time, previously developed by the author's team (publications from 2016 to 2022), are being used here.

Most of the results have been already published by the same team in 2016, 2017, 2020 and 2021. In this manuscript, very few new data have been added. The authors have put together measurements of intra-Golgi transport kinetics and Golgi residence time of many cargos. The quantitative results are supported by a large number of Golgi mini-stacks/cells analyzed. They are discussed with regard to the intra-Golgi transport models being debated in the field, namely the cisternal maturation/progression model and the stable compartments model. However, over the past decades, the cisternal progression model has been mostly accepted thanks to many experimental data.

The authors show that different cargos have distinct intra-Golgi transport kinetics and that the Golgi residence time of glycosyltransferases is high. From this and the experiment using brefeldinA, the authors suggest that the rim progression model, adapted from the stable compartments model, fits with their experimental data.

Strengths:

The major strength of this manuscript is to put together many quantitative results that the authors previously obtained and to discuss them to give food for thought about the intraGolgi transport mechanism.

The analysis by fluorescence microscopy of intra-Golgi transport is tough and is a tour de force of the authors even if their approach show limitations, which are clearly stated. Their work is remarkable in regards to the numbers of Golgi markers and secretory cargos which have been analyzed.

Weaknesses:

As previously mentioned, most of the data provided here were already published and thus accessible for the community. Is there is a need to publish them again?

The authors' discussion about the intra-Golgi transport model is rather simplistic. In the introduction, there is no mention of the most recent models, namely the rapid partitioning and the rim progression models. To my opinion, the tubular connections between cisternae and the diffusion/biochemical properties of cargos are not enough taken into account to interpret the results. Indeed, tubular connections and biochemical properties of the cargos may affect their transit through the Golgi and the kinetics with which they reach the TGN for Golgi exit.

Nocodazole is being used to form Golgi mini-stacks, which are necessary to allow intra-Golgi measurement. The use of nocodazole might affect cellular homeostasis but this is clearly stated by the authors and is acceptable as we need to perturb the system to

conduct this analysis. However, the manual selection of the Golgi mini-stack being analyzed raises a major concern. As far as I understood, the authors select the mini-stacks where the cargo and the Golgi reference markers are clearly detectable and separated, which might introduce a bias in the analysis.

The terms 'Golgi residence time' is being used but it corresponds to the residence time in the trans-cisterna only as the cargo has been accumulated in the trans-Golgi thanks to a 20°C block. The kinetics of disappearance of the protein of interest is then monitored after 20°C to 37°C switch.

Another concern also lies in the differences that would be introduced by different expression levels of the cargo on the kinetics of their intra-Golgi transport and of their packaging into post-Golgi carriers.

Please see below for our replies to intra-Golgi transport models, the Golgi residence time, and different expression levels of cargos.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

The data shown by the authors to measure differential intra Golgi velocities based on previously established methodology make a case for a stable compartment model, however more data is needed to make a complete story and the clarity of presentation can be improved.

We sincerely appreciate the reviewer's insightful, detailed, and constructive feedback. Your thoughtful comments have helped us refine our analyses, clarify key points, and strengthen the overall quality of our manuscript. We are grateful for the time and effort you have dedicated to reviewing our work and providing valuable suggestions. Your input has been instrumental in improving both the scientific rigor and presentation of our findings. Thank you for your thorough and thoughtful review.

Main points:

(1) Along with the studies in yeast, which authors describe in this paper, the main evidence for cisternal maturation model in mammalian cells comes from Bonfanti *et al.*, ([https://doi.org/10.1016/S0092-8674\(00\)81723-7](https://doi.org/10.1016/S0092-8674(00)81723-7)), which used EM to visualize a wave of Collagen through Golgi stacks. It is therefore important this work needs to include collagen as one of the cargos tested. Can the authors use the RUSH-Col1AGFP (see: <https://doi.org/10.1083/jcb.202005166>) as a cargo to monitor intra-Golgi velocities?

I understand that Hela cells are not professional collagen-secreting, but the authors can use U2OS cells to measure collagen export and two other extreme (slow and fast) cargos to validate the same trend in intra-Golgi transport velocities is seen in other cell lines. This will address three concerns: a. This is not a Hela-specific phenomenon; b. Transport of large cargoes like collagen agree with their proposal; c. To see if the same cargo has the same (similar) intra-Golgi speed and the trend between different cargoes is conserved across cell lines.

Due to the difficulty of manipulating and imaging the procollagen-I RUSH reporter, we selected the collagenX-RUSH reporter (SBP-GFP-collagenX) instead. Our previous study (Tie *et al.*, eLife, 2028) demonstrated that SBP-GFP-collagenX assembles as a large molecular weight particle, each having ~ 190 copies of SBP-GFP-collagenX. With an estimated mean size of ~ 40 nm, these aggregates are not as large as FM4 aggregates and procollagen-I (> 300 nm) and, therefore, are not excluded from conventional transport vesicles, which typically have a size

of 50 – 100 nm. However, collagenX has distinct intra-Golgi transport behaviour from conventional secretory cargos – while conventional secretory cargos localize to the cisternal interior, collagenX partitions to the cisternal rim (Tie et al., eLife, 2028).

We studied the intra-Golgi transport of SBP-GFP-collagenX in HeLa cells via GLIM and side averaging. The new results are included in Figure 3 of the revamped manuscript. CollagenX has similar intra-Golgi transport kinetics as conventional secretory cargos, displaying the first-order exponential function in LQ vs. time and velocity vs. time plots.

The side-averaging images are consistent with previous and current results. collagenX displays a double-punctum during the intra-Golgi transport, indicating a cisternal rim localization, as expected for large secretory cargos. Therefore, our new data demonstrated that cisternal rim partitioned large-size secretory cargos might follow intra-Golgi transport kinetics similar to those of cisternal interior partitioned conventional secretory cargos.

We tried SBP-GFP-CD59 and SBP-GFP-Tac-TC, cargos with fast and slow intra-Golgi transport velocities, respectively, in 293T cells. Results are included in Figure 2, Supplementary Figure 2, and Table 1 of the revamped manuscript. We found that SBP-GFPTac-TC showed similar t_{intra} s, 17 and 14 min, respectively, in HeLa and 293T cells. Considering our previous finding that glycosylation has an essential role in the Golgi exit (Sun et al., JBC, 2020), the distinct intra-Golgi transport kinetics of SBP-GFP-CD59 (t_{intra} s, 13 and 5 min, respectively, in HeLa and 293T cells) might be due to its distinct luminal glycosylation between HeLa and 293T cells. Supporting this hypothesis, SBP-GFP-Tac-TC does not have any glycosylation sites due to the truncation of the Tac luminal domain.

(2) RUSH assay has its own caveats which authors also refer to in the manuscript. Authors should test their model by using pulse chase approaches by SNAP tagged constructs which will allow them to do pulse chase assays without the requirement to release cargo as a wave (see: doi: 10.1242/jcs.231373). It is not necessary to test all the cargoes but the two on the ends of the spectrum (slow and fast). To avoid massive overexpression, authors could express the proteins using weaker promoters. Authors could also use this approach to simultaneously measure the two cargoes by tagging them with CLIP and SNAP tags and doing the pulse chase simultaneously (see: DOI: 10.1083/jcb.202206132). In this case it may be difficult to stain both GM130 and TGN, but authors could monitor the rate of segregation from the GM130 signal.

During the RUSH assay, the sudden release of a large amount of secretory reporters does not occur under native secretory conditions and, consequently, might introduce artifacts. The reviewer suggests using pulse-chase labeling of SNAP (or CLIP)-tagged secretory cargos, which occurs in a steady state and hence more closely resembles native secretory transport. This is an excellent suggestion. However, we have not yet tested this method due to the following concerns.

The standard protocol involves blocking existing reporters, pulse-labeling newly synthesized reporters, and chasing their movement along the secretory pathway. However, the typical 20minute pulse labeling period used in the two references would be too long, as a substantial portion of the reporters would already reach the *trans*-Golgi or exit the Golgi before the chase begins. Conversely, reducing the pulse labeling time would significantly weaken the GLIM signal.

(3) While the intra-Golgi velocities are different for different cargoes tested, authors should show a control that the arrival of the cargoes from ER to the cis-Golgi follows similar kinetics or if there are differences there is no correlation with the intra-Golgi velocities. In other words, do cargoes which show slow intra-Golgi velocities also take more time to reach the cis-Golgi and vice versa.

In nocodazole-induced Golgi ministacks, the ER exit site, ERGIC, and *cis*-Golgi are spatially closely associated. At the earliest measurable time point—5 minutes after biotin treatment—we observed that the secretory cargo had already reached the *cis*-Golgi (Figure 2 and Supplementary Figure 2). The rapid ER-to-*cis*-Golgi transport exceeds the temporal resolution of our current protocol, making it difficult to address the reviewer’s question (see our reply to Minor Points (2) of Reviewer #2 for more detailed discussion on this).

(4) Were the different cargos traveling (at different speeds) through Golgi at the rims, or in the middle of ministack, or by vesicles?

Please also refer to our reply to Question 1 of Reviewer #1. For the nocodazole-induced Golgi ministack, we previously investigated the lateral cisternal localization of RUSH secretory reporters using our en face average imaging (Tie et al., eLife, 2018). We found that small or conventional cargos (such as CD59 and E-cadherin) partition to the cisternal interior while large cargos (collagenX and FM4-CD8a) partition to the cisternal rim during their intra-Golgi transport. Using GLIM, we showed that the intra-Golgi transport kinetics of collagenX is similar to that of small cargos as both follow the first-order exponential function (Figure 3A-C). Therefore, cisternal rim partitioned large size secretory cargos might have intra-Golgi transport kinetics similar to those of cisternal interior partitioned conventional secretory cargos.

(5) Figure 4, under both nocodazole and BFA treatment for 30mins, would the stacks have the same number (274 nm per LQ) as thickness? Or does it shrink a little? Considering extended BFA treatment reduced intact Golgi ministacks. This is important to understand the LQ numbers of those Golgi proteins. Besides, can they include one ERGIC marker in this assay, would it be approaching cis-Golgi? Images used for quantification in Figure 4 should be shown in the main figure.

We define the axial size of the Golgi ministack as the axial distance from the GM130 to the GalT-mCherry, $d_{\text{(GM130-GalT-mCherry)}}$, measured using the Gaussian centers of their line intensity profiles. As the reviewer suggested, we measured the axial size of the ministack during the nocodazole and BFA treatment. Indeed, we found a decrease in the ministack axial size from 300 ± 10 nm at 0 min to 190 ± 30 nm at 30 min of BFA treatment. This observation is further confirmed by our side average imaging. The new data is presented in Fig. 6G.

Our study focuses on changes in the organization of the Golgi ministack. So, we didn’t include ERGIC53 in the current analysis. Instead, we quantified the axial distance between GalT-mCherry and CD8a-furin, $d_{\text{(GalT-mCherry-CD8a-furin)}}$, and found that it decreased from 200 ± 20 nm at 0 min to 100 ± 30 nm at 30 min of BFA treatment, suggesting the collapse of the TGN. The collapse of the TGN is further visualized by our side average imaging. The new data is presented in Fig. 6H.

Therefore, our new data demonstrates that the Golgi ministack shrinks, and the TGN collapses under BFA treatment.

Minor points:

(1) The LQ data come from confocal/airy scan images, but no such images were shown in this paper. The authors can't assume every reader to have prior knowledge of their previous work. It will be beneficial to have one example image and how the LQ was measured.

As advised by the reviewer, we have prepared Supplementary Figure 1 to provide a brief illustration of the principle behind GLIM and image processing steps involved.

(2) The cargos used in this paper need to be introduced: what are they, how were they used in previous literature. Especially the furin constructs come out of the blue (also see point 7).

As suggested by the reviewer, we have included a schematic diagram in Fig. 1 of the revised manuscript to illustrate all RUSH reporters and their corresponding ER hooks. In this diagram, we also highlight the key sequence differences in the cytosolic tails of different furin mutants.

Additionally, we have added references for each RUSH reporter at the beginning of the Results and Discussion section.

(3) There are two categories of exocytosis, constitutive and regulated. It important to state that the phenomenon observed is in cells predominantly showing only constitutive secretion.

As the reviewer advised, we have added the following sentences in the section titled “Limitations of the study”.

“Third, all RUSH reporters used in this study are constitutive secretory cargos. As a result, the intra-Golgi transport dynamics observed here might not reflect those of regulated secretion, which involves the synchronized release of a large quantity of cargo in response to a specific signal.”

(4) All the cargoes show a progressive reduction in instantaneous velocities from cis to medial to trans. Authors should discuss how do they mechanistically explain this. Is the rate of vesicle production progressively decreasing from cis to trans and if so, why?

As our imaging methods cannot differentiate vesicles from the cisternal rim, we could not tell if the vesicle production rate had changed during the intra-Golgi transport. We have provided an explanation of the progressive reduction of the intra-Golgi transport velocity in the Results and Discussion section. Please see the text below.

“The progressive reduction in intra-Golgi transport of secretory cargo might result from the enzyme matrix’s retention at the trans-Golgi. As the secretory cargos progress along the Golgi stack from the cis to the trans-side, more and more cargos become temporarily retained in the trans-Golgi region, gradually reducing their overall intra-Golgi transport velocity. If the release or Golgi exit of these cargos from the enzyme matrix follows a constant probability per unit time, i.e., a first-order kinetics process, the rate of cargo exiting from the Golgi should follow the first-order exponential function. Since the mechanism underlying intra-Golgi transport kinetics reflects fundamental molecular and cellular processes of the Golgi, further experimental data are essential to rigorously test this hypothesis.”

(5) The supp file 1 nicely listed the raw data for plotting, and n for numbers of ministacks. Could the authors also show number of cells or experiment repeats?

In the revamped version of the Supplementary File 1, we have added the cell number for each LQ measurement.

(6) This recent work used novel multiplexing methods to show that nocodazole-treated cells had similar protein organization as in control may be cited. It also showed the effect of BFA. [https://www.cell.com/cell/abstract/S0092-8674\(24\)00236-8](https://www.cell.com/cell/abstract/S0092-8674(24)00236-8).

We have added this reference to the Introduction section to support that nocodazole-induced Golgi ministacks have a similar organization as the native Golgi. However, our BFA treatment was combined with the nocodazole treatment, while this paper's BFA treatment does not contain nocodazole.

(7) Figure 1G-J, authors should show a schematic to show the difference between different furin constructs. Also, LQ values in Fig 1I start from 1. Authors may need to include even earlier timepoints.

As suggested by the reviewer, we have shown the domain organization of wild type and mutant furin RUSH reporters in Figure 1, highlighting key amino acids in the cytosolic tail. Please also see our reply to Minor Points (2) of Reviewer #1.

In the revised manuscript, Fig. 1I (SBP-GFP-CD8a-furin-AC #1) has been updated to become Fig. 2J. In this dataset, the first time point was selected at a relatively late stage (20 min), resulting in an initial LQ value of 0.92. However, this should not pose an issue, as SBP-GFP-CD8a-furin-AC reaches a plateau of ~ 1.6 . The number of data points is sufficient to capture the rising phase and fit the first-order exponential function curve with an adjusted $R^2 = 0.99$. Furthermore, we have four independent datasets in total on the intra-Golgi transport of SBPGFP-CD8a-furin-AC (#1-4), demonstrating the consistency of our measurements.

(8) Figure 2A need to show the data points, not just the lines.

In the revamped manuscript, Fig. 2A has been updated to become Fig. 4A. The plot of Fig. 4A is calculated based on Equation 3.

$$\frac{dLQ}{dt} = \frac{\ln 2}{t_{\text{intra}}} (y_0 - LQ) \quad (\text{Equation 3})$$

So, it does not have data points. However, t_{intra} is calculated based on the experimental LQ vs. t kinetic data.

(9) Imaging and camera settings like exposure time, pixel size, etc should be reported in Methods.

As suggested by the reviewer, we have supplied this information in the Materials and Methods section of the revised manuscript.

(1) The exposure time and pixel size for the wide-field microscopy:

“The image pixel size is 65 nm. The range of exposure time is 400 – 5000 ms for each channel.”

(2) The exposure time and pixel size for the spinning disk confocal microscopy: “The image pixel size is 89 nm. The range of exposure time is 200 – 500 ms for each channel.”

(3) The pixel dwelling time and pixel size for the Airyscan microscopy:

“For side averaging, images were acquired under 63× objective (NA 1.40), zoomed in 3.5× to achieve 45 nm pixel size using the SR mode. The pixel dwelling time is 1.16 μs .”

Reviewer #2 (Recommendations For The Authors):

We sincerely appreciate the reviewer's insightful, detailed, and constructive feedback. Your thoughtful comments have helped us refine our analyses, clarify key points, and strengthen the overall quality of our manuscript. We are grateful for the time and effort you have dedicated to reviewing our work and providing valuable suggestions. Your input has been instrumental in improving both the scientific rigor and presentation of our findings. Thank you for your thorough and thoughtful review.

Minor points:

(1) Equation 2: A should be in front of the $\ln 2$. It's already resolved in equation 3, so likely only needs changing in the text

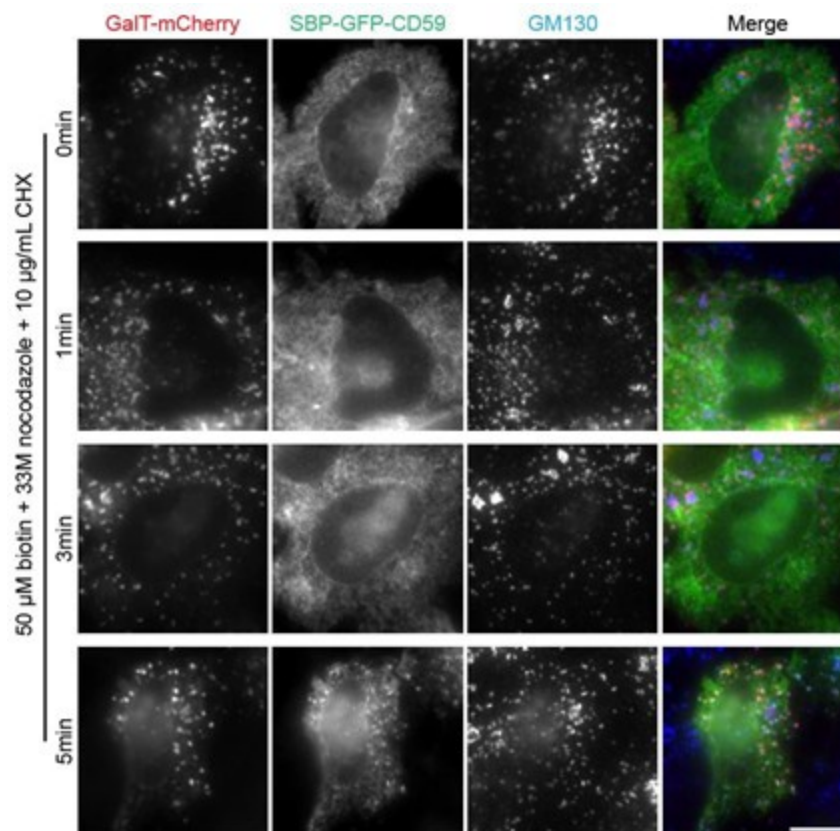
As suggested by the reviewer, we have changed it accordingly.

$$\frac{dLQ}{dt} = \frac{d \ln 2}{\ln 2} e^{\left(\frac{\ln 2}{\ln 2} t \right)} \quad (\text{Equation 2})$$

(2) Line 152: Why is there a lack of experimental data? High ER background and low golgi signal make it difficult to select ministacks: would be good to see examples of these images. Is 0 a relevant timepoint as cargo is still at the ER? Instead would a timepoint <5' be better demonstrate initial arrival in fast cargo, and 0' discarded?

We observed that RUSH reporters typically do not exit the ER in < 5 min of biotin treatment, resulting in a high ER background and low Golgi signal. Example images of SBP-GFP-CD59 are shown below (scale bar: 10 μ m). Possible reasons include: 1) the time required for biotin diffusion into the ER, 2) the time needed to displace the RUSH hook from the RUSH reporter, and 3) the time for recruitment of RUSH reporters to ER exit sites. As a result, we could not obtain LQs for time points earlier than 5 min during the biotin chase.

Author response image 1.



Despite the challenge in measuring LQs at early time points, 0 is still a relevant time point. At $t = 0$ min, RUSH reporters should be at the ER membrane near the ER exit site, a definitive pre-Golgi location along the Golgi axis, although we still don't have a good method to determine its LQ .

(3) Table 1 Line 474: 1-3 independent replicates: is there a better way of incorporating this into the table to make it more streamlined? It would be useful to see each cargo as a mean with error. Is there a more demonstrative way to present the table, for example (but does not have to be) fastest cargo first (t_{intra}) as in Table 2?

As suggested by the reviewer, we revised Table 1. We calculated the mean and SD of t_{intra} and arranged our RUSH reporters in ascending order based on their t_{intra} values.

(4) Line 264 / Fig 3B: It's unclear to me why the VHH-anti-GFP-mCherry internalisation approach was used, when the cells were expressing GFP, that could be used for imaging. Also, this introduces a question over trafficking of the VHH itself, to access the same compartments as the GFP-proteins are localised. It would be useful to describe the choice of this approach briefly in the text.

Here, the surface-labeling approach is used to investigate if GFP-Tac-TC possesses a Golgi retrieval pathway after its exocytosis to the plasma membrane. When VHH-anti-GFP-mCherry is added to the tissue culture medium, it binds to the cell surface-exposed GFP-fused MGAT1, MGAT2, Tac, Tac-TC, CD8a, and CD8a-TC. Next, VHH-anti-GFP-mCherry traces the internalized GFP-fused transmembrane proteins. The surface-labeling approach has two advantages in this case. 1) It is much more sensitive in revealing the minor number of GFPtransmembrane

proteins at the plasma membrane and endosomes, which are usually drowned in the strong Golgi and ER background fluorescence in the GFP channel. 2) While the GFP fluorescence distribution has reached a dynamic equilibrium, the surface labeling approach can reveal the endocytic trafficking route and dynamics.

As the reviewer suggested, we added the following sentence to describe the choice of the cell surface labeling – “By binding to the cell surface-exposed GFP, VHH-anti-GFP-mCherry serves as a sensitive probe to track the endocytic trafficking itinerary of the above GFP-fused transmembrane proteins”.

Regarding the trafficking of VHH-anti-GFP-mCherry itself, in HeLa cells that do not express GFP-fused transmembrane proteins, VHH-anti-GFP-mCherry can be internalized by fluid-phase endocytosis. However, the fluid-phase endocytosis is negligible under our experimental condition, as we previously demonstrated (Sun et al., JCS, 2021; PMID: 34533190).

(5) 446 Typo "internalization"

It has been corrected.

Reviewer #3 (Recommendations For The Authors):

Below are my recommendations for the authors to improve their manuscript:

We sincerely appreciate the reviewer's insightful, detailed, and constructive feedback. Your thoughtful comments have helped us refine our analyses, clarify key points, and strengthen the overall quality of our manuscript. We are grateful for the time and effort you have dedicated to reviewing our work and providing valuable suggestions. Your input has been instrumental in improving both the scientific rigor and presentation of our findings. Thank you for your thorough and thoughtful review.

(1) Line 48: Tie et al. 2016 is cited. Please add references to original work showing that cargos transit from cis to trans Golgi cisternae.

After reviewing the literature, we identified two references that provide some of the earliest morphological evidence of secretory cargo transit from the *cis*- to the *trans*-Golgi:

(1) Castle et al, JCB, 1972; PMID: 5025103

(2) Bergmann and Singer, JCB, 1983; PMID: 6315743

The first study utilized pulse-chase autoradiographic EM imaging to track secretory protein movement, while the second employed immuno-EM imaging to observe the synchronized release of VSVGtsO45. Accordingly, we have removed Tie et al., 2016 and replaced it with these newly identified references.

(2) I would suggest to cite earlier (in the Introduction) the rapid partitioning and rim progression models.

As suggested, we have moved the rapid partitioning and rim progression models to the Introduction section.

(3) Figure 1: LQ vs. time plot for SBP-GFP-CD8a-furinAC (panel I, 0.9 to 1.75 in 150 min) is different from Fig 7G of Tie et al. 2016 (LQ 0-1.5 in 100 min). Please comment on why those 2 sets of data are different.

We appreciate the reviewer for pointing out this error. In our previous publication (Tie et al., MBoC, 2016), we presented a total of four datasets on SBP-GFP-CD8a-furin-AC. However, in the earlier version of our manuscript, we mistakenly listed only three datasets, inadvertently omitting Fig. 7G from Tie et al., MBoC, 2016.

In the revised version, we have now included Fig. S2T (SBP-GFP-CD8a-furin-AC #4), which corresponds to Fig. 7G from Tie et al., MBoC, 2016.

(4) As mentioned in the public review, I think measurement of the expression level of the cargos is necessary to compare their transport kinetics.

The reviewer raises a valid concern that is challenging to address. All our data were obtained by imaging overexpressed reporters, and we assume that their overexpression does not significantly impact the Golgi or the secretory pathway. Our previous studies have demonstrated that overexpression does not substantially affect LQs (Figure S2 of Tie et al., MBoC, 2016, and Figure S1 of Tie et al., JCB, 2022).

We acknowledge this concern as one of the limitations in our study at the end of our manuscript:

“First, our approach relied on the overexpression of fluorescence protein-tagged cargos. The synchronized release of a large amount of cargo could significantly saturate and skew the intra-Golgi transport.”

(5) To my opinion, cisternal continuities would also affect retrograde transport (accelerate) (by diffusion for instance) and not only retrograde transport. Please comment on how this would affect intra-Golgi transport kinetics.

We believe the reviewer is suggesting “cisternal continuities would also affect retrograde transport (accelerate) (by diffusion for instance) and not only anterograde transport.”

Transient cisternal continuities have been reported to facilitate the anterograde transport of large quantities of secretory cargos (Beznoussenko et al., 2014; PMID: 24867214) (Marsh et al., 2004; PMID: 15064406) (Trucco et al., 2004; PMID: 15502824). However, we are not aware of any reports demonstrating that such continuities facilitate the retrograde transport of secretory cargo, although Trucco et al. (2004) speculated that Golgi enzymes might use these connections to diffuse bidirectionally (anterograde and retrograde direction). For this reason, we did not discuss this scenario in our manuscript.

(6) Lines 188-190: I don't understand why the rapid partitioning model is excluded. Please detail more the arguments used for this statement.

Below is the section from the Introduction that addresses the reviewer's question.

“This model (rapid partitioning model) suggests that cargos rapidly diffuse throughout the Golgi stack, segregating into multiple post-translational processing and export domains, where cargos are packed into carriers bound for the plasma membrane. Nonetheless, synchronized traffic waves have been observed through various techniques, including EM (Trucco et al., 2004) and advanced light microscopy methods we developed, such as GLIM and side-averaging (Tie et al., 2016; Tie et al., 2022). These findings suggest that the rapid partitioning model might not accurately represent the true nature of the intra-Golgi transport.”

(7) I would suggest replacing the 'Golgi residence time' by another name as it reflects mainly the time of Golgi exit if I am not mistaken.

We believe the term “Golgi residence time” more accurately reflects the underlying mechanism – retention. The same approach to measure the Golgi residence time can also be applied to Golgi enzymes such as ST6GAL1. Its slow Golgi exit kinetics ($t_{1/2} = 5.3$ hours) (Sun et al., JCS, 2021) should be primarily due to a strong Golgi retention at its steady state Golgi localization.

In contrast, the conventional secretory cargos’ Golgi exit times are usually much shorter ($t_{1/2} < 20$ min) (Table 2) due to weaker Golgi retention. In a broader sense, the Golgi exit kinetics of a secretory cargo should be influenced by its Golgi retention. Furthermore, we have consistently used the term “Golgi residence time” in our previous publications. So, we propose maintaining this terminology in the current manuscript.

(8) Lines 300-306: I would suggest that the authors remove this part as it is highly speculative and not supported by data.

We have relocated this discussion to the section titled "Our data supports the rim progression model, a modified version of the stable compartment model."

Our enzyme matrix hypothesis offers a potential explanation for key observations, including the differential cisternal localization of small and large cargos and the interior localization of Golgi enzymes. Cryo-FIB-ET has shown that the interior of Golgi cisternae is enriched with densely packed Golgi enzymes (Engel et al., PNAS, 2015; PMID: 26311849), supporting this hypothesis.

Additionally, this hypothesis helps explain the gradual reduction in intra-Golgi transport velocities of secretory cargos, as requested by Reviewer #1 (Minor Points 4). For these reasons, we propose retaining this discussion in the manuscript.

(9) In Figure 3B, percentage of MGAT2-GFP cells with anti-GFP signal at the Golgi is of 41% while Sun et al. 2021 reported 25%, please comment this difference. Reply:

We included more cells for the quantification. The percentage of cells showing Golgi localization of VHH-anti-GFP-mCherry is now 32% ($n = 266$ cells). The observed difference, 32% vs. 25% (Sun et al., JCS, 2021), is likely due to uncontrollable variations in experimental conditions, which might have influenced the endocytic Golgi targeting efficiency.

(10) The effects of brefeldinA are pleiotropic as it disassembles COPI and clathrin coats but also induces tubulation of endosomes. I would recommend using Golgicide A, which is more specific.

We agree with the reviewer that Golgicide A might be more specific as an inhibitor of Arf1. We will certainly consider using this inhibitor next time.

<https://doi.org/10.7554/eLife.98582.2.sa0>